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The effects of lectins on the release of EDRF from rabbit aorta

Joseph F. Kleha, Pierre Devesly & [1]Anthony Johns

Department of Pharmacology, Berlex Laboratories, Cedar Knolls, New Jersey, 07927 U.S.A.

The effects of the lectins, wheat germ agglutinin (WGA) and concanavalin A (Con A) on endothelium intact pre-contracted rabbit aorta were investigated. WGA (8.3 μM) produced endothelium-dependent relaxations, while Con A (4.3 μM) produced a partially endothelium-dependent relaxation. The endothelium-dependent relaxations to WGA were completely reversed by N-acetylglucosamine, haemoglobin and methylene blue and were partially reversed by N^G-monomethyl-L-arginine (L-NMMA). It is suggested that WGA works as an agonist in releasing endothelium-derived relaxing factor (EDRF) from the endothelium of rabbit aorta by interacting with a glycosylated receptor on endothelial cells.

Keywords: Wheat germ agglutinin; concanavalin A; EDRF; rabbit aorta; N-acetylglucosamine; haemoglobin; methylene blue; endothelial cells

Introduction Lectins have been used to characterize and isolate cell surface glycoproteins for many cell types including endothelial cells (Hedo, 1984). Lectins have the ability to bind to specific saccharide groups of glycoproteins and this binding can be reversed and blocked by specific sugars (Grulich-Henn *et al.*, 1988). Some lectins have been used to label specifically the vascular endothelial cells without labelling the underlying smooth muscle cells (Raub *et al.*, 1990).

One of the many functions of the endothelium is the mediation of vasodilatation, which involves the release of a potent vasodilator referred to as endothelium-derived relaxing factor (EDRF) (Furchgott, 1984). EDRF is very labile with rapid biolcgical action. The responses to EDRF are similar to the responses of nitrovasodilators in that they both stimulate the enzyme guanylate cyclase increasing cellular levels of guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP) leading to vascular smooth muscle relaxation (Furchgott, 1984). Endothelium-dependent vasodilators also stimulate the endothelium to release prostacyclin (PGI_2). Investigators have suggested that in cultured endothelial cells the release of EDRF and PGI_2 is coupled (De Nucci *et al.*, 1988).

Thrombin stimulates synthesis and release of PGI_2 from human umbilical vein endothelial cells (Weksler *et al.*, 1978) and in these cells WGA has been demonstrated to block thrombin-induced increase in intracellular free calcium, [^{3}H]-arachidonate release, and PGI_2 release (Grulich-Henn *et al.*, 1987). We have shown that WGA does not block the thrombin, bradykinin, or acetylcholine receptor on the native endothelium but binds to its own receptor and causes the release of EDRF which relaxes vascular smooth muscle.

Methods Male New Zealand White rabbits were anaesthetized with sodium pentobarbitone. The aorta was removed and placed in cooled physiological salt solution (PSS) of the following composition (mM): NaCl, 140, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, D-glucose 10, HEPES/Na HEPES 5, pH 7.4 plus EDTA 10 μM at 37°C. Rings of approximately 3 mm in width were used with the endothelial layer intact along with endothelial denuded tissues. The endothelium was removed by gently rubbing the intimal surface of the blood vessel with a wooden toothpick. The tissues were set up under 1 g of tension in PSS aerated with room air at 37°C; the tension was adjusted during the first 2 h of equilibration. The tension was measured with an isometric force transducer (Grass FT03C) and displayed on a polygraph (Beckman R-611). The tissues were contracted with 80 mM KCl to determine the maximum tension of the tissue. The presence of endothelium was determined by the addition of acetylcholine (ACh), 300 nM, to pre-contracted tissues.

The effects of the different lectins were tested on the tissues contracted with phenylephrine (300 nM PhE on endothelial intact tissues and 100 nM PhE on tissues with the endothelium removed).

Results The effects of WGA and Con A were tested up to 8.3 μM and 4.3 μM, respectively. The effect of WGA in the presence and absence of endothelium is shown in Figure 1a and b. WGA was added to endothelium-intact and endothelium-denuded tissues starting at 1 μg ml^{-1} (0.028 μM) and ending at 300 μg ml^{-1} (8.3 μM). WGA caused an endothelium-dependent relaxation with a half maximum inhibitory concentration (IC_{50}) of 0.9 $\pm$ 0.2 μM ($n = 6$). No relaxation was measured when WGA was added to the endothelium-denuded tissues even at high concentrations of 8.3 μM WGA.

Con A caused both endothelium-intact and endothelium-denuded tissue to relax after a Phe sustained contraction. At higher concentrations (4.3 μM), Con A caused an 80.1 $\pm$ 3.5% ($n = 6$) relaxation in the presence of endothelium and a 21.0 $\pm$ 3.6% ($n = 6$) relaxation in the absence of endothelium.

In tissues pretreated with atropine (1 μM), WGA (8.3 μM) was equally effective in causing relaxation of precontracted endothelium-intact aortic rings, suggesting that WGA is not working through the acetylcholine receptor. Also in tissues relaxed with WGA (1 μM, approximately 50% relaxation), thrombin (0.5 u ml^{-1}), bradykinin (10 nM) or acetylcholine (300 nM) were able to relax the tissue further without any apparent inhibition of the response.

The effects of various interventions on the relaxation to WGA are shown in Figure 2. These interventions demonstrate that the relaxation to WGA is due to the release of EDRF from the endothelium. In 6 experiments, haemoglobin (10 μM), caused a 108 $\pm$ 5% reversal of the WGA-induced relaxation (Figure 2c). Methylene blue (10 μM) caused a 112 $\pm$ 3% reversal of the WGA-induced relaxation (Figure 2d). The guanylate cyclase inhibitor LY83583 (6-anilino-5,8-quinolinedione) at a concentration of 3 μM caused a 95 $\pm$ 4% reversal of the WGA-induced relaxation (Figure 2e). The inhibitor of EDRF synthesis, N^G-monomethyl-L-arginine (L-NMMA) at 1 mM attenuated the response to WGA by 55 $\pm$ 2% (Figure 2f). N-acetyl glucosamine (20 mM) caused a 99 $\pm$ 3% reversal of the WGA-induced relaxation (Figure 2a). The effect of the sugar, N-acetyl glucosamine, was specific because after

[1] Author for correspondence.

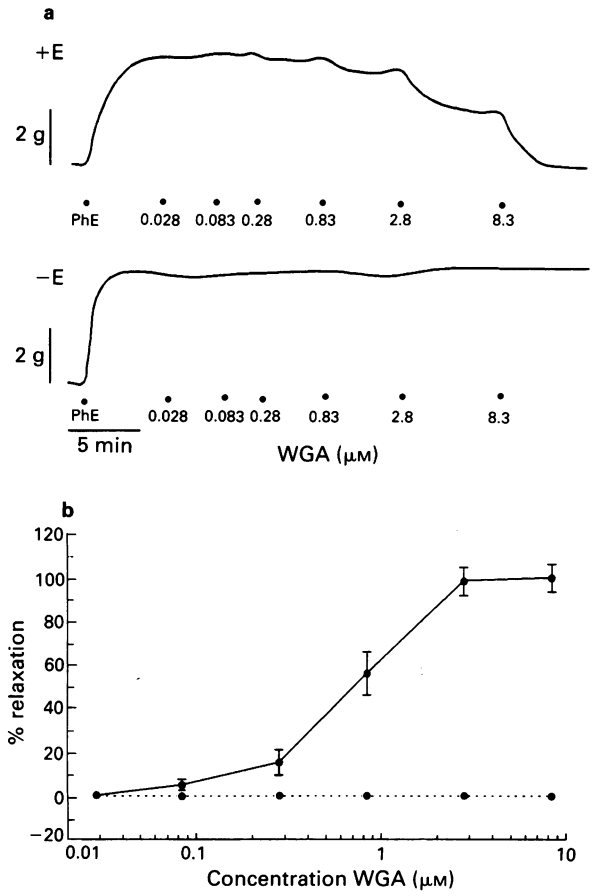

Figure 1 (a) Representative isometric tension recordings of paired rabbit aortic rings with endothelium (+E) and without endothelium (−E) contracted with phenylephrine (PhE) showing endothelium-dependent relaxations to wheat germ agglutinin (WGA) added cumulatively. (b) Effect of increasing concentrations of WGA on the contractile responses of rabbit isolated aortic rings. Responses to WGA with and without endothelium were investigated in tissues from the same animal. Data are expressed as percentage relaxation of the response to phenylephrine (300 nM PhE with endothelium and 100 nM without endothelium), and shown as mean ($n = 6$) with s.e.mean indicated by vertical bars.

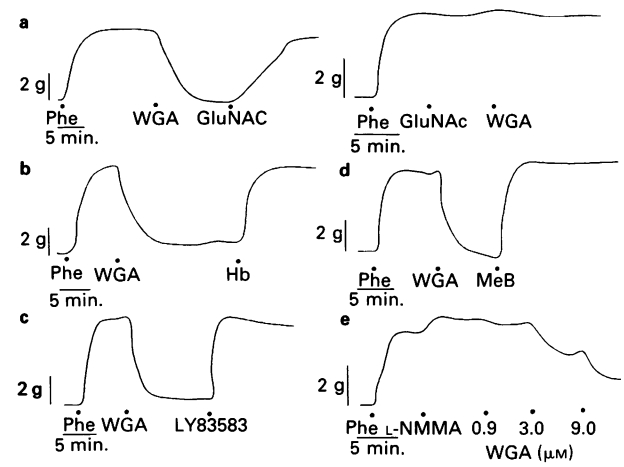

Figure 2 Representative isometric tension recordings of rabbit aorta with the endothelium intact showing the effects of various interventions on the relaxation to wheat germ agglutinin (WGA). The effects of N-acetyl glucosamine (GluNAc) at 20 mM on the effects of 8.3 μM WGA is shown in (a). The effect of 10 μM haemoglobin (Hb) (b), 10 μM methylene blue (MeB) (c) and 10 μM LY83583 (d) on the relaxation to WGA are shown. The relaxation response to WGA in the presence of 1 mM N^G-monomethyl-L-arginine (L-NMMA) is shown in (e). All tissues precontracted with phenylephrine (PhE).

reversal of the WGA-induced relaxation, acetylcholine (300 nM) could still produce 100% relaxation ($n = 6$).

Discussion In endothelium-intact rabbit aorta contracted with PhE (<50% max.) WGA and Con A relaxed the blood vessel. The effect of Con A was partially endothelium-dependent, whereas the response to WGA required a functionally intact endothelium. The endothelium-dependent relaxation to WGA was concentration-dependent with an IC_{50} of 0.9 ± 0.2 μM. L-NMMA attenuate the endothelium-dependent vascular relaxations induced by various EDRF agonists (Johns *et al.*, 1990). Relaxations induced by WGA were attenuated by L-NMMA and completely inhibited by methylene blue, haemoglobin (Martin *et al.*, 1984), and LY83583 (Mulsch *et al.*, 1988). The effect of WGA is therefore consistent with many other endothelium-dependent vasodilators and suggests the relaxation is due to the release of EDRF (Furchgott, 1984). The relaxation to WGA was reversed by N-acetyl glucosamine which is specific for WGA recognition (Grulich-Henn *et al.*, 1988). This sugar was ineffective against acetylcholine-induced relaxation. It is concluded that the endothelium 'recognizes' WGA as an agonist and the specific interaction with the vascular endothelium results in the release of EDRF. Whether the interaction of WGA is with a lectin receptor or with the glycosylation site on another receptor has yet to be determined.

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Characterization of nitric oxide synthases in non-adrenergic non-cholinergic nerve containing tissue from the rat anococcygeus muscle

[1]Jane A. Mitchell, Hong Sheng, Ulrich Förstermann & Ferid Murad

Department 47S, AP10/2, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A. and Department of Pharmacology Northwestern University Medical School, Chicago, IL 60611, U.S.A.

Tissue homogenates prepared from rat anococcygeus muscle converted L-arginine to L-citrulline indicating the presence of nitric oxide (NO) synthase. NO synthase activity was also found in crude and partially-purified soluble and particulate fractions prepared from the homogenates. Both soluble and particulate NO synthase were dependent on NADPH, 5,6,7,8-tetrahydrobiopterin and calcium, and inhibited by N^G-nitro-L-arginine. Tissue homogenates or crude cytosolic and membrane fractions from rat vas deferens, which does not contain NO releasing non-adrenergic non-cholinergic neurones, had no NO synthase activity.

Keywords: Nitric oxide; arginine; cyclic GMP; N^G-nitro-L-arginine; endothelium-derived relaxing factor; guanylyl cyclase; 5,6,7,8-tetrahydrobiopterin

Introduction Electrical stimulation of autonomic nerves to various tissues produces relaxations which are not mediated by adrenergic or cholinergic transmitters. These non-adrenergic non-cholinergic nerves (NANC; Gillespie, 1972) release an unidentified transmitter which in some tissues, such as the rat anococcygeus, resembles NO or a related molecule (Gillespie & Sheng, 1990). In brain tissue, the NO-like material is synthesized by a cytosolic Ca^{2+}-dependent isoform of NO synthase that has recently been purified (Bredt & Snyder, 1990; Schmidt *et al.*, 1991). Vascular endothelium also releases a relaxing factor (EDRF), with characteristics similar to NO, which is formed from L-arginine (Palmer *et al.*, 1988) by a particulate isoform of NO synthase (Förstermann *et al.*, 1991; Mitchell *et al.*, 1991). Here we show that NANC innervated rat anococcygeus muscle contains both soluble and particulate NO synthase activity.

Methods Male Wistar rats were anaesthetized with CO_2 gas and killed by decapitation. The anococcygeus muscle or vas deferens was removed and immediately placed in liquid nitrogen. For each enzyme preparation between 30 and 50 frozen muscles were homogenized in ice-cold buffer 1 (Tris/HCl 50 mM; EDTA 0.1 mM; EGTA 0.1 mM; 2-mercaptoethanol 12 mM; leupeptin 2 μM; pepstatin A 1 μM and phenylmethylsulphonyl fluoride 1 mM) with a tissue homogenizer (Polytron; Switzerland). The homogenate was filtered through muslin before being homogenized a second time on ice with a glass tissue grinder and teflon pestle (25 strokes). The homogenate was centrifuged at 100,000 *g* for 1 h at 5°C. The supernatant fraction (crude cytosol) was then either assayed directly or applied to a DEAE anion exchange column (DEAE-Sephacel; Pharmacia; 0.5 ml). The column was washed with 10 volumes of buffer 1 and eluted with buffer 1 containing 0.3 M NaCl. This procedure removes contaminating haemoglobin and concentrates protein. The 100,000 *g* pellet was either resuspended by homogenization and assayed directly or washed with buffer 1 containing 1 M KCl (5 min exposure at 5°C) before being centrifuged at 100,000 *g* for 30 min at 5°C. The supernatant was discarded and the KCl washed membrane fraction resuspended in buffer 1. This procedure removes any loosely associated soluble proteins from the particulate fraction. Protein concentrations were measured by use of the Bradford reagent (Bio-Rad, Richmond, CA, U.S.A.) with bovine serum albumin as standard. Bovine aortic endothelial cells were cultured and homogenized as described previously (Förstermann *et al.*, 1991; Mitchell *et al.*, 1991).

Enzymatic activity was measured by the ability of cytosolic or particulate fractions of anococcygeus muscle to form [^{3}H]-L-citrulline from [^{3}H]-L-arginine (DuPont/NEN) (Bredt & Snyder, 1990; Schmidt *et al.*, 1991) or by guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation in rat foetal lung fibroblast (RFL-6) cells as previously described (Förstermann *et al.*, 1991; Schmidt *et al.*, 1991). All tissue culture products were purchased from Gibco Laboratories (Grand Island, NY). Tetrahydrobiopterin (BH_4) was obtained from Dr B. Schirks Laboratories (Jona, Switzerland). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistics Results are presented as the mean ± s.e.mean, where *n* is the number of values. The statistical difference between groups was evaluated by Student's unpaired *t* test. $P < 0.05$ was taken to be statistically significant.

Results The specific NO synthase activity in homogenates prepared from rat anococcygeus was approximately half of that found in homogenates of bovine aortic endothelial cells (Table 1). The specific activity of the particulate fraction from rat anococcygeus increased following washing with KCl (presumably this step removes some inhibitory material) and was then unaffected by a second KCl wash (data not shown). The specific NO synthase activity of cytosolic fractions increased following DEAE anion exchange chromatography (Table 1). The cytosolic fraction (1 μg protein ml^{-1}), treated with phosphocellulose (Schmidt *et al.*, 1991) to remove haemoglobin, stimulated cyclic GMP levels in RFL-6 cells more than 3 fold (from 0.7 ± 0.2 to 3.0 ± 0.2 pmol cyclic GMP/10^6 RFL-6 cells 3 min^{-1}) in the presence of L-arginine (100 μM), NADPH (100 μM) and superoxide dismutase (SOD; 20 u ml^{-1}). This increase was reduced by more than 50% to 1.3 ± 0.1 pmol cyclic GMP/10^6 RFL-6 cells 3 min^{-1} ($n = 3$) in the absence of SOD or in the presence of 3 μM haemoglobin (1.2 ± 0.5 pmol cyclic GMP/10^6 RFL5 cells 3 min^{-1}; $n = 3$). No activity was found in homogenate, cytosol or membrane

[1] Author for correspondence at: 47S, AP10/2, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A.

Table 1 Nitric oxide (NO) synthase activity in preparations from various tissues

Tissue	*Preparation*	*Citrulline* (pmol mg^{-1} protein $30\,min^{-1}$)	n
BAE	Homogenate	40.7 ± 3.7	3
Rat AC	Homogenate	19.6 ± 1	9
Rat AC	Cytosol	17.6 ± 2	14
Rat AC	Pellet	33.1 ± 10	6
Rat AC	KCl-washed pellet	78.5 ± 26	17
Rat AC	DEAE-eluted cytosol	171.0 ± 16.5	3
Rat vas	Homogenate	0	3
Rat vas	Cytosol	0	3
Rat vas	KCl-washed pellet	0	3

The NO synthase activity found in preparations from cultured bovine aortic endothelial cells (BAE), rat anococcygeus (Rat AC), or rat vas deferens (Rat vas) was measured with the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine (pmol mg^{-1} protein $30\,min^{-1}$). In each case the following co-factors were present: NADPH (1 mM), L-arginine (10 μM), [^{3}H]-L-arginine (35 nM), tetrahydrobiopterin (3 μM) and calcium (2 mM). Citrulline formation was measured over a 30 min time course at 25°C (n = 3–17).

fractions (n = 3) prepared from rat vas deferens. Although this is a similar uro-genital non-vascular smooth muscle preparation it does not contain NO releasing NANC nerves (Sheng, H. unpublished observations). The cytosolic and particulate forms of NO synthase from rat anococcygeus appeared similar in their regulation and requirement of co-factors. Both enzymes were dependent on Ca^{2+} and NADPH and, to differing extents, partially dependent on BH_4 (Figure 1). In addition both enzymes were strongly inhibited by the specific NO synthase inhibitor N^G-nitro-L-arginine (10 μM; Hobbs & Gibson, 1990), (Figure 1).

Discussion Our results show that the rat anococcygeus muscle contains both cytosolic and particulate NO synthase. The particulate activity did not decline following repeated washes with 1 M KCl indicating that it represents an integral membrane protein or is anchored in the membrane. As described for NO synthase from brain (Mayer *et al.*, 1990; Schmidt *et al.*, 1991) both soluble and particulate enzymes from rat anococcygeus required NADPH and BH_4. The finding that the particulate enzyme had a greater requirement for BH_4 than the soluble could be explained by higher levels of endogenous BH_4 in the soluble fraction.

It is now well-established that brain tissue contains soluble but not particulate NO synthase (Bredt & Snyder, 1991; Schmidt *et al.*, 1991). NO synthase from NANC tissue appears to be different in that substantial enzymatic activity was found to be associated with the particulate fraction.

The data presented here add further to the evidence that the NANC transmitter is NO (or a related molecule) formed from L-arginine. An alternative explanation could be that an unknown NANC transmitter activates NO synthase in smooth muscle cells. This seems unlikely as preparations from rat vas deferens had little or no enzyme activity. In addition the NO synthase in smooth muscle cells is known to be Ca^{2+}-independent and only appears following induction with endotoxin and/or cytokines (Rees *et al.*, 1990). As NANC-derived NO synthase is activated by Ca^{2+} we propose that field stimulation results in Ca^{2+} influx into the respective neurones which activates NO synthase, thereby controlling the amount of neurotransmitter released.

Purification of NANC derived NO synthase will allow for the generation of antibodies to the protein which can then be used for histochemical localization of the enzyme within the tissue.

The authors wish to thank Ms Zei-Jing Huang for her excellent tissue culture services and Mr Bing Cheng for kindly supplying the [^{3}H]-L-arginine. In addition the authors would like to thank Ms Kathy Kohlaas for her help with the citrulline assay and Ms Jayne Azzarello for technical assistance.

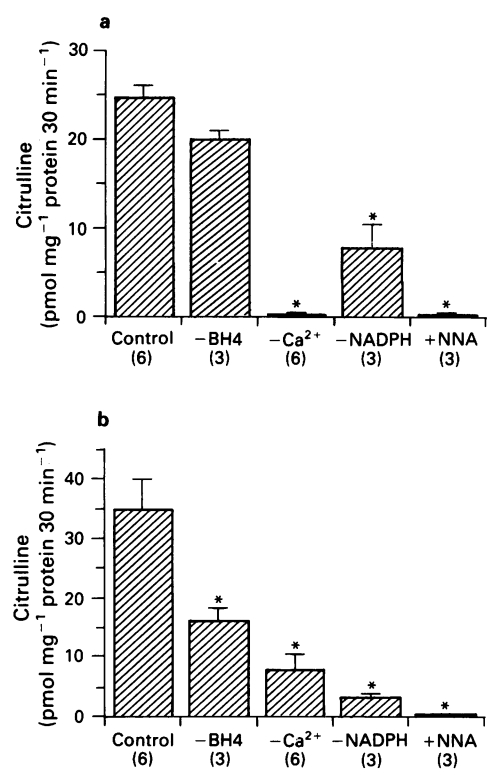

Figure 1 Effect of removal of various co-factors on the activity of crude cytosolic (a) and KCl-washed particulate (b) nitric oxide (NO) synthase isolated from rat anococcygeus. Control activity was measured by [^{3}H]-L-citrulline formation from [^{3}H]-L-arginine in the presence of NADPH (1 mM), L-arginine (10 μM) tetrahydrobiopterin (BH_4; 3 μM) and calcium (2 mM). In the absence of BH_4 or NADPH enzyme activity was reduced. Significant reduction, $P < 0.05$. When either calcium was removed and EGTA (0.1 mM) added (−Ca^{2+}), or N^G-nitro-L-arginine added (10 μM; +NNA) to the preparation NO synthase activity was virtually abolished. The columns represent means ± s.e.mean from 3–6 experiments; number of experiments shown in parentheses.

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Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist, CP-96,345

[1]I.J.M. Beresford, P.J. Birch, R.M. Hagan & S.J. Ireland

Department of Neuropharmacology, Glaxo Group Research Ltd., Park Road, Ware, Hertfordshire, SG12 0DP

The affinity of the non-peptide antagonist CP-96,345 for tachykinin NK_1 receptors has been estimated in a range of species by use of both radioligand binding and functional assays. CP-96,345 was 30–120 fold less active at NK_1 receptors in rat and mouse than in the other species examined, including man. These results demonstrate the existence of species variations in NK_1 receptors.

Keywords: Tachykinin NK_1 receptor; NK_1 receptor antagonist; species variants; subtype, CP-96,345

Introduction Receptors for the mammalian tachykinins, substance P (SP), neurokinin A and neurokinin B, have been classified on pharmacological criteria into three subtypes, termed NK_1, NK_2 and NK_3 (see review by Guard & Watson, 1991). This classification has been substantiated by the cloning of all three receptors (Nakanishi, 1991). However, functional studies *in vitro* using early peptide antagonists suggested that subtypes of the NK_1 receptor may exist (Brown *et al.*, 1985a,b) and, recently, in NK_1 receptor binding assays, the affinity of the non-peptide tachykinin NK_1 receptor antagonist CP-96,345 has been shown to be species-dependent (Gitter *et al.*, 1991; Snider *et al.*, 1991).

To investigate further the possible existence of species subtypes of the NK_1 receptor, we have measured the ability of racemic CP-96,345 to inhibit binding of [^{3}H]-SP to brain cortex membranes prepared from eight different species and have determined whether estimates of affinity at [^{3}H]-SP binding sites correlate with those determined at NK_1 receptors using functional assays *in vitro*.

Methods *[^{3}H]-substance P binding* [^{3}H]-SP binding assays were performed essentially as described by Dam & Quirion (1986). Cerebral cortical membranes (8–15 mg wet weight per assay tube) were incubated with [^{3}H]-SP (0.5–0.7 nM, specific activity 34 Ci mmol^{-1}, DuPont) at 22°C for 40 min. Non-specific binding was defined as that remaining in the presence of physalaemin (1 μM).

Smooth muscle preparations Rings of rabbit thoracic aorta (male New Zealand White rabbits, 2–3 kg, Froxfield) or sections of guinea-pig ileum longitudinal smooth muscle (male Dunkin-Hartley guinea-pigs, 300–500 g, Porcellus) were prepared as described by Regoli *et al.* (1984). Preparations were mounted in organ baths (37°C) filled with either Krebs-Henseleit medium containing indomethacin (1 μM) (aorta) or Tyrode solution containing atropine, indomethacin, mepyramine, methysergide and ondansetron, all at 1 μM (ileum). Mechanical activity was recorded isometrically.

[1] Author for correspondence.

Neonatal rat spinal cord Spinal cord was excised from C.D. rat pups (1–8 days *post partum*, Glaxo), hemisected sagitally and superfused (2 ml min^{-1}) with modified Krebs-Henseleit medium (containing $MgSO_4$ 0.7 mM and $CaCl_2$ 1.2 mM) at room temperature. Depolarization responses were recorded extracellularly from lumbar (L3–L5) ventral roots (see Brown *et al.*, 1985a).

Experimental design Aorta preparations were pre-contracted with phenylephrine (0.1 μM). Concentration-relaxation response curves to substance P methylester (SPOMe) were constructed by cumulative addition. For ileum and spinal cord, concentration-response curves to SPOMe were constructed non-cumulatively by use of serially-increasing concentrations. Experiments to determine the apparent affinity of antagonists were undertaken as described previously (see Ireland *et al.*, 1991).

Data analysis Binding data were analysed by the curve-fitting programmes ALLFIT and LIGAND. From functional assays, the apparent affinity of antagonist (pK_B) was estimated as described previously (Ireland *et al.*, 1991).

Drugs Racemic CP-96,345 [*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine] was synthesized in the Department of Medicinal Chemistry, Glaxo Group Research, Ware, Herts. Physalaemin and SPOMe were supplied by Peninsula and Cambridge Research Biochemicals, respectively.

Results *[^{3}H]-substance P binding* CP-96,345 potently inhibited binding of [^{3}H]-SP to rabbit, guinea-pig, human, bovine, hamster and gerbil cerebral cortices with similar nanomolar potencies (Table 1). In contrast, CP-96,345 was 30–120 fold less potent in rat and mouse tissues (Table 1). In comparison, the NK_1 agonist physalaemin was equipotent in all species (Table 1). Saturation analysis of [^{3}H]-SP binding (0.02–10 nM) to rabbit, guinea-pig and rat cerebral cortex indicated that [^{3}H]-SP bound to single populations of binding sites with equilibrium dissociation constants (K_D) of 165 ± 2, 122 ± 2 and 106 ± 24 pM, respectively (n = 4). Maximal

Table 1 Comparison of the potencies (pIC_{50}) of CP-96,345 and physalaemin to inhibit binding of [^{3}H]-substance P ([^{3}H]-SP) to cerebral cortical membranes prepared from different species

	Rabbit	*Guinea-pig*	*Man*	*Bovine*	*Hamster*	*Gerbil*	*Rat*	*Mouse*
CP-96,345	8.62 ± 0.06	8.50 ± 0.08	8.46	8.86 ± 0.12	8.40 ± 0.01	8.51 ± 0.11	6.77 ± 0.08	6.92 ± 0.08
Physalaemin	8.13 ± 0.05	7.92 ± 0.10	n.d.	8.10 ± 0.01	8.35 ± 0.06	7.99 ± 0.03	8.12 ± 0.08	8.25 ± 0.09

Results are mean pIC_{50} values ± s.e.mean of 3–10 experiments, except man (n = 1). Slopes of displacement curves were not significantly different from unity.

binding capacities were calculated to be 103 ± 11, 25 ± 1 and 64 ± 10 fmol mg^{-1} protein ($n = 4$) in rabbit, guinea-pig and rat cortex, respectively. Using the K_D determinations, pKi values for CP-96,345 were calculated to be 9.30 ± 0.08 ($n = 10$), 9.18 ± 0.12 ($n = 5$) and 7.65 ± 0.06 ($n = 5$) in rabbit, guinea-pig and rat, respectively.

Functional responses CP-96,345 behaved as a reversible competitive antagonist of responses induced by SPOMe in the rabbit aorta, guinea-pig ileum and neonatal rat spinal cord. Thus, in the presence of CP-96,345, concentration-response curves were displaced to the right in a concentration-dependent and parallel manner (Figure 1). Further, Schild plots constructed from the antagonism data had gradients not significantly different from unity. The estimated values were 0.89 (95% confidence limits 0.66–1.11, $n = 20$), 0.78 (0.40–1.16, $n = 15$) and 0.94 (0.80–1.07, $n = 9$) in aorta, spinal cord and ileum, respectively (Figure 1). The apparent affinity of CP-96,345 was similar in the rabbit aorta and guinea-pig ileum (pK_B 8.81 ± 0.06 ($n = 20$) and 8.89 ± 0.02 ($n = 9$), respectively). In contrast, CP-96,345 was markedly less potent in the neonatal rat spinal cord (pK_B 7.13 ± 0.10 ($n = 15$)). CP-96,345 (100 nM) had no effect on contractions induced by either carbachol or bradykinin in guinea-pig ileum or by phenylephrine in rabbit aorta (data not shown).

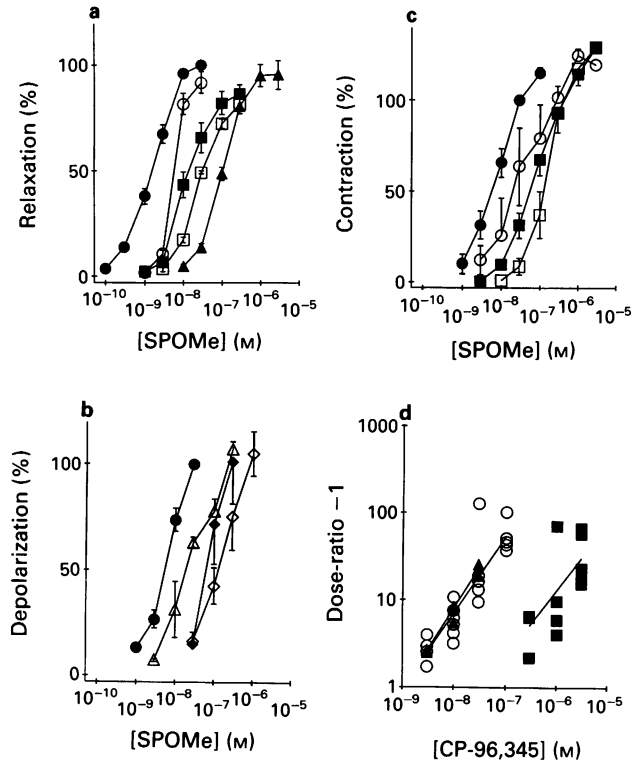

Figure 1 Antagonism by CP-96,345 of responses to substance P methyl ester (SPOMe) in the rabbit thoracic aorta (a), neonatal rat spinal cord (b), or guinea-pig ileum (c). Data are expressed as a mean percentage of the response to SPOMe (30 nM). Symbols indicate controls (●), or the presence of CP-96,345 at 3 (○); 10 (■); 30 (□); 100 (▲); 300 (△); 1000 (◆) or 3000 (◇) nM. Each point is mean of single determinations in at least 3 separate preparations; vertical bars show s.e.mean. (d) Schild plots for CP-96,345 antagonism of SPOMe-induced responses in rabbit thoracic aorta (○), guinea-pig ileum (▲) or neonatal rat spinal cord (■). Data were derived from the experiments illustrated in Figure 1a–c. Each point represents data obtained from a separate preparation.

Discussion The observation that the non-peptide NK_1 receptor antagonist, CP-96,345 was approximately 30–120 fold less potent at inhibiting [^{3}H]-SP binding in rat or mouse cerebral cortex than other mammalian species (including man) is in agreement with previous studies suggesting that this compound can discriminate species variants of NK_1 receptors (Gitter *et al.*, 1991; Snider *et al.*, 1991). These differences are unlikely to arise from differential affinities of [^{3}H]-SP for cortical binding sites since the K_D value for the ligand was very similar in rat, rabbit and guinea-pig cortex. In addition, the NK_1 agonist, physalaemin, was equipotent in all species tested.

Importantly, the observed differences in binding affinities were reflected in antagonist potencies at functional NK_1 receptors in representative isolated preparations. Thus, there was good agreement between estimates of apparent affinity in functional preparations and binding studies conducted in tissue from the same species: in rat spinal cord and rat cortex, CP-96,345 was approximately 40 fold weaker than in rabbit aorta and rabbit cortex or guinea-pig ileum and guinea-pig cortex.

The present results demonstrate that the affinity of CP-96,345 for functional NK_1 receptors is species-dependent. They are also consistent with the suggestion that NK_1 receptors can be resolved into two groups, those in rabbit, guinea-pig, human, cow, hamster and gerbil being distinct from those in rat and mouse. The possibility of further subdivision of the NK_1 receptor either between or within species remains to be addressed.

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SPECIAL REPORT

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Local intracoronary infusions of bradykinin profoundly reduce the severity of ischaemia-induced arrhythmias in anaesthetized dogs

Agnes Vegh, Laszlo Szekeres & [1]*James R. Parratt

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

Bradykinin in a dose (25 ng kg^{-1} min^{-1}) which did not alter coronary flow, or saline, were infused into a small branch of the left anterior descending coronary artery in dogs anaesthetized with chloralose and urethane, for 10 min prior to coronary artery occlusion and throughout the 25 min occlusion period. The degree of inhomogeneity of conduction and epicardial ST-segment changes were measured in the ischaemic zone with a composite electrode. In control dogs, coronary artery occlusion led to severe arrhythmias with an incidence of ventricular fibrillation of 47% and tachycardia of 80% and with a mean of 528 ± 140 ventricular premature beats. In marked contrast, those dogs administered bradykinin had no ventricular fibrillation or tachycardia and the number of premature beats was significantly less (53 ± 19). ST-segment changes were also much less in these dogs. These results raise the possibility that bradykinin might contribute to the protective effects of preconditioning and acts as an 'endogenous myocardial protective substance'.

Keywords: Bradykinin; myocardial ischaemia; arrhythmias; ventricular fibrillation; ST-segment changes; reperfusion; preconditioning

Introduction Brief periods of coronary artery occlusion reduce the severity of the ultrastructural changes (Murry *et al.*, 1986) and the ventricular arrhythmias (Vegh *et al.*, 1990) that occur when the same artery is later occluded for a longer period. This phenomenon is known as preconditioning. The mechanisms remain to be elucidated but one of several possibilities is that brief preconditioning periods of ischaemia, trigger the release from the heart of 'endogenous myocardial protective substances' which then 'precondition' the myocardium by protecting it against a later, more severe, ischaemic stress (Vegh *et al.*, 1991).

We have recently described, in cardiac and coronary vascular tissue, an acid-optimum enzyme capable of releasing kinins from kininogen under conditions of ischaemia (Zeitlin *et al.*, 1989). This raises the possibility that plasma kinins may be involved, as endogenous myocardial protective substances in preconditioning. However, although inhibition of breakdown by angiotensin converting enzyme (ACE) inhibitors results in cardiac protection during ischaemia (Linz *et al.*, 1990) we could find no report of the effects of bradykinin on the severity of the early arrhythmias that occur during the first minutes of coronary artery occlusion. In the present paper we describe the results, in anaesthetized dogs, of infusing a low concentration of bradykinin directly into a small branch of a coronary artery on arrhythmias, on the degree of inhomogeneity of conduction and on the ST-segment changes that result from a prolonged occlusion of that artery.

Methods Twenty-four adult mongrel dogs, with a mean weight of 23.8 ± 1.6 kg, were anaesthetized with a mixture of chloralose and urethane (60 and 200 mg kg^{-1} intravenously) and ventilated with room air by use of a Harvard respirator at a rate and stroke volume sufficient to maintain arterial blood gases and pH within normal limits (Vegh *et al.*, 1990). The temperature was maintained at 37 ± 0.5°C by means of a heating pad. A thoracotomy was performed at the fifth intercostal space and the left anterior descending (LAD) coronary artery dissected free just proximal to the main diagonal branch and a thread placed loosely around it. A smaller branch of the LAD proximal to this proposed site of occlusion was catheterized for the intracoronary infusion of bradykinin or saline. Epicardial ST-segment changes and the degree of inhomogeneity of conduction were measured in the area distal to the occlusion with the composite electrode described by Vegh *et al.* (1987). In some of the experiments coronary blood flow was measured on either the LAD or on the left circumflex coronary artery (or both) with an electromagnetic flow probe (Statham SP 2202; 2.0 mm) and/or a 2.4 mm Doppler flow probe (Triton Technology, San Diego, U.S.A.). Arterial and left ventricular (LV) pressures, and LV$dP/dt$ were measured with Statham P23Db pressure transducers and all parameters, together with a limb lead ECG, were recorded on an 8 channel Medicor R-81 Recorder. The area at risk was measured with patent blue V dye at the end of the experiment and expressed as a percentage of the free left ventricular wall. The mean value was 39.1 ± 1.7%. Ventricular arrhythmias during ischaemia and reperfusion were analysed statistically as previously described (Vegh *et al.*, 1990).

Fifteen of the 24 dogs acted as controls and 9 were infused with a dose of bradykinin (25 ng kg^{-1} min^{-1}) which, from previous experience (Lochner & Parratt, 1964) was at the limit of that required to increase coronary blood flow. The protocol was to infuse saline for 20 min at a rate of 0.075 ml min^{-1} and then bradykinin (or saline) for 10 min preceding coronary artery occlusion and then throughout the 25 min occlusion period. The myocardium was reperfused at the end of this period.

Results Neither the intracoronary infusion of saline nor bradykinin elicited significant haemodynamic changes (e.g. arterial pressure 141 ± 6 mmHg systolic and 96 ± 5 mmHg diastolic during the saline infusion and 144 ± 5/98 ± 4 mmHg after 10 min of bradykinin infusion; heart rate 152 ± 5 to 149 ± 4 beats min^{-1}; LVEDP 6.7 ± 0.8 to 6.2 ± 0.7 mmHg; LV$dP/dt_{max}$ 2355 ± 111 to 2381 ± 119 mmHg s^{-1}). Coronary blood flow was hardly affected by this dose of bradykinin (change less than 5 ml min^{-1} from control values of 50–55 ml min^{-1} diastolic and 10–15 ml min^{-1} systolic). To increase flow, significantly higher dose of bradykinin (0.33 and 1.24 μg kg^{-1} min^{-1}) or single bolus injections of acetylcholine (0.05 μg kg^{-1}) were required.

Ventricular ectopic activity was pronounced in the control dogs (Figure 1) with a high incidence (47%) of ventricular

[1] Author for correspondence.

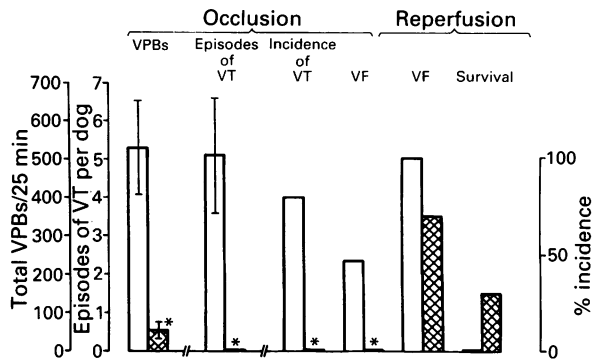

Figure 1 Ventricular arrhythmias during a 25 min occlusion of the left anterior descending coronary artery in anaesthetized dogs and following abrupt reperfusion at the end of that period. Bradykinin, infused into a small branch of the artery proximal to the occlusion in a dose of 25 ng kg^{-1} min^{-1} significantly reduced the incidence and the severity of these arrhythmias. Open columns are the controls (n = 15) and cross-hatched columns are from the bradykinin-treated animals (n = 9). * $P < 0.05$ (Mann-Whitney U test or Fisher's Exact Probability Test (for changes in the incidence of events)).

fibrillation (VF), a large number of ventricular premature beats (VPBs) in the survivors (528 ± 140) and several periods of ventricular tachycardia (VT; 5.1 ± 1.6 episodes per dog). In marked contrast, those dogs infused with bradykinin had little ventricular ectopic activity over the 25 min occlusion period (53 ± 19 VPBs, and there was no VT or VF in any of the dogs; Figure 1). Changes in the degree of inhomogeneity for the first 5 min of occlusion were similar (65 ± 5, 88 ± 10, 150 ± 17 and 154 ± 22 ms at 0, 1, 3 and 5 min of occlusion respectively versus 66 ± 3, 77 ± 5, 120 ± 13 and 154 ± 22 ms during the bradykinin infusion; NS). Changes in ST-segment elevation on occlusion were less marked during the bradykinin infusion (3.6 ± 0.9, 6.6 ± 1.2 and 7.9 ± 1.2 mV at 1, 3 and 5 min versus 1.7 ± 0.4 ($P < 0.05$), 3.9 ± 0.5 ($P < 0.05$) and 6.1 ± 0.9 (NS) at the same times during the bradykinin infusion).

Discussion A number of recent studies have shown that increased endogenous bradykinin levels are a major component of the beneficial effects of ACE inhibitors under conditions of experimental myocardial ischaemia since these drugs also inhibit kininase II. Perhaps the most conclusive evidence comes from a recent study (Martorana *et al.*, 1990) showing that cardioprotective effects of ACE inhibitors, such as an ability to reduce myocardial infarct size, are suppressed by bradykinin antagonists. Less attention has been given to the possibility that bradykinin, besides reducing ultrastructural damage resulting from ischaemia might also reduce the severity of ischaemia-induced arrhythmias. Tobe *et al.*, 1991 infused bradykinin, in a dose of 3.3 μg kg^{-1} min^{-1} by way of a Swan-Ganz catheter in the pulmonary artery, to anaesthetized pigs during balloon occlusion of a coronary artery (and reperfusion 45 min later). Two weeks later the dogs were re-anaesthetized and ventricular tachyarrhythmias were induced by use of programmed electrical stimulation. Electrical stability (assessed from the filtered QRS deflection using signal-averaging electrocardiography) was greater in those dogs that had been given bradykinin during the coronary artery occlusion-reperfusion period and it was less easy to induce ventricular tachycardia in these hearts. Myocardial creatine phosphokinase loss was also less in those animals administered bradykinin, signifying reduced myocardial damage.

Our present studies are concerned not with late ischaemic arrhythmias, but with those life-threatening arrhythmias that occur within minutes of coronary artery occlusion. These early ventricular arrhythmias were dramatically reduced by the local intra-arterial administration of bradykinin in a dose that hardly modified coronary blood flow. We have, as yet, no explanation for this profound modification of arrhythmia severity. Possibilities include prostanoid (prostacyclin?) and nitric oxide release, both of which contribute to the beneficial effects of preconditioning, (Vegh *et al.*, 1990; 1991), increased myocardial glucose uptake, which could decrease arrhythmia severity perhaps by reducing potassium loss (Bernier & Hearse, 1988) or a reduced severity of ischaemia (as indicated by the reduced ST-segment changes in the present study and by decreased myocardial lactate production in the studies of Linz *et al.*, 1990). Whatever the explanation, these results raise the possibility that bradykinin, released locally as a consequence of myocardial ischaemia, might participate in the protective effects of preconditioning.

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Differences in inositol phosphate production in blood vessels of normotensive and spontaneously hypertensive rats

[1]E. Vila, I.M. Macrae & J.L. Reid

University Department of Medicine and Therapeutics, Gardiner Institute, Western Infirmary, Glasgow G11 6NT

1 Total inositol phosphate formation was measured in labelled femoral and iliac arteries and veins of 14 week-old spontaneously hypertensive rats (SHR) and age-matched Wistar Kyoto (WKY) controls, either unstimulated or in the presence of noradrenaline.

2 Basal levels of [^{3}H]-inositol phosphates and [^{3}H]-phosphatidylinositol were significantly enhanced in SHR femoral artery, but not in the other 3 vessels, compared with WKY.

3 Noradrenaline stimulated phosphoinositide hydrolysis in all four vessels of SHR and WKY. Pretreatment with prazosin (10^{-7}–10^{-6} M) but not with yohimbine (10^{-7} M), inhibited the noradrenaline-induced inositol phosphate formation indicating an α_1-adrenoceptor-mediated response.

4 In the femoral artery of SHR compared to WKY, [^{3}H]-inositol phosphate accumulation induced by noradrenaline (10^{-7}–10^{-5} M) was significantly reduced when expressed relative to basal values although the response to higher concentrations (10^{-4}–10^{-3} M) was not altered. In contrast, a significant reduction of inositol phosphates was seen only with 10^{-7} M noradrenaline when absolute values were compared. In the other three vessels, no difference in noradrenaline-induced [^{3}H]-inositol phosphate formation was observed between strains.

5 These data suggest that phosphoinositide hydrolysis-mediated by α_1-adrenoceptors may be reduced in some but not all blood vessels of adult SHR.

Keywords: α_1-adrenoceptors; hypertension; phosphoinositide hydrolysis; noradrenaline

Introduction

Essential hypertension is characterized by an increase in peripheral vascular resistance (Webb & Vanhoutte, 1979; Webb, 1984). Abnormal responses to a variety of vasoconstrictor and vasodilator agents have been shown in vascular smooth muscle from different animal models of hypertension, including the spontaneously hypertensive rat (SHR) (Holloway & Bohr, 1973; Berecek *et al.*, 1980; Whall *et al.*, 1980). Contractions of helical strips of the aorta from SHR have been found to decrease (Shibata *et al.*, 1973; Aidulis *et al.*, 1990) or not change (Hallback *et al.*, 1971) in response to noradrenaline, as compared with normotensive animals. Further, the sensitivity to noradrenaline is not modified in femoral small arteries, is decreased in rat tail artery (Mulvany *et al.*, 1982) and is increased in mesenteric vessels (Mulvany *et al.*, 1980) of SHR compared to Wistar Kyoto rats (WKY) if the uptake of noradrenaline by the nerve terminal is inhibited.

It is extensively accepted that the sympathetic nervous system controls vascular tone predominantly through α_1-adrenoceptors. The stimulation of these receptors by agonists activates a phosphodiesterase, phospholipase C, and leads to hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate to generate two second messengers, inositol 1,4,5,trisphosphate and 1,2-diacylglycerol. Inositol-1,4,5-trisphosphate is known to release calcium from intracellular stores of smooth muscle cells leading to contraction (Somlyo *et al.*, 1985).

The differences in vascular contraction observed in a variety of animal models of high blood pressure compared to normotensive animals have led to the suggestion that they may be ascribed to differences in the cellular processes linked to activation of α_1-adrenoceptors or to differences in the number of receptors. In that sense, several authors have demonstrated changes in vascular inositol phosphate formation in various rat models of hypertension. Eid & De Champlain (1988) showed that in the femoral and mesenteric arteries of DOCA-salt hypertensive rats the noradrenaline-induced inositol phosphate production was increased compared to controls. However Heagerty *et al.* (1986) demonstrated that in the aorta of 5-week old SHR, before major blood pressure and vascular changes have occurred, both basal and noradrenaline-induced inositol phosphate formation in the presence of lithium was increased compared with age-matched WKY rats. This suggests that in the early stages of hypertension the system was overactive. In the same study at 19 weeks of age, when hypertension was established, the noradrenaline-induced inositol phosphate accumulation was reduced in SHR compared with age-matched WKY rats. In addition, Ek *et al.* (1989) have reported that phosphoinositide turnover was impaired in the aorta of 12–16 week-old SHR while Eid & De Champlain (1988) have demonstrated an increase in basal and noradrenaline-stimulated inositol monophosphate production in atria and ventricles of DOCA-salt hypertensive rats. However, the importance of all these findings in relation to our understanding of hypertension remains unclear. The aim of this study was to measure basal and stimulated levels of [^{3}H]-inositol phosphate formation in the femoral and iliac arteries and veins of adult SHR and WKY rats.

Methods

Experiments were carried out on 14 week-old male SHR and WKY rats (Harlan Olac, Bicester, U.K.).

Assay for total inositol phosphates

The animals were killed by a sharp blow to the head and the blood vessels (femoral and iliac arteries and veins) were removed, cleaned of adherent tissue and immediately placed on ice. The vessels were incubated for 30 min at 37°C in Krebs solution (composition, mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 0.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1) with 1.5% bovine serum albumin (BSA) continuously bubbled with 5% CO_2:95% O_2. The Krebs solution was changed every 10 min. Subsequently, the blood vessels were transferred

[1] Author for correspondence at permanent address: Departament de Farmacologia i Psiquiatria, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain.

and incubated for a further 15 min in Krebs solution containing 10^{-2} M LiCl and 2×10^{-6} M imipramine. Two vessels were transferred to each assay tube containing 450 μl of Krebs solution with 10^{-2} M LiCl and 2×10^{-2} M imipramine. Each sample was weighed, 0.5 μCi of [^{3}H]-myoinositol added and incubated at 37°C for 3 h under an atmosphere of 95% O_2:5% CO_2.

Noradrenaline (10^{-7}–10^{-3} M) or the vehicle ascorbic acid (0.1%) was added 30 min before stopping the reaction. Antagonists were added 15 min before noradrenaline. Blank tubes lacking only tissue, and control tubes lacking agonist were assayed simultaneously with all experiments. The reaction was terminated by the addition of $CH_3OH/CHCl_3/HCl$ (40:20:1 V/V/V) and the sample sonicated for 45 min. After the addition of 0.63 ml of $CHCl_3$ and 1.26 ml distilled water, the samples were centrifuged at 2500 g for 10 min to facilitate phase separation. The aqueous layer was removed from the tubes for assay of inositol phosphates. Each sample was neutralized and run through a column containing Dowex anion exchange resin in the formate form. The columns were washed with 15 ml of unlabelled *myo*-inositol (5 mM). The total [^{3}H]-inositol phosphates were eluted by washing with 0.1 M formic acid in 1 M ammonium formate. The eluant was collected in scintillation vials and scintillant added. Samples were counted in a liquid scintillation counter.

Assay for phosphoinositides

The lipid layer remaining after removal of the aqueous phase was used for measurement of the [^{3}H]-phosphoinositides: 3 ml of $CHCl_3/CH_3OH$ (2:1, V/V) and 2 ml of CH_3OH/KCl (1 M)-*myo*-inositol (0.01 M) (1:1, V/V) were added to each sample, mixed and centrifuged at 250 g for 5 min. The aqueous layer was removed and discarded. The washing process with CH_3OH/KCl-*myo*-inositol was repeated twice. The lipid phase was then removed and placed in a scintillation vial and left to evaporate overnight. Scintillant was added and the samples counted in a liquid scintillation counter.

Drugs and isotopes

[^{3}H] *myo*-inositol was purchased from Amersham International plc and ECOSCINT scintillant fluid from National Diagnostics. All other materials were obtained from the Sigma Chemical Company.

Analysis of results

Basal [^{3}H]-inositol phosphates were calculated as counts per minute (c.p.m.) per mg of tissue and stimulated [^{3}H]-inositol phosphates as a percentage of unstimulated values. Due to the small size of the vessels used, each sample belongs to a different animal. Therefore group sizes varied and are given in the tables and figures which describe the results. In all the studies n is the number of samples, with every sample being the mean of two duplicates except for the body and tissue sample weight where n is the number of animals used. Comparisons between hypertensive and normotensive rats were carried out by use of ANOVA and Student's t test with a Bonferroni correction for multiple comparisons. Two-sample and unpaired t tests were used to analyse the differences between normotensive and hypertensive basal inositol phosphates, body and tissue weights, respectively. All values are expressed as mean ± s.e.mean.

Results

The mean body weight and the mean tissue sample weight for normotensive and hypertensive rats are shown in Table 1. Body weight was significantly greater in SRH compared to age-matched WKY rats. However, the mean sample weight for each vessel was not significantly different between strains.

Basal and noradrenaline (10^{-5} M)-stimulated levels of [^{3}H]-inositol phosphates, expressed as c.p.m.mg^{-1}, in the four vessels are shown in Table 2. In the femoral artery the unstimulated basal value was significantly higher ($P < 0.05$) in SHR compared to WKY rats but no difference was observed between basal values in the other vessels studied.

Noradrenaline (10^{-5} M) induced a significant increase in [^{3}H]-inositol phosphate accumulation in all four vessels of SHR and WKY (Table 2). The type of α-adrenoceptor involved in this activation is addressed in Figures 1 and 2. As shown, pretreatment of the tissues with the selective α_1-adrenoceptor antagonist prazosin (10^{-7}–10^{-6} M) dramatically inhibited the inositol phosphate accumulation induced by noradrenaline (10^{-5} M), whereas pretreatment with the selective α_2-adrenoceptor antagonist, yohimbine (10^{-7} M), resulted in no significant inhibitory effect. The selectivity of this blockade indicates an α_1-adrenoceptor-mediated response in both strains of rats.

Figure 3 illustrates the concentration-response curve to noradrenaline for phosphoinositide metabolism in the femoral artery and in the femoral vein from SHR and WKY rats. Concentrations of 10^{-7}–10^{-5} M noradrenaline elicited significantly smaller responses (expressed as % of unstimulated basal values) in the femoral artery of SHR compared to femoral arteries of WKY rats. No significant differences were observed with higher doses of agonist (10^{-4} and 10^{-3} M). When the results obtained in the femoral artery were expressed as absol-

Table 1 Mean body weight (g) plus mean of tissue sample weights (2 vessels/sample)

	n	*Body weight*	*FA*	*FV*	*IA*	*IV*
WKY	108	278 ± 28	5.24 ± 1.2	5.07 ± 0.9	8.15 ± 0.12	6.14 ± 0.12
SHR	113	298 ± 25*	5.07 ± 1.0	4.84 ± 0.9	7.90 ± 0.09	5.73 ± 0.13

n = number of animals; FA = femoral artery; FV = femoral vein; IA = iliac artery; IV = iliac vein. * $P < 0.05$ by unpaired Student's t test.

Table 2 Basal and noradrenaline (10^{-5} M)-stimulated [^{3}H]-inositol phosphate formation, expressed as c.p.m.mg^{-1}, in 14 week-old SHR and WKY rats

	WKY				*SHR*			
	n	*Basal*	n	*NA*	n	*Basal*	n	*NA*
Femoral artery	20	169 ± 17	11	679 ± 87.1***	19	229 ± 22†	10	684 ± 91.8***
Femoral vein	19	249 ± 30.3	9	357 ± 36.5**	18	285 ± 35.6	11	393 ± 46.4**
Iliac artery	21	117 ± 17	10	166 ± 21.3*	20	114 ± 14.5	10	192 ± 22.8**
Iliac vein	21	190 ± 20.6	10	278 ± 26.8**	20	182 ± 33	11	278 ± 41.4**

*** $P < 0.001$; ** $P < 0.02$; * $P < 0.05$ within strains; † $P < 0.05$ between strains by two-sample Student's t test; n = number of samples.

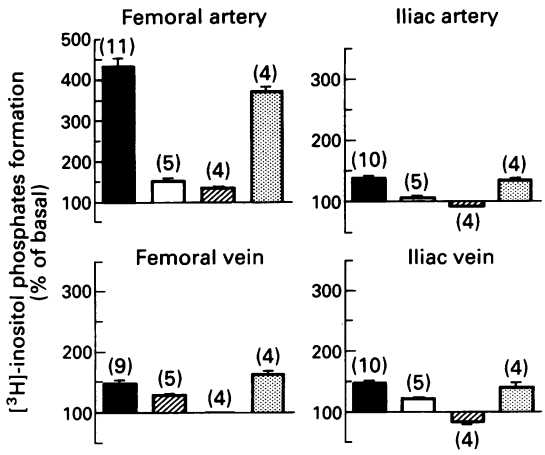

Figure 1 Effect of α-adrenoceptor blockade on noradrenaline (10^{-5} M)-induced accumulation of [^{3}H]-inositol phosphates in several blood vessels of 14 week-old WKY rats. The solid columns represent control, the open columns in the presence of prazosin (10^{-7} M), the hatched columns in the presence of prazosin (10^{-6} M) and the stippled columns in the presence of yohimbine (10^{-7} M). Results are expressed as mean ± s.e.mean. Values for each treatment are expressed as a percentage of the unstimulated value. The number of samples is indicated in parentheses.

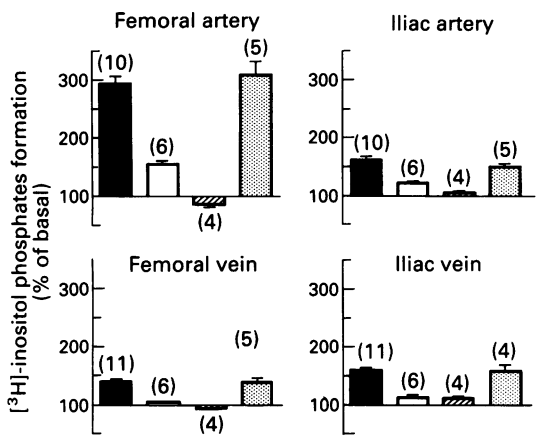

Figure 2 Effect of α-adrenoceptor blockade on noradrenaline (10^{-5} M)-induced accumulation of [^{3}H]-inositol phosphates in several blood vessels of 14-week-old SHR. The solid columns represent control, the open columns in the presence of prazosin (10^{-7} M), the hatched columns in the presence of prazosin (10^{-6} M) and the stippled columns in the presence of yohimbine (10^{-7} M). Results are expressed as mean ± s.e.mean. Values for each treatment are expressed as a percentage of the unstimulated value. The number of samples is indicated in parentheses.

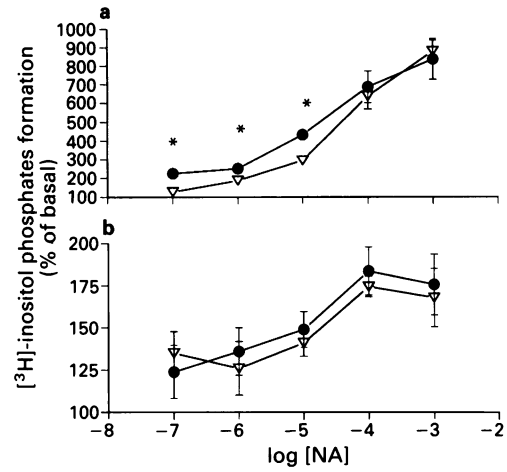

Figure 3 Concentration-response curve for noradrenaline-induced accumulation of [^{3}H]-inositol phosphate formation in the femoral artery (a) and femoral vein (b) of 14 week-old SHR (●) and WKY (▽) rats. Results are expressed as mean with s.e.mean shown by vertical bars. * $P < 0.05$ by ANOVA with a Bonferroni correction.

ute values (Table 3) no difference was observed except with the lowest dose of NA (10^{-7} M) where the value obtained in the WKY rats was greater than in age-matched SHR. In the femoral vein (Figure 3b) as well as in the iliac artery and vein (results not shown) neither the concentration-response curve to noradrenaline nor the absolute values showed difference between SHR and age-matched WKY rats.

Table 4 shows the unstimulated levels of [^{3}H]-phosphoinositides in the four vessels. In the femoral artery, but not in the other three vessels from SHR, [^{3}H]-phosphoinositide values were greater ($P < 0.05$) than in age-matched WKY.

Discussion

Extensive studies have been made of phosphatidylinositol turnover in many tissues including the rat aorta but few detailed studies in small blood vessels have been described. The present study demonstrates that noradrenaline increases phosphoinositide hydrolysis in femoral and iliac arteries and veins of 14 week-old SHR and WKY rats via α_1-adrenoceptors. The population of α-adrenoceptors mediating contraction in the rat femoral artery has been well characterized as exclusively of the α_1 subtype on the basis of the affinity for agonists and antagonists, but in femoral veins both α_1- and α_2-adrenoceptors subtypes seem to be involved

Table 3 Noradrenaline-induced [^{3}H]-inositol phosphate accumulation, expressed as c.p.m.mg^{-1} of tissue, in the femoral artery of 14 week SHR and WKY rats

	Noradrenaline (M)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
SHR	281 ± 18.6* (6)	391 ± 7.1 (6)	684 ± 91.8 (10)	1458 ± 157 (6)	2008 ± 160 (6)
WKY	380 ± 31.9 (6)	433 ± 34.7 (6)	679 ± 36.5 (11)	1160 ± 143 (6)	1409 ± 322 (6)

Number of experiments in parentheses. * $P < 0.05$ between strains by two-sample Student's t test.

Table 4 Basal [^{3}H]-phosphoinositides, expressed as c.p.m.mg^{-1}, in 14 week-old SHR and WKY rats

	WKY	n	*SHR*	n
Femoral artery	1123.8 ± 137.3	9	1987 ± 293*	9
Femoral vein	761 ± 42	4	563 ± 89.5	4
Iliac artery	455.6 ± 69.9	4	590 ± 69.2	4
Iliac vein	351.9 ± 33	4	268 ± 46	4

n = number of experiments. * $P < 0.05$ between strains.

(Edvinsson *et al.*, 1989). It has also been demonstrated, in the femoral veins of Wistar rats, that phosphoinositide turnover is linked to activation of α_1- but not α_2-adrenoceptors (Stubbs *et al.*, 1988). These results are in good agreement with those obtained in this study which confirms that contraction induced by α_1-adrenoceptor stimulation in the femoral artery and vein is associated with phosphoinositide turnover. In the vas deferens and caudal artery (Fox *et al.*, 1985) noradrenaline appears to be more potent in activating [^{3}H]-*myo*-inositol metabolism than in activating contraction possibly due to a greater receptor reserve for contraction than for inositol phosphate formation. The femoral artery of Sprague-Dawley rats (Edvinsson *et al.*, 1989) contracts in response to noradrenaline (10^{-7}–10^{-3} M) with the maximum contraction obtained between 10^{-4} M and 10^{-3} M. These results correlate well with the results of this study. The concentration-response curve for formation of [^{3}H]-inositol phosphates to noradrenaline (10^{-7}–10^{-3} M) shows a maximum response between 10^{-4} and 10^{-3} M. However, Edvinsson *et al.* (1989) found that the femoral vein had a greater affinity for noradrenaline than the femoral artery. Therefore when compared to the concentration-response curve for inositol phosphate accumulation, noradrenaline seems to be less potent for activation of [^{3}H]-*myo*-inositol metabolism than contraction in the rat femoral vein. Nevertheless when the α_2-adrenoceptor antagonist rauwolscine (10^{-6}–10^{-5} M) was present, the contractile concentration-response curve was clearly shifted to the right and the maximum obtained was again at 10^{-3} M (Edvinsson *et al.*, 1989). Taking account that [^{3}H]-inositol phosphate accumulation induced by noradrenaline in the femoral vein is exclusively mediated via α_1-adrenoceptor stimulation, the results obtained in the femoral vein agree with those obtained in the contractile studies when rauwolscine was present. To date no one has reported, either the subtype of α-adrenoceptors or the cellular response involved in the contractile response of iliac artery and vein in response to noradrenaline. A study carried out using the iliac artery of Wistar rats demonstrated that prazosin but not yohimbine produced a shift to the right of the contractile concentration-response curve of noradrenaline (Vila, Daly & McGrath, unpublished results) suggesting that the population of postjunctional α-adrenoceptors in this vessel belong to the α_1-adrenoceptor subtype. These results agree with those obtained in the present study and demonstrate that the contraction induced by α_1-adrenoceptors in the iliac artery is associated with phosphoinositide turnover. In addition the fact that noradrenaline induced an increase of [^{3}H]-inositol phosphates in the iliac vein, which was blocked by prazosin, suggests that this vessel contains a population of α_1-adrenoceptors linked to phosphoinositide metabolism.

In spite of the great amount of information available on basal and noradrenaline-induced inositol phosphate levels in the aorta of SHR and WKY rats only a few reports on phosphoinositide turnover in small blood vessels of hypertensive and normotensive rats are available. Basal values of inositol phosphates in the femoral artery, but not in the other three vessels studied, were significantly higher in the SHR than in age-matched WKY rats. These results partly agree with the enhanced basal activity of the phosphatidylinositol cycle demonstrated in the heart but not in the femoral artery (Eid & De Champlain, 1988) or the mesenteric artery (Takata *et al.*, 1989) of DOCA-salt rats compared to controls. In the aorta of 5 week-old SHR the basal activity was higher than in age-matched WKY rats but this difference was no longer apparent when the hypertension was established (Heagerty *et al.*, 1986). Our results concerning the basal values of inositol phosphates could be explained by the fact that unstimulated [^{3}H]-phosphatidylinositol levels in the femoral artery but not in the other three vessels were higher in the SHR than in control animals. However we cannot exclude the possibility of a greater phosphoinositide turnover in the femoral artery of 14 week-old SHR. Since the studies in all the blood vessels were always carried out in parallel, we do not have any explanation for the higher basal [^{3}H]-*myo*-inositol incorporation into lipid membrane of the SHR femoral but not the iliac artery or the veins other than different blood vessels behave in a different way. Our results are partly in accord with those of Durkin *et al.* (1989) who, using a highly sensitive method to measure the tissue levels of phospholipids in rat resistency arteries, showed that radiolabelled phospholipids but not inositol phosphates were greater in the resting state of 12 week-old SHR rats. In contrast Ek *et al.* (1989) did not see any difference between adult SHR and WKY either in the incorporation of ^{32}P into aortic inositol lipid or [^{3}H]-*myo*-inositol into inositol phosphates.

The stimulation of inositol phosphate accumulation induced by noradrenaline was similar in both strains of rats except in the femoral artery where the results obtained varied depending on the mode of analysis used. When the agonist-induced inositol phosphate accumulation is expressed relative to the basal value the results obtained seem to suggest a decrease in noradrenaline-induced [^{3}H]-inositol phosphate accumulation in adult SHR compared to age-matched WKY. Alternatively, if absolute values for noradrenaline-stimulated tissue are compared (Table 3) a significant difference is only apparent at the lowest concentration. Ek *et al.* (1989) failed to show an increase of [^{3}H]-inositol phosphates by noradrenaline in SHR whereas labelling was increased 219% in the aorta of WKY. Furthermore, a decrease of inositol phosphate accumulation induced by noradrenaline was reported in the aorta of 19 week-old but not in 5 week-old SHR (Heagerty *et al.*, 1986). In addition, isometric contractions of aortic rings as well as incorporation of ^{32}P-phosphate into phosphatidic acid induced by noradrenaline was greater in WKY than in age-matched SHR (Ek *et al.*, 1989; Aidulis *et al.*, 1990). In another study carried out in neuronal cells in primary culture from the hypothalamic brainstem areas of 1 day-old normotensive and hypertensive rat brain it has been observed that noradrenaline-induced phosphoinositide turnover was significantly lower in SHR neuronal cultures than in WKY cultures pointing to a difference genetically determined not secondary to hypertension (Crews *et al.*, 1988). In contrast Eid & De Champlain (1988) have reported that there is an increase of inositol phosphate accumulation in the femoral arteries of DOCA-salt hypertensive rats following noradrenaline-stimulation. Similarly, the results in precapillary resistance arteries from 5 and 12 week-old SHR and WKY rats reveal that the level of 1,4,5-inositol trisphosphate was increased in SHR (Durkin *et al.*, 1990).

In summary, our results demonstrate that in the femoral and iliac arteries and veins of SHR and WKY phosphoinositide hydrolysis is linked to α_1-adrenoceptors. In addition, enhancement in the basal levels of inositol phosphates as well as in [^{3}H]-phosphoinositides was observed in the femoral artery of SHR, indicating a greater incorporation of [^{3}H]-*myo*-inositol into the membrane lipid in this femoral vessel. The significant difference in basal levels of inositol phosphates between strains complicate the analysis and interpretation of agonist-stimulated results. However, irrespective of the way results are calculated, there is evidence of a reduced response in the femoral artery of SHR suggesting a reduced α_1-adrenoceptor-mediated phosphoinositide response in some but not all blood vessels of adult SHR.

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Characterization of the biological effects of 2-methylthio-ATP on rat hepatocytes: clear-cut differences with ATP

[1]Stefaan Keppens & Henri De Wulf

Afdeling Biochemie, Campus Gasthuisberg, Fakulteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

1 In several tissues, 2-methylthio adenosine triphosphate (2MeSATP) is a very potent P_{2Y}-purine agonist. In rat hepatocytes, 2MeSATP half-maximally activated glycogen phosphorylase at 20 nM and was therefore about 25 times more effective than ATP (K_a 0.5–0.8 μM). This strong glycogenolytic potency of 2MeSATP suggests on its own the presence of P_{2Y}-purinoceptors in liver.

2 Displacement of the radioligand ATPα[^{35}S] from its receptor however occurred at much higher concentrations of 2MeSATP than was anticipated on the basis of its glycogenolytic potency.

3 The interaction of 2MeSATP with the receptor, characterized with ATPα[^{35}S] as radioligand, cannot be considered as a pure competitive interaction.

4 2MeSATP did not share the ability of ATP to counteract the effect of glucagon on the adenosine 3':5'-cyclic monophosphate levels.

5 2MeSATP barely increased the levels of inositol trisphosphate (IP_3).

6 The glycogenolytic effect of 2MeSATP was completely abolished by pretreatment of the hepatocytes with phorbol myristic acetate.

7 It is tentatively concluded that 2MeSATP and ATP are interacting with different P_2 purinoceptors.

Keywords: Purinoceptors; rat liver; glycogenolysis; glycogen phosphorylase; 2-methylthio ATP

Introduction

Extracellular adenosine 5'-triphosphate (ATP) induces liver glycogenolysis in an adenosine 3':5'-cyclic monophosphate (cyclic AMP) independent, Ca^{2+}-mediated way (Keppens & De Wulf, 1985; Charest *et al.*, 1985; Sistare *et al.*, 1985; Buxton *et al.*, 1986; Okajima *et al.*, 1987). ATP interacts with specific receptors at the plasma membrane (Keppens & De Wulf, 1986), linked in a pertussis-toxin insensitive way (Okajima *et al.*, 1987) to a membrane bound phospholipase-C, generating inositol trisphosphate (IP_3) and diacylglycerol (Charest *et al.*, 1985). According to the classification proposed by Burnstock (Burnstock & Kennedy, 1985) the receptors mediating the glycogenolytic effect are classified as P_{2Y}-purinoceptors (Keppens & De Wulf, 1986). Apart from the glycogenolytic effect, ATP also shares with the other calcium-dependent glycogenolytic agonists the ability to counteract cyclic AMP increases induced either by glucagon (Keppens & De Wulf, 1985) or by forskolin (Okajima *et al.*, 1987). This cyclic AMP lowering effect is due to a pertussis-toxin sensitive inhibition of adenylate cyclase (Okajima *et al.*, 1987).

The ATP analogue, 2-methylthio ATP (2MeSATP) has been shown to be a very potent ($\geqslant$ATP) P_{2Y}-purine agonist in guinea-pig taenia coli, rabbit portal vein and endothelium of several species (see Gordon, 1986, for references), turkey erythrocytes (Boyer *et al.*, 1989) and bovine aortic endothelial cells (Allsup & Boarder, 1990).

This study describes some of the effects of 2MeSATP in rat liver parenchymal cells and compares them with the effects of ATP.

Methods

We have used adult male Wistar-strain albino rats (200–250 g body wt.) that were fed *ad libitum* with a standard laboratory chow. ATPαS (adenosine 5'-[α-thio]triphosphate), [^{3}H]-inositol and ATPα[^{35}S] (adenosine 5'-[α-[^{35}S]thio]triphosphate) were purchased from Amersham International, Amersham, Bucks.; ATP, phorbol 12-myristic 13-acetate (phorbol) and collagenase type 1 were from Sigma Chemical Co., St. Louis, MO, U.S.A.; 2-methylthio-ATP was from Research Biochemicals Incorporated, Natick, MA, U.S.A., Dowex 1 × 4 was from Bio-Rad Laboratories GmbH, Munchen, Germany. The sources of other chemicals have been given previously (Keppens *et al.*, 1989). Inositol (1,4,5)trisphosphate ([4,5-^{32}P(N)]) was from NEN Research Products, Dupont, Brussels, Belgium.

Isolation and incubation of the hepatocytes was carried out as previously described (Vandenheede *et al.*, 1976). Briefly, the liver was removed from the animal, cleared of blood and then perfused for about 10 min with a Krebs-Henseleit bicarbonate buffer without calcium. Subsequently, collagenase (30 mg 100 ml^{-1} of perfusion buffer) and calcium (2.5 mM) were added and the perfusion was continued for another 25–30 min. The hepatocytes were harvested and incubated at 37°C in a Krebs-Henseleit buffer containing 10 mM glucose in closed plastic vials saturated with 95% O_2, 5% CO_2 (v/v). Rat liver plasma membranes were purified as described by Cantau *et al.* (1980). Binding studies were performed as described by Keppens & De Wulf (1986). Cyclic AMP was determined with a competitive protein-binding technique (Gilman, 1970) by use of an assay kit from The Radiochemical Centre (Amersham, Bucks.). Loading of the cells with [^{3}H]-inositol and determination of inositolbisphosphate (IP_2) and inositoltrisphosphate (IP_3) were carried out according to the classical methods, recently reviewed by Blackmore & Exton (1989).

In brief, liver cells were preincubated for at least 1 h with 0.1 mM [^{3}H]-inositol (15 mCi mmol^{-1}); they were then washed twice in normal Krebs-Henseleit buffer, supplemented with 1 mM cold inositol. Samples of 1 ml of the hepatocyte suspension (5 × 10^6 cells ml^{-1}) were then incubated in 20 ml plastic vials, gassed with O_2/CO_2 (95%/5%, v/v) and constantly shaken (80 cycles min^{-1}). After 10 min, 10 mM LiCl was added and the incubation was continued for another 5 min. The cells were then treated with the different agonists and at the indicated time, the incubation was stopped by 14% TCA and 5 mM EDTA. After ether extraction of TCA, 12.5 mM tetraborate was added and 0.5 ml of the sample was put on a column containing 1 ml of a Dowex 1 × 4 resin. Inositol was washed from the column by 16 ml of 60 mM ammoniumformate/5 mM disodiumtetraborate; inositolphosphate by 8 ml of 200 mM ammoniumformate/100 mM formic

[1] Author for correspondence.

acid; inositolbisphosphate (IP_2) by 8 ml of 400 mM ammoniumformate/100 mM formic acid and inositoltrisphosphate (IP_3) by 8 ml of 1 M ammoniumformate/100 mM formic acid (checked with a standard of ^{32}P labelled IP_3). For a detailed description of this method see Blackmore & Exton (1989). Radioactivity in the eluates was determined by scintillation counting.

Results

Figure 1 shows the concentration-dependency for the activation of glycogen phosphorylase by 2MeSATP. Half-maximal effect (K_a) was obtained at 20 nM, a value below the K_a value of about 0.5–0.8 μM that we obtain systematically for ATP. In order to validate further 2MeSATP as a P_{2Y}-purine agonist, we next studied the binding affinity for the receptor previously characterized with ATPα[^{35}S] as radioligand (Keppens & De Wulf, 1986). Figure 2 shows the displacement of ATPα[^{35}S] from its receptor by different concentrations of 2MeSATP or ATP. The concentration of 2MeSATP needed to displace ATPα[^{35}S] from its receptor was nearly 1000 fold higher than needed to activate phosphorylase (Figure 1). Furthermore, as illustrated in the inset of Figure 2, the curvilinear displacement curve obtained with 2MeSATP does not fit with a simple competitive type of interaction precluding an exact determination of the K_D, in clear contrast to the situation obtained with ATP (straight line with a slope = 1 and a $K_D = 2\,\mu$M). These unexpected binding characteristics of 2MeSATP prompted us to explore further some of the effects of 2MeSATP and to compare them with those of ATP. We therefore checked whether 2MeSATP, like ATP, would be able to counteract cyclic AMP increases induced by glucagon. From Figure 3 it is obvious that 2MeSATP was not able to reduce the cyclic AMP levels, even at concentrations (up to 0.1 mM) which were much higher than those needed to activate phosphorylase ($\approx$20 nM, Figure 1) and even after longer incubation periods of up to 5 min (not shown). It was checked that 2MeSATP on its own did not increase cyclic AMP levels (not shown).

We next studied the effect of 2MeSATP on the levels of IP_2 and IP_3. Figure 4 compares the levels of IP_2 and IP_3 obtained 30 s after ATP or 2MeSATP treatment. It is clear that the 2-methylthio derivative was not a very potent phosphatidylinositolbisphosphate (PIP_2) degrading agonist (Figure 4). The low levels of IP_2 and IP_3 obtained after 2MeSATP treatment in combination with the full activation of phosphorylase (Figure 1) is a situation which is analogous to the α_1-adrenoceptor effect on rat liver, poor PIP_2 degradation (Exton, 1985) being associated with full activation of phosphorylase. Since phorbol myristic acetate is known to block the effect of α_1-adrenoceptor agonists (Exton, 1985), we studied the effect of phorbol treatment of the hepatocytes on the glycogenolytic effect of 2MeSATP. Figure 5 shows the complete suppression of phosphorylase activation by 2MeSATP after a 5 min pretreatment of the hepatocytes with the phorbol ester whereas only a minor inhibitory effect on the glycogenolytic capacity of ATP was observed.

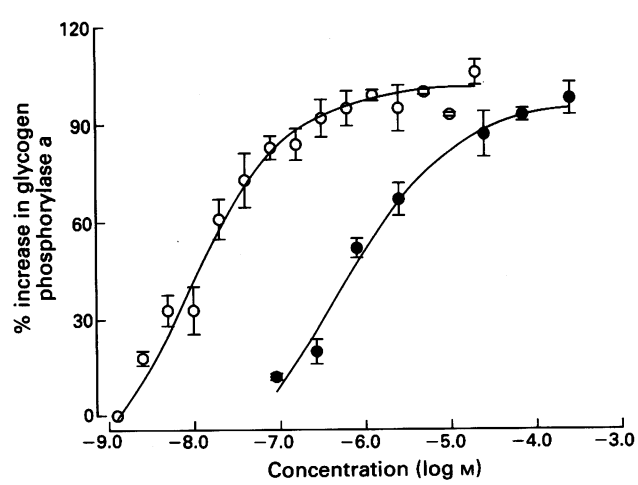

Figure 1 Concentration-response curve of phosphorylase activation by 2MeSATP and ATP in rat hepatocytes. Rat hepatocytes were preincubated for 20–30 min with 10 mM glucose and then treated for 20 s with increasing concentrations of 2MeSATP (○) or ATP (●). Phosphorylase activity was then assayed and is expressed as percentage increase above control. Values shown are means of 6 to 8 independent experiments; s.e.mean shown by vertical bars. Phosphorylase activities ranged from basal values of 20–30 to maximal values of 100–110 mu mg^{-1} of protein.

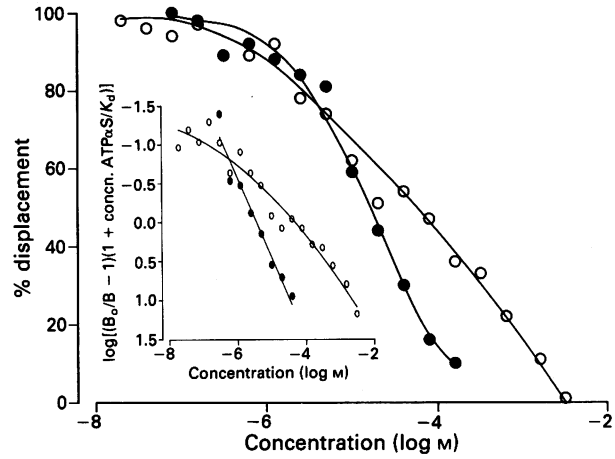

Figure 2 Concentration-dependent displacement of ATPα[^{35}S] from its receptor by 2MeSATP and by ATP. Liver plasma membranes were treated with 50 nM ATPα[^{35}S] for 1 min in the presence of increasing concentrations of 2MeSATP (○) or ATP (●). Values are expressed as a percentage of specific binding observed in the absence of competitor. The inset shows the plot of log[(B_0/B − 1)(1 + concn. ATPαS/K_D)] versus the logarithm of the concentration of 2MeSATP (○) or of ATP (●). B_0 and B are respectively the amount of bound ATPα[^{35}S] in the absence or presence of 2MeSATP (or ATP), concn. ATPαS is the concentration (50 nM) of ATPα[^{35}S] used and K_D (0.1 μM) is the dissociation constant for ATPα[^{35}S]. The K_D value for ATP is 2 μM. The data shown are typical of 9 other experiments.

Discussion

2MeSATP is, to our knowledge, the most potent ATP analogue able to activate glycogen phosphorylase in rat

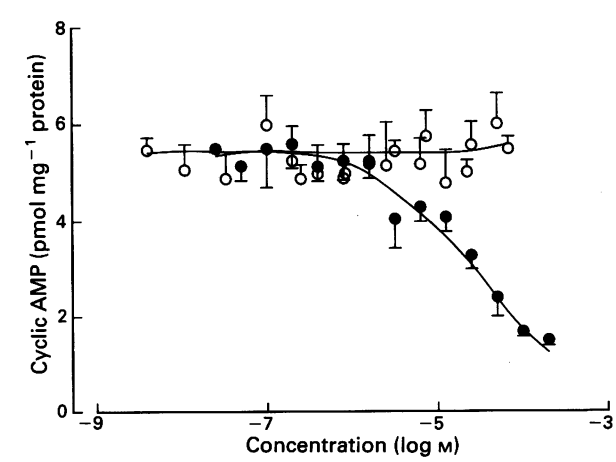

Figure 3 Effect of ATP and 2MeSATP on the cyclic AMP levels after glucagon. Hepatocytes, treated for 1 min with 20 nM glucagon, were challenged with increasing concentrations of ATP (●) or 2MeSATP (○); 1 min thereafter cyclic AMP was assayed. Data shown are means of 7 independent experiments; vertical bars show s.e.mean.

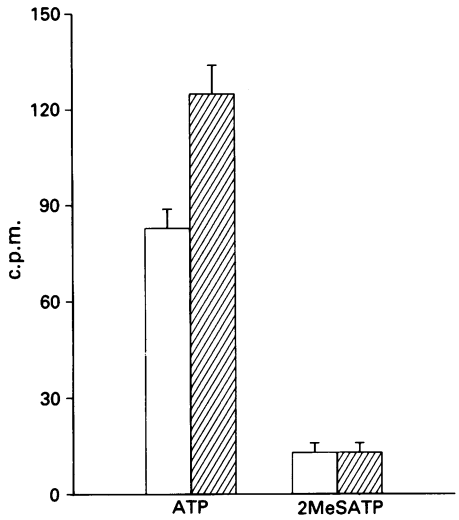

Figure 4 Effect of ATP and 2MeSATP on the levels of inositolbisphosphate (IP_2) and inositoltrisphosphate (IP_3) in rat hepatocytes. Rat hepatocytes were treated for 30 s with 20 μM ATP or 2MeSATP. Results are expressed as percentage increases above control and are given as means for (11) experiments. Open columns: IP_3, hatched columns: IP_2; vertical bars show s.e.mean.

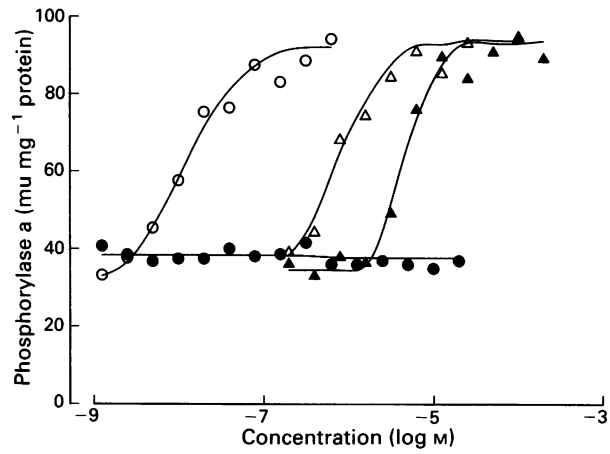

Figure 5 Effect of phorbol treatment of hepatocytes on the glycogen phosphorylase activation by 2MeSATP or ATP. Hepatocytes, treated for 5 min with either 1% dimethylsulphoxide (DMSO, open symbols) or 1.6 μM phorbol in 1% DMSO (closed symbols), were challenged with increasing concentrations of ATP (triangles) or 2MeSATP (circles). Results shown are representative of 3 independent experiments.

hepatocytes. Activation was obtained in a cyclic AMP-independent way suggesting that it is mediated via P_2-purinoceptors. Since liver contains specific P_{2Y}-purinoceptors, mediating the glycogenolytic effect of extracellular ATP (Keppens & De Wulf, 1986) and since in other tissues the high potency of 2MeSATP ($\gg$ ATP) is taken as a characteristic of the P_{2Y}-purinoceptors (Gordon, 1986), it could be concluded that in rat liver also the effect of 2MeSATP was due to interaction with this type of receptor. From our previous studies (Keppens & De Wulf, 1986) we know that for a number of ATP analogues, there is a good correlation between their glycogenolytic effect and their affinity for the receptor and that both effects (binding to the receptor and activation of phosphorylase) are obtained in the same concentration-range. For 2MeSATP this was not the case. Indeed, displacement of the radioligand ATPα[^{35}S] from the receptor was only obtained at nearly a 1000 fold higher concentration of 2MeSATP (Figure 2) than needed to activate phosphorylase (Figure 1) and was not the result of a simple competition of 2MeSATP with ATPα[^{35}S] for the receptor (inset of Figure 2). These data suggest that rat liver P_2-purinoceptors are not a homogeneous population of receptors. This could be in agreement with the data of Okajima *et al.* (1987) reporting the presence of two different P_2-purinoceptors, one mediating the glycogenolytic effect, the other mediating the inhibition of adenylate cyclase. Only the latter effect can be abolished by pertussis toxin treatment of the hepatocytes. In clear contrast to ATP, 2MeSATP did not interact with this latter type of receptors since it was not able to prevent the glucagon effect on cyclic AMP levels.

The glycogenolytic effect of ATP has been shown to be mediated by an increased degradation of PIP_2, giving rise to IP_3, which is responsible for the increased cytosolic calcium levels (Charest *et al.*, 1985). The very poor increase in the levels of IP_2 and IP_3 induced by 2MeSATP treatment of the hepatocytes was certainly unexpected but further illustrated the difference between ATP and 2MeSATP. The low level of IP_3 might however, as in the case of the α_1-adrenoceptor activation of phosphorylase (Exton, 1985), be sufficient to mediate the glycogenolytic response of 2MeSATP. The analogy with the α_1-adrenoceptor effect is further illustrated by the fact that phorbol treatment, reported to prevent the α_1-adrenoceptor initiated glycogenolysis in rat hepatocytes (Exton, 1985), also abolished the glycogenolytic effect of 2MeSATP. The differential effect of phorbol against 2MeSATP and against ATP is another clear indication that 2MeSATP does not belong to the group of purines that we earlier characterized as P_{2Y}-purine agonists (Keppens & De Wulf, 1985; 1986).

The present study clearly shows that the classification of the purinoceptors in different subclasses depends on the parameter used. Indeed, the activation of phosphorylase by 2MeSATP suggests interaction with P_{2Y}-purinoceptors; however, if one considers the IP_3 levels, instead, no such suggestion arises.

We tentatively conclude that, apart from purinoceptors of the P_{2Y}-subclass, liver possesses other P_2-purinoceptors.

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Ruthenium-red inhibits CGRP release by capsaicin and resiniferatoxin but not by ouabain, bradykinin or nicotine in guinea-pig heart: correlation with effects on cardiac contractility

[1]Anders Franco-Cereceda, Ya-Ping Lou & Jan M. Lundberg

Department of Pharmacology, Karolinska Institutet, Box 60 400, S-104 01 Stockholm, Sweden

1 The possible influence of ruthenium-red (RR) on contractility and outflow of calcitonin gene-related peptide (CGRP)-like and neuropeptide Y (NPY)-like immunoreactivity (LI) from the heart of the guinea-pig induced by capsaicin, resiniferatoxin, nicotine, ouabain or bradykinin was studied *in vitro*.

2 In the isolated right atrium, exposure to capsaicin evoked an increase in contractile rate and tension simultaneously with an enhanced outflow of CGRP-LI, indicating release from the atria. Repeated administration of capsaicin induced tachyphylaxis. Incubation with RR markedly attenuated the capsaicin-evoked release of CGRP-LI while no clear-cut effects were seen on contractile tension or rate.

3 In the isolated whole heart, perfusion with capsaicin induced an increased outflow of CGRP-LI and stimulated heart rate, while a negative inotropic effect was observed. A second administration of capsaicin to the same preparations failed to influence the CGRP-LI outflow and in these experiments the positive chronotropic effect was absent while the negative inotropic action remained unchanged. Capsaicin-perfusion in the presence of RR failed to induce any increased outflow of CGRP-LI from the hearts or changes in contractile activity. However, after 1 h of rinsing with Tyrode solution repeated capsaicin perfusion in the absence of RR caused a clear-cut (60% of control) release of CGRP-LI and contractile responses were restored.

4 Perfusion with resiniferatoxin evoked a RR-sensitive, clear-cut increased CGRP-LI output without any effects on contractile force or heart rate. Repeated administration of resiniferatoxin induced tachyphylaxis with respect to outflow. Capsaicin perfusion after resiniferatoxin did not influence cardiac rate, force or CGRP-LI outflow suggesting development of cross-tachyphylaxis.

5 Perfusion with RR did not influence the outflow of CGRP-LI or contractility changes evoked by perfusion with nicotine, ouabain or bradykinin. In addition, the release of NPY-LI by nicotine remained unchanged in the presence of RR. Furthermore, the positive chronotropic effect of human CGRP alpha remained intact in the presence of RR.

6 It is concluded that RR selectively inhibits capsaicin- and resiniferatoxin-induced excitation of cardiac sensory nerves as revealed by inhibition of both CGRP-LI release and the cardiostimulatory action of capsaicin. RR also seems to protect the cardiac capsaicin-sensitive fibres from the development of tachyphylaxis to capsaicin. Finally, RR prevents the capsaicin-evoked negative inotropic effect which is not related to activation of sensory nerves.

Keywords: Capsaicin; CGRP; guinea-pig heart; resiniferatoxin; ruthenium red; sensory nerves

Introduction

Calcitonin gene-related peptide (CGRP) (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983) is a neuropeptide with 37 amino-acid residues that is co-localized with substance P (SP) in C-fibre afferents in the heart of several species, including the guinea-pig, rat and man (Lundberg *et al.*, 1985; Franco-Cereceda *et al.*, 1987a). CGRP (Franco-Cereceda *et al.*, 1987b), and SP (Hoover, 1987) have been shown to be released by capsaicin from the guinea-pig isolated heart and release of CGRP seems to explain the cardio-stimulatory effects of capsaicin (Franco-Cereceda & Lundberg, 1985; see Franco-Cereceda, 1988). Thus, the heart responds to sensory nerve activation by increasing the outflow of CGRP-like immunoreactivity (LI) which allows studies on the influence of various drugs on sensory nerves and contractility. There are a variety of agents known by electrophysiological experiments to activate cardiac C-fibre afferents (see Bishop *et al.*, 1983) and several of these, including nicotine, bradykinin and ouabain, evoke a release of CGRP-LI from the isolated heart in combination with various changes in contractile activity (Franco-Cereceda *et al.*, 1987b).

It was recently reported that ruthenium-red (RR), an inhibitor of transmembrane calcium fluxes (Moore, 1971; Swansson *et al.*, 1974), attenuates the excitatory effects of capsaicin on sensory nerves (Maggi *et al.*, 1988; Franco-Cereceda *et al.*, 1989a). Since the release of CGRP-LI evoked by both capsaicin and nicotine in the guinea-pig heart is Ca^{2+}-dependent (Franco-Cereceda *et al.*, 1989b) we have in this study further investigated the possible influence of RR on the stimulatory effects of capsaicin, ouabain, bradykinin and nicotine on the outflow of CGRP-LI from the guinea-pig heart to reveal whether RR exerts a general depressant function on cardiac C-fibres or if the action is restricted to capsaicin-induced excitation. In addition, we have studied the possible effects of resiniferatoxin, a potent capsaicin-like irritant (Szallasi & Blumberg, 1989), on cardiac contractility and CGRP-LI outflow and have investigated whether its effects are modified by RR. Neuropeptide Y (NPY) which is also released from the heart upon nicotine-exposure (Franco-Cereceda *et al.*, 1987b) has been measured simultaneously as a marker for sympathetic nerve activation (Lundberg *et al.*, 1982).

Methods

Adult guinea-pigs (weighing approximately 300 g) were killed by a blow to the head. The aorta was quickly cannulated and 100 iu heparin in 5 ml of Tyrode solution (composition, mM:

[1] Author for correspondence.

NaCl 137, $NaHCO_3$ 11.9, KCl 2.7, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and glucose 5.6) was injected into the coronary circulation to rinse out remaining blood. The heart ($n = 60$) was then excised and perfused with a pressure of 6.7 kPa (70 cmH_2O) according to Langendorff (see Broadley, 1978). The Tyrode solution was warmed to 37°C and aerated with 95% O_2 and 5% CO_2 immediately before entering the coronary circulation. A diastolic tension of 10 mN was applied via a suture at the apex of the ventricles and mechanical activity was measured with Grass FT03C transducers and recorded on a Grass Polygraph, model 7B, connected to a tachygraph unit. The preparations were allowed to equilibrate for 30 min before the administration of drugs. Drugs were adminstered for 5 min by use of a roller-perfusion pump connected to the aortic cannula. From another group of animals the right atrium ($n = 15$) was excised, mounted in 2 ml organ baths and perfused with Tyrode solution at 2 ml min^{-1}. The atria were given a resting tension of 5 mN and the contractile activity was measured as for the whole heart preparations (see above).

Perfusate fractions (5 min) from both the atria and whole hearts were collected in a beaker placed on ice immediately below the preparations and acetic acid was added to give a final concentration of 0.2 M. The samples were thereafter desalted with SEP-Pak C_{18} cartridges, lyophilized and redissolved in 0.5 ml buffer for the determination of CGRP- and NPY-LI by radioimmunoassay (RIA). The content of CGRP-LI was determined with antiserum RAS 6009 (Peninsula, Ca, U.S.A.; see Franco-Cereceda *et al.*, 1989) and NPY-LI was analysed by use of antiserum N1 (Theodorsson-Norheim *et al.*, 1985).

In separate experiments, RR (2.5×10^{-6} M for the atria and 5×10^{-6} M for the whole hearts) was added to the perfusion solution 30 min before exposure to the different drugs. The concentrations were chosen based on preliminary experiments which had revealed that this was the highest concentration without any negative effects on cardiac contractility *per se* in the corresponding atrial and whole heart experiments.

Drugs

Capsaicin, ouabain-octahydrate, nicotine-bitartrate (Sigma, St. Louis, MO, U.S.A.); resiniferatoxin (Chemicals for Cancer Res. Inc., MN, U.S.A.); human CGRP alpha, bradykinin (Peninsula, Belmont, CA, U.S.A.); ruthenium red (Serva, Heidelberg, F.R.G.) were used. Capsaicin and resiniferatoxin (10 mg ml^{-1}) were dissolved in 60% ethanol and further diluted in Tyrode solution.

Statistical evaluation

Statistical analyses were performed by Student's t test. Values are expressed either as absolute values or as percentage of a control period immediately before administration of drugs. $P < 0.05$ was considered significant.

Results

After 30 min of equilibration, the basal CGRP- and NPY-LI outflow, contractile tension, heart rate and perfusate volume were 31 ± 3 fmol per fraction, 37 ± 9 fmol per fraction, 16.6 ± 0.5 mN, 206 ± 8 beats min^{-1} and 24 ± 4 ml 5 min^{-1}, respectively in the control group which were not significantly different compared to 27 ± 5 fmol per fraction, 34 ± 4 fmol per fraction, 18.4 ± 1.1 mN, 212 ± 10 beats min^{-1} and 22 ± 5 ml 5 min^{-1}, respectively in the group perfused with Tyrode solution containing RR.

In the atrial preparations the CGRP-LI outflow, frequency and tension after 30 min equilibration in the control group were 4 ± 1 fmol per fraction, 211 ± 30 beats min^{-1} and 9 ± 2 mN, respectively compared to 6 ± 1 fmol per fraction, 203 ± 19 beats min^{-1} and 10 ± 3 mN, respectively in the RR-perfused group.

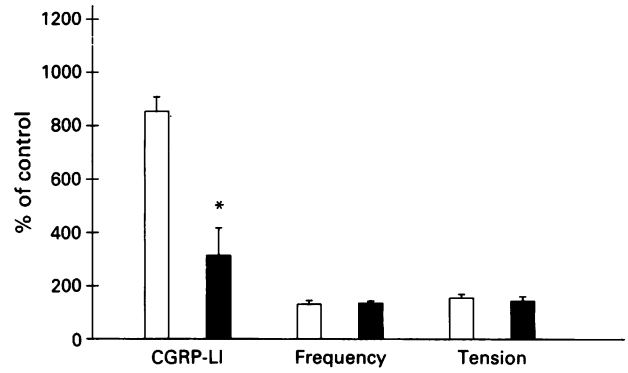

Figure 1 Effects of 5 min of exposure to capsaicin (10^{-6} M) on calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow, frequency and tension in the guinea-pig isolated spontaneously beating right atrium. Open columns represent control experiments and filled columns experiments after 30 min perfusion with Tyrode solution containing ruthenium-red (2.5×10^{-6} M). * $P < 0.05$, using Student's t test. Values are given as means (s.e.mean shown by vertical bars) and expressed as percentage of the control period immediately before capsaicin administration. ($n = 4$–9 animals in each group.)

Capsaicin-exposure caused a clear-cut positive inotropic and chronotropic effect in the isolated spontaneously beating right atrium. Thus, the rate increased to $131 \pm 4\%$ (to 274 ± 30 beats min^{-1}) and the peak tension to $155 \pm 17\%$ (15 ± 4 mN) of control. Simultaneously the outflow of CGRP-LI increased by $854 \pm 134\%$ (to 33 ± 7 fmol per fraction) (Figure 1). A second application of capsaicin after 15 min induced tachyphylaxis with regards to both the stimulatory effects of capsaicin as well as the release of CGRP-LI. Pre-incubation with RR (2.5×10^{-6} M) for 30 min significantly reduced (by 43%, $P < 0.05$), but did not completely abolish, the release of CGRP-LI induced by capsaicin. In these experiments, the stimulatory effects on atrial contractility by capsaicin remained unchanged (Figure 1).

In the whole heart preparations, capsaicin-perfusion caused a concentration-dependent increase in heart rate and CGRP-LI release in combination with a decrease in contractile tension (Figure 2). Thus, capsaicin increased the frequency to $110 \pm 3\%$, $117 \pm 3\%$, $130 \pm 6\%$, the CGRP-LI outflow by $260 \pm 49\%$, $1384 \pm 54\%$, $1852 \pm 58\%$, and decreased the

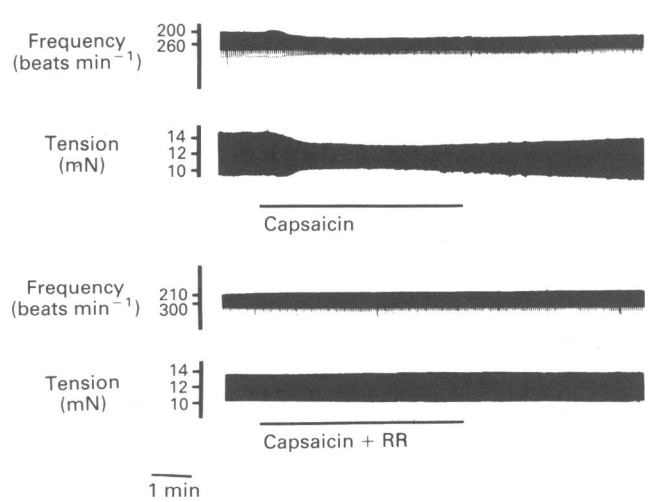

Figure 2 Original tracings showing the effects of 5 min (bar) of perfusion with capsaicin (10^{-6} M) on the guinea-pig isolated heart in a control experiment (top panel) and after pretreatment for 30 min with ruthenium red (RR; 5×10^{-6} M; bottom panel). Contractile parameters are given as frequency (beats min^{-1}) and tension (mN).

Table 1 Effects on the outflow of calcitonin gene-related peptide-like (CGRP-LI) and neuropeptide Y-like immunoreactivity (NPY-LI) and on frequency, tension and perfusate volume (expressed as % of control) of a 5-min exposure of the guinea-pig isolated whole heart to capsaicin, resiniferatoxin, nicotine, ouabain and bradykinin

	CGRP-LI	*NPY-LI*	*Frequency*	*Tension*	*Perf. volume*
Capsaicin					
(10^{-8} M)	240 ± 49*	—	110 ± 3	98 ± 3	104 ± 7
+RR	118 ± 11	—	95 ± 4	141 ± 15	93 ± 5
(10^{-7} M)	1383 ± 54***	—	117 ± 3*	87 ± 10	101 ± 7
2nd	159 ± 32	—	100 ± 4	90 ± 6	102 ± 2
+RR	215 ± 22	—	91 ± 11	97 ± 7	95 ± 12
(10^{-6} M)	1852 ± 58***	—	130 ± 6**	71 ± 6*	111 ± 6
2nd	97 ± 4	—	92 ± 2	78 ± 3	105 ± 4
+RR	399 ± 109	—	96 ± 3	100 ± 10	91 ± 14
Resiniferatoxin					
(10^{-8} M)	410 ± 31**	—	103 ± 5	103 ± 4	99 ± 4
2nd	94 ± 15	—	97 ± 5	101 ± 3	99 ± 6
+RR	104 ± 38	—	96 ± 6	108 ± 4	85 ± 11
Nicotine					
(10^{-4} M)	429 ± 62**	1076 ± 314**	137 ± 8***	257 ± 18**	108 ± 9
2nd	386 ± 28	—	136 ± 10	210 ± 24	105 ± 9
+RR	515 ± 24	1173 ± 190	130 ± 6	218 ± 17	98 ± 5
Ouabain					
(10^{-4} M)	370 ± 46*	—	139 ± 7*	182 ± 18*	93 ± 10
+RR	411 ± 70	—	130 ± 4	217 ± 31	99 ± 4
Bradykinin					
(5×10^{-6} M)	339 ± 40**	—	112 ± 4	99 ± 7	112 ± 7
2nd	346 ± 84	—	119 ± 7	104 ± 17	111 ± 6
+RR	295 ± 56	—	116 ± 6	112 ± 10	108 ± 4

The effects of a second administration (2nd) or preincubation with ruthenium-red (RR; 5×10^{-6} M) are also shown.
— indicates experiments not performed (NPY-LI). Values are given as means ± s.e.mean and represent 3–6 animals in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's t test for paired samples, comparing the values at the first administration with a control period immediately before administration of drugs.

tension to 98 ± 3%, 87 ± 10%, 71 ± 6% of control at 10^{-8} M, 10^{-7} M, and 10^{-6} M, respectively (Table 1). When repeating the capsaicin administration (10^{-7} M or 10^{-6} M) after 60 min of rinsing only the negative inotropic effect remained. In the presence of RR (5×10^{-6} M), the effects of capsaicin (10^{-8} M, 10^{-7} M or 10^{-6} M) on heart rate, contractile force and release of CGRP-LI were significantly reduced (Figures 3, 4, Table 1). However, when the capsaicin-perfusion (10^{-6} M) was repeated in experiments after 60 min of rinsing with RR-free Tyrode solution, the contractile changes induced by capsaicin were similar to the effects observed in the control group at the first capsaicin-administration (not shown) while the release of CGRP-LI was 60% of control (Figure 3).

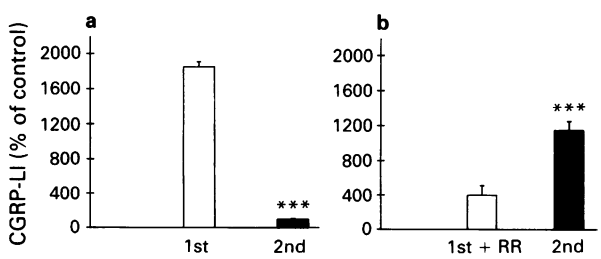

Figure 3 Effects of 5 min exposure to capsaicin (10^{-6} M) on the outflow of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from the isolated Langendorff-perfused guinea-pig heart. Panel (a) shows the effects of a repeated administration (2nd) of capsaicin 60 min after the first exposure (1st); (b) shows the effects of a second exposure to capsaicin in Tyrode solution 60 min after the first exposure to capsaicin was performed in the presence of ruthenium-red (RR, 5×10^{-6} M). *** $P < 0.001$, using Student's t test for paired samples. Values are given as means (vertical bars show s.e.mean) and are expressed as percentage of a control period immediately before capsaicin-perfusion. (n = 4–8 animals in each group.)

Perfusion with resiniferatoxin (10^{-8} M) evoked a clear-cut increase in CGRP-LI outflow but no changes in heart rate or contractile force were observed (Table 1, Figure 5). A second administration of resiniferatoxin failed to influence the basal CGRP-LI outflow. RR (5×10^{-6} M) completely abolished the stimulation of CGRP-LI outflow by resiniferatoxin. Capsaicin-exposure (10^{-6} M) after resiniferatoxin revealed the development of cross-tachyphylaxis with regards to heart rate (104 ± 6% of basal) and contractile force (99 ± 7% of basal) while a small increase in CGRP-LI outflow was still detected (171 ± 27% of basal compared to 1852 ± 54% of basal in control experiments).

Nicotine (10^{-4} M) perfusion for 5 min markedly stimulated both the contractile rate and force of the hearts i.e. the frequency increased to 137 ± 8% and the tension to 257 ± 8% of control. Simultaneously there was a large release of CGRP-LI (by 429 ± 62%; Figure 5) and also of NPY-LI (by 1076 ± 314%) (Table 1). There was no apparent tachyphylaxis to the effects of nicotine when repeating the administration after 20 min of rinsing (Table 1). In these experiments RR failed to evoke any changes in either the contractile response or release of CGRP-LI (Figure 5) and NPY-LI induced by nicotine (Table 1).

Ouabain (10^{-4} M) exposure also increased the tension (to 182 ± 18%) and frequency (to 139 ± 7%) of the isolated perfused hearts (Table 1). However, this stimulation of cardiac contractility was usually followed by severe arrhythmias which, in spite of prolonged rinsing, made it impossible to repeat the ouabain administration to the same hearts. No change in the response to ouabain was observed as compared to the controls after perfusion with Tyrode solution containing RR (Table 1).

Bradykinin-perfusion (5×10^{-6} M) evoked a small increase in heart rate (to 112 ± 4% of control) without any influence on contractile force (99 ± 7%) (Table 1). The outflow of

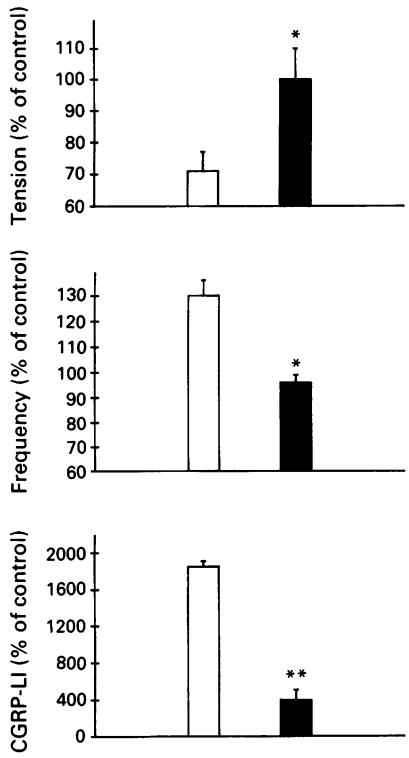

Figure 4 Effect on calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow, frequency and tension (expressed as means and given as percentage of a control period immediately before capsaicin-administration) by 5 min perfusion with capsaicin (10^{-6} M) in the Langendorff-perfused whole heart; s.e.mean shown by vertical bars. Open columns represent controls and filled columns represent experiments performed after 30 min pretreatment with ruthenium-red (5×10^{-6} M) in a separate group of animals. * $P < 0.05$; ** $P < 0.01$ using Student's t test. (n = 4–8 animals in each group.)

CGRP-LI was increased to 339 ± 40% of control by bradykinin. Repeated administration of bradykinin after 20 min of rinsing did not induce tachyphylaxis. In addition, RR was ineffective in modulating the response to bradykinin (Figure 5, Table 1).

Perfusion with capsaicin, resiniferatoxin, nicotine, ouabain or bradykinin did not induce any major changes in the perfusate volume.

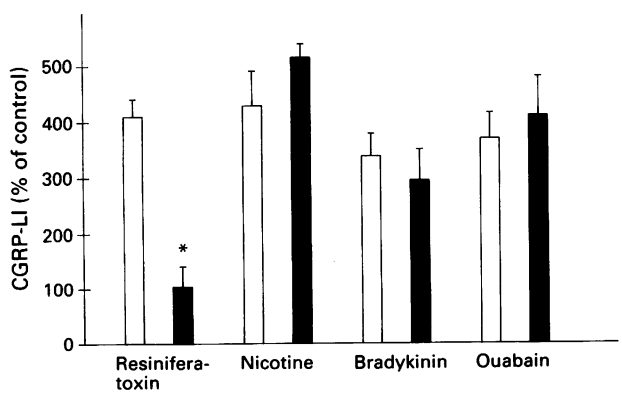

Figure 5 Effect on calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow from the isolated Langendorff-perfused heart by 5 min exposure to resiniferatoxin (10^{-8} M), nicotine (10^{-4} M), ouabain (10^{-4} M) or bradykinin (5×10^{-6} M). Open columns represent control experiments and filled columns experiments performed in hearts pretreated 30 min with Tyrode solution containing ruthenium-red (5×10^{-6} M). Values are given as means (s.e.mean shown by vertical bars) and expressed as percentage of a control period immediately before the administration of the drugs. * $P < 0.05$; Student's t test. (n = 3–5 animals in each group.)

Bolus administration of human CGRP alpha (1.3 nmol) to the Langendorff-perfused hearts increased the frequency to 158 ± 9% of control while no consistent effect on the contractile tension was observed. The stimulatory response to CGRP remained unchanged (165 ± 4%) after 30 min perfusion with Tyrode solution containing RR.

Discussion

This study demonstrates that RR is a selective antagonist of the excitatory effects of capsaicin and resiniferatoxin in the heart with respect to the release of the neuropeptide, CGRP. Furthermore, RR also protects the heart from the cardiostimulatory actions and negative inotropic effects of capsaicin as well as the development of capsaicin-desensitization.

In the present investigation, activation of cardiac sensory nerves was indirectly demonstrated as revealed by the release of CGRP-LI by capsaicin, resiniferatoxin, nicotine, ouabain and bradykinin (see Franco-Cereceda *et al.*, 1988) in agreement with electrophysiological studies using direct recordings of cardiac afferents (see Bishop *et al.*, 1983). Additionally, nicotine released NPY-LI suggesting activation of cardiac sympathetic nerve fibres (Franco-Cereceda, 1988).

Release of neurotransmitters is generally considered to require the presence of extracellular Ca^{2+} (Smith & Winkler 1972; Gamse *et al.*, 1981). Capsaicin evokes an influx of extracellular Ca^{2+} (Jancsó *et al.*, 1984; Zernig *et al.*, 1984) which probably accounts for its peptide-releasing properties and in the guinea-pig Langendorff perfused heart, the effects of both capsaicin and nicotine on peptide release are Ca^{2+}-dependent (Franco-Cereceda *et al.*, 1987b). However, the capsaicin-induced neuro-excitatory effects are not influenced by nifedipine (Rang *et al.*, 1987) or D-600 (Marsh *et al.*, 1987) and therefore do not seem to involve either L- or N-type voltage-sensitive Ca^{2+}-channels (Miller *et al.*, 1987). RR inhibits transmembrane Ca^{2+}-fluxes in neuronal tissue (Swansson *et al.*, 1974) and inhibits Ca^{2+}-dependent transmitter release (Rahaminoff & Alnaes, 1973; Tapia & Meza-Ruiz, 1977). The effect of RR on neuronal Ca^{2+}-channels is not known, but based on the present experiments it is likely that capsaicin, but not nicotine, ouabain or bradykinin, activates Ca^{2+}-mechanisms that are sensitive to RR.

Resiniferatoxin is a naturally-occurring diterpene with structural similarity to capsaicin and like capsaicin, it causes hypothermia, neurogenic inflammation and pain in the rat (see Szallasi & Blumberg, 1989). Electrophysiological recordings have shown cross-tachyphylaxis between resiniferatoxin and capsaicin but not to other activators of peripheral nerve fibres, e.g. bradykinin and heat (Dray *et al.*, 1990). Our experiments confirm these findings and furthermore illustrate the ability of resiniferatoxin to release CGRP-LI from the guinea-pig heart in a RR-sensitive manner, thus mimicking the effects of capsaicin. No cardioexcitatory effects of resiniferatoxin (10^{-8} M) were detected in spite of CGRP release but nor does capsaicin evoke any clear-cut functional effects at similar CGRP outflow.

The basal outflow of CGRP-LI was not influenced by perfusion with RR although it is significantly reduced in Ca^{2+}-free Tyrode-perfusion (Franco-Cereceda *et al.*, 1989b). The Ca^{2+}-dependency of the spontaneous CGRP-outflow therefore argues against a predominant action of RR in elevating the free cytosolic Ca^{2+}-concentration by inhibition of mitochondrial Ca^{2+}-uptake (Moore, 1971) which actually would promote transmitter release (Bernath & Vizi, 1987). Furthermore, the CGRP-LI release induced by nicotine, ouabain and bradykinin remained unchanged in the presence of RR and the action of RR is therefore unlikely to be explained by an increase in intracellular Ca^{2+}-concentration since this would inhibit the influx of extracellular Ca^{2+} and attenuate peptide release. This assumption is further supported by the finding that RR at the concentration used here, did not influence basal heart rate or contractile force, two parameters which are

extremely sensitive to changes in free intracellular Ca^{2+} concentrations (see Langer, 1975). The Ca^{2+} entry-blocking properties of RR could explain the protective effect of RR against capsaicin desensitization. Capsaicin exposure is associated with a marked accumulation of intracellular Ca^{2+} in cultured sensory neurones which can be blocked by low concentrations of RR (Wood *et al.*, 1988). Thus, this protective effect of RR may simply reflect the fact that after inhibition of peptide-release in the presence of RR, a second application of capsaicin in the absence of RR will again cause influx of extracellular Ca^{2+} and release CGRP since the peptide-vesicles are still accessible. It should be emphasized in this context, that capsaicin-tachyphylaxis in the guinea-pig isolated atrium can under some conditions be reversed by repeated rinsing (Franco-Cereceda & Lundberg, 1985) and does not necessarily imply an irreversible functional state of the tissue. The contractile effects of exogenous CGRP remained unchanged in the presence of RR indicating that RR did not interfere with CGRP-metabolism or post-junctional CGRP mechanisms. To what extent RR could interfere with possible capsaicin binding sites is not known.

RR inhibited both the positive chronotropic and the negative inotropic effect of capsaicin. The stimulation of heart rate by capsaicin is most probably mediated by a release of CGRP (Franco-Cereceda & Lundberg, 1985; Franco-Cereceda *et al.*, 1989b, see, Franco-Cereceda, 1988) while the inhibitory effect of capsaicin on ventricular contractility is probably of non-neuronal nature (see Franco-Cereceda, 1988). The reason why RR also reversed the negative inotropic effect of capsaicin is unclear but suggests some common mechanism for capsaicin-evoked release of mediators from sensory nerves and inhibition of myocyte contractility. This could possibly be explained by the fact that RR inhibits capsaicin-induced accumulation of intracellular Ca^{2+} which in turn would attenuate cardiac ventricular contractility (Zernig *et al.*, 1984). The partial, but not complete, reduction of CGRP-LI release in the atria probably explains why the capsaicin-induced stimulation of atrial rate and contractility were not significantly reduced by RR. Furthermore, after repeated capsaicin-exposure when RR was present during the first application, the contractile changes of the whole heart were similar to control, although the CGRP-LI release was reduced by 40%. A relatively moderate CGRP-release may thus be sufficient to induce a maximal functional response (see also Franco-Cereceda *et al.*, 1989).

There were no effects on the perfusate volume by the different drugs tested in the present study. This is likely to be due to the fact that the constant pressure Langendorff model used with a high basal flow does not seem to reflect easily decreases in coronary vascular tone as revealed by an increased perfusate volume (see Franco-Cereceda *et al.*, 1989b).

In conclusion, the present data reveal that RR exerts selective inhibitory effects on capsaicin- and resiniferatoxin-induced functional responses and peptide-release from cardiac C-fibres compared to nicotine, bradykinin and ouabain. RR may thus be a useful tool in studying the mechanisms for selective activation of cardiac capsaicin-sensitive sensory nerves under different experimental conditions.

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Endothelium-dependent mesenteric vasorelaxant effects and systemic actions of endothelin (16-21) and other endothelin-related peptides in the rat

Stephen A. Douglas & [1]C. Robin Hiley

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 The rat isolated superior mesenteric bed, perfused with Krebs-Henseleit solution containing 10 μM indomethacin and precontracted with 100 μM methoxamine, was used to study the vasorelaxation produced by some fragments of endothelin-1, by two alanyl-substituted analogues, and by human and porcine proendothelins. The systemic cardiovascular effects of some of these peptides were also studied in anaesthetized rats.

2 Endothelin (16-21) was an endothelium-dependent vasodilator about 10 times less potent than acetylcholine and its amide was about 500 times less potent than the free acid. Human proendothelin-1 and porcine proendothelin-1 also caused endothelium-dependent relaxations but neither [Ala^{3,11}]endothelin-1 nor [Ala^{1,3,11,15}]endothelin-1 produced significant reductions in the methoxamine-induced tone. Sodium nitroprusside was approximately 200 times less potent than acetylcholine in the presence of the endothelium and was the only vasorelaxant to be active after destruction of the endothelium by perfusion with 0.3% CHAPS; in the absence of the endothelium it was 3.7 times more potent as a vasodilator than in its presence.

3 Porcine proendothelin-1 had weak contractile activity in the isolated mesenteric bed but porcine endothelin (22-39), endothelin (16-21) and endothelin (16-21) amide did not have vasoconstrictor actions.

4 Endothelin-3, [Ala^{3,11}]endothelin-1, [Ala^{1,3,11,15}]endothelin-1 and endothelin (16-21) all had systemic blood pressure effects in the anaesthetized rat. Although endothelin (16-21) did not cause vasoconstriction *in vitro*, it increased mean arterial pressure *in vivo* but was at least 100 times less potent than endothelin-3. Despite causing no vasorelaxation *in vitro*, [Ala^{1,3,11,15}]endothelin-1 and [Ala^{3,11}]endothelin-1 induced short-lived systemic depressor responses which were followed by pressor responses. Endothelin-3 was more potent as a systemic depressor agent than as a pressor agonist and, as a vasodepressor agent, it was 3–4 times more potent than either of the alanyl-substituted analogues. Endothelin-3, [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 were equipotent vasopressor agents. Porcine endothelin (22-39) had no systemic blood pressure effects.

5 This study shows directly that the presence of both disulphide bonds is required in the endothelins for them to be able to cause endothelium-dependent relaxation in the mesenteric vascular bed and that this response is mediated by different receptors from those causing contraction of the vascular smooth muscle in the mesentery. Vasorelaxation caused by endothelin (16-21) and its amide suggests that there is a receptor on the endothelium similar to one reported in the guinea-pig trachea. Finally, the ability of endothelin peptides to cause systemic vasodepressor responses is not related to their ability to cause endothelium-dependent relaxation, at least in the mesenteric circulation, and, for endothelin (16-21), its systemic pressor response is not likely to be the result of vasoconstriction in the mesenteric bed.

Keywords: Endothelin; endothelin (16-21); proendothelin-1; endothelin-1 analogues; acetylcholine; sodium nitroprusside; rat mesenteric bed; vasodilatation; endothelium; systemic depressor response

Introduction

Most members of the endothelin/sarafotoxin family of peptides are potent systemic pressor agents (Inoue *et al.*, 1989; Gardiner *et al.*, 1990a,b) and have vasoconstrictor actions at low concentrations in many blood vessels and vascular beds (Lovenberg & Miller, 1990). They consist of 21 amino acids linked by two cystine disulphide bridges between positions 1–15 and 3–11 (Yanagisawa *et al.*, 1988; Kloog *et al.*, 1988; Inoue *et al.*, 1989) and their vasoconstrictor activity is characterized by requiring the terminal tryptophan residue to be the free acid (Nakajima *et al.*, 1989) and, preferably, in the L-conformation (Kimura *et al.*, 1988). The overall systemic response to these peptides, however, includes a characteristic, short-lived vasodepressor phase before the long-lasting pressor effect develops (Wright & Fozard, 1988; Gardiner *et al.*, 1989a) although the precise mechanism underlying this initial systemic depressor response is currently unknown.

Despite the controversy surrounding the mechanism involved in mediating the systemic vasodepressor responses observed to the endothelin/sarafotoxin peptides, endothelium-dependent relaxation is known to occur *in vitro* in response to endothelin-3 (Warner *et al.*, 1989; Douglas & Hiley, 1990), endothelin-1, endothelin-2, mouse vasoactive intestinal contractor and sarafotoxin S6b (Douglas & Hiley, 1990). The order of potency of these peptides in causing endothelium-dependent relaxation appears to be different from that for their vasoconstrictor effect; endothelin-3 is the least potent at causing vascular smooth muscle contraction but is the most potent as a vasorelaxant (Douglas & Hiley, 1990). This suggests that different receptors may be mediating the two responses. This proposition receives further support from the observation that, although all the peptides shown to cause endothelium-dependent relaxation in the rat isolated mesenteric arterial bed also showed modulation by the endothelium of their pressor responses (Douglas & Hiley, 1990), two peptides, [Ala^{1,15}]endothelin-1 and [Ala^{3,11}]endothelin-1, in which one or other of the two disulphide bridges was absent

[1] Author for correspondence.

showed no endothelium-dependence in their vasoconstrictor effects (Randall *et al.*, 1989). However, whether or not these two alanyl-substituted analogues possess vasodilator activity has not yet been directly assessed.

Confirmation of the existence of more than one receptor for the endothelins has come with the cloning of two separate receptor proteins (Arai *et al.*, 1990; Sakurai *et al.*, 1990) one of which, termed the ET_A receptor, has a much greater affinity for endothelin-1 than endothelin-3 whilst the other, the ET_B receptor, shows no great differences in affinity for endothelin-1 and other members of this peptide family (Vane, 1990). A receptor cloned from the A10 rat vascular smooth muscle cell line shows 90% homology with the ET_A receptor and binds endothelin-1 about 100 times better than it does endothelin-3 (Lin *et al.*, 1991). Other interesting, functional, evidence for receptor heterogeneity in the responses to the endothelins has been obtained by Maggi *et al.* (1989a,b). They showed that the C-terminal hexapeptide of the endothelins was inactive in the rat aorta but was a full agonist in the guinea-pig bronchus although endothelin-1, endothelin-2, endothelin-3 and sarafotoxin S6b were all potent full agonists in both tissues. However, the absence of an antagonist to the actions of the endothelins has prevented the certain identification of the receptors at which endothelin (16-21) acts as being identical with those for the parent 21 amino acid peptides.

The present study aimed to investigate further the question of heterogeneity in functional endothelin/sarafotoxin receptors by studying the relaxant actions of the C-terminal hexapeptide and of two 21 amino acid analogues in which one ([$Ala^{3,11}$]endothelin-1) or both ([$Ala^{1,3,11,15}$]endothelin-1) of the disulphide bridges were absent; these studies were performed on the isolated mesentery and in anaesthetized rats. Since the putative precursors of endothelin-1 in the pig and man, i.e. porcine proendothelin-1 (of 39 amino acids) and human proendothelin-1 (with 38 amino acid residues), have pressor activity it was also decided to determine if these precursors, as well as the proendothelin fragment, porcine proendothelin (22-39), had vasorelaxant activity (using acetylcholine and sodium nitroprusside for comparison). The relationship between the vasorelaxant actions of these peptides and the systemic depressor response that occurs following the i.v. administration of these peptides in the anaesthetized rat was also examined.

A preliminary account of some of these findings was given to the joint meeting of the British Pharmacological Society and Association Française des Pharmacologistes in Lyon during April 1991 (Douglas & Hiley, 1991b).

Methods

The isolated, Krebs-Henseleit perfused, superior mesenteric arterial bed of the rat

Adult male Wistar rats (250–400 g; Bantin & Kingman, Hull) were given a single i.p. injection of sodium pentobarbitone (60 mg kg^{-1}: Sagatal; RMB Animal Health Ltd, Dagenham) and heparin (1000 u kg^{-1}; Paines & Byrne, Greenford). After laparotomy, the superior mesenteric artery was identified and cleaned of adherent tissue to allow perfusion of the mesenteric arterial bed essentially according to the method of McGregor (1965). The artery was ligated proximal to the abdominal aorta and cannulated with polythene PP50 tubing (Portex, Hythe). The mesenteric bed was perfused at 37 ± 1°C and 4 ml min^{-1} with oxygenated (95% O_2:5% CO_2) Krebs-Henseleit solution of the following composition (mM): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 and D-glucose 5.5. The Krebs-Henseleit solution contained 10 μM indomethacin and 5 g l^{-1} bovine serum albumin. The perfused mesentery was separated from the gastrointestinal tract, placed on a Perspex block maintained at 37 ± 1°C and covered with Nescofilm (Nippon Shoji Kaishi Ltd, Osaka, Japan) to avoid surface damage.

The pressure in the mesentery was recorded from a T-piece, placed in the perfusion circuit near the point at which the cannula was inserted into the artery, by means of a Bell & Howell Type 4-422-0001 pressure transducer coupled to a Grass model 7D polygraph. Zero pressure was set at zero flow and, since the flow rate was kept constant, the changes in perfusion pressure were directly proportional to changes in vascular resistance.

When required, the endothelium was removed by perfusion of the vascular bed with a 0.3% (w/v) solution in distilled water of the detergent CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate) for 120–150 s (Hiley *et al.*, 1987). For this procedure the flow rate was reduced to 2 ml min^{-1} and, afterwards, the tissue was allowed 45 min to re-equilibrate with the Krebs-Henseleit solution. In all preparations, the presence or absence of a functional endothelium was assessed by determining whether or not acetylcholine opposed noradrenaline-induced vasoconstriction (Randall *et al.*, 1989). The flow rate was returned to 4 ml min^{-1} before the construction of a dose-response curve for the dilatation caused by a peptide, acetylcholine or sodium nitroprusside. The vasorelaxant actions of the drugs were assessed by recording reductions in the tone established by infusion of 100 μM methoxamine (final perfusate concentration) into the preparation. Agonist administration was in bolus doses of 40 μl or less.

Experiments to determine the pressor activity of peptides in the mesenteric bed were carried out at a flow rate 2 ml min^{-1} using Krebs-Henseleit solution containing indomethacin but in the absence of methoxamine and bovine serum albumin (Randall *et al.*, 1989). After a 30 min equilibration period, peptides were administered into the perfusion circuit in volumes of 100 μl or less. Doses were given 5 min apart or 5 min after the tissue perfusion pressure had returned to the resting basal value following the previous dose of peptide, which ever was the longer.

Systemic blood pressure responses

Male Wistar rats (200–400 g) were anaesthetized with 60 mg kg^{-1} sodium thiopentone (Trapanal; BYK Gulden, Konstanz, Germany) and 30 mg kg^{-1} sodium pentobarbitone (Sagatal; RMB, Dagenham) given i.p. Cannulae were placed in the trachea, to allow artificial respiration (20 ml kg^{-1} at 54 breaths min^{-1}), and the left jugular vein. The right common carotid artery was cannulated for measurement of arterial pressure with a Bell & Howell transducer (Type 4-422-0001) connected to a Grass Model 79D polygraph. Heart rate was derived from the ECG using subcutaneous needle electrodes and a Grass 7P4 ECG preamplifier. Body temperature was maintained at 37 ± 1°C with a homeothermic blanket (BioScience, Sheerness). A 20 min equilibration period was allowed before peptides were given through the jugular vein cannula as bolus doses in volumes of 100 μl or less. All doses were washed through the cannula with 250 μl saline (0.9% NaCl, w/v) and each animal was used only to examine the effects of a single peptide.

Statistical and data analysis

Values are given as the mean ± s.e.mean. The values of n show the number of preparations in which the individual doses used to construct a dose-response curve were examined; since some doses of an agonist were used on all the preparations, the greatest value of n shows the total number of preparations used. Means were compared with Student's unpaired t test.

Where possible, dose-response curves were analysed by fitting the logistic equation:

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

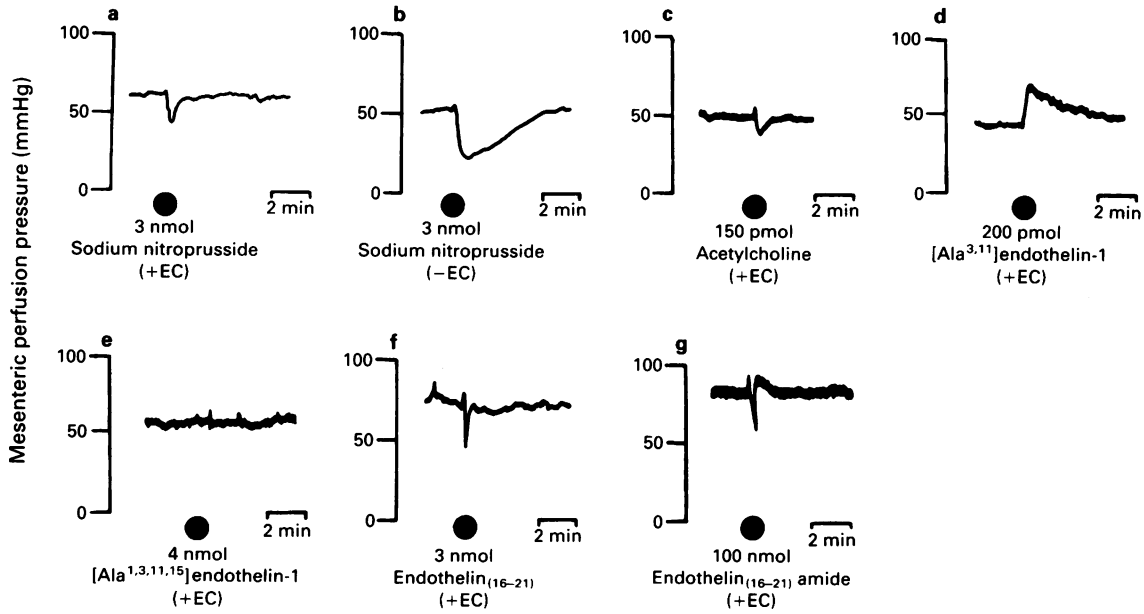

Figure 1 Traces showing representative relaxation responses in the isolated perfused mesenteric bed precontracted with 100 μM methoxamine to (a and b) 3 nmol sodium nitroprusside, (c) 150 pmol acetylcholine, (d) 200 pmol [Ala^{3,11}]endothelin-1, (e) 4 nmol [Ala^{1,3,11,15}]endothelin-1, (f) 3 nmol endothelin (16–21) and (g) 100 nmol endothelin (16–21) amide. +EC indicates that a functional endothelium was present and −EC shows an experiment in which the endothelium had been removed with CHAPS.

where R is the response, A the dose of drug, R_{max} the maximum response, n_H the slope function and ED_{50} the dose giving the half maximal response. A modified Marquardt procedure, implemented in Harwell routine VB01A on an IBM 3084Q mainframe computer, was used to carry out the curve fitting (Randall *et al.*, 1989).

Drugs and peptides

All solutions were made fresh daily. (−)-Noradrenaline bitartrate (Koch-Light, Haverhill) was dissolved in distilled water containing 1 mg ml^{-1} ascorbic acid (Fisons, Loughborough) to give a solution of 5 mg ml^{-1}; subsequent dilutions were made in saline. A 1 mg ml^{-1} solution of indomethacin (Sigma Chemical Company, Poole) was made in 5% (w/v) sodium bicarbonate and this was added to the Krebs-Henseleit solution to give the required concentration. Sodium nitroprusside (Koch-Light), methoxamine hydrochloride (Sigma) and acetylcholine iodide (Sigma) were dissolved in saline and solutions of sodium nitroprusside were protected from light. Fraction V bovine serum albumin and CHAPS were from Sigma.

[Ala^{3,11}]endothelin-1, porcine proendothelin (22-39) and endothelin (16-21) were from Peninsula Laboratories (Europe) Ltd, St. Helens. Human proendothelin-1 and porcine proendothelin-1 were from the Peptide Institute, Osaka, Japan. [Ala^{1,3,11,15}]endothelin-1 and endothelin (16-21) amide were synthesized at the Marion Merrell Dow Research Institute, Strasbourg. All the peptides were dissolved in distilled water to give 100 μM stock solutions. The concentrations of these solutions were checked by absorbance spectrophotometry at 280 nm using the following molar extinction coefficients (for a 10 mm path length): porcine proendothelin (22-39), 1400; endothelin (16-21) and endothelin (16-21) amide, 5600; [Ala^{1,3,11,15}]endothelin-1, 7000; [Ala^{3,11}]endothelin-1, 7125; human proendothelin-1 and porcine proendothelin-1, 8650.

Results

Vasorelaxant actions of acetylcholine and sodium nitroprusside

In the presence of endothelium, both acetylcholine and sodium nitroprusside decreased methoxamine-induced tone in the mesenteric bed (Figure 1) in a dose-related manner (Figure 2). Sodium nitroprusside was about 200 fold less potent than acetylcholine as a vasodilator but it caused a significantly greater maximal degree of reversal of the methoxamine-induced tone (Table 1). Figure 1 shows that the duration of the relaxant response to acetylcholine (1–2 min) was less than that to sodium nitroprusside (5–6 min).

Perfusion of the mesentery with 0.3% CHAPS abolished the functional integrity of the endothelium without affecting the

Table 1 Comparison of the potency and maximal responses for the vasodepressor effects of endothelin (16–21) and porcine proendothelin-1 with those of acetylcholine and sodium nitroprusside

	R_{max} (% methoxamine induced tone)	ED_{50} (pmol)	n_H	n
Acetylcholine	60.8 ± 2.1	7.3 ± 1.4	1.0 ± 0.1	5–6
Sodium nitroprusside +EC	86.8 ± 8.7**/†††	1560 ± 690*/†	0.6 ± 0.1	5–6
Sodium nitroprusside −EC	95.0 ± 1.3***/†††	414 ± 49***/†††	1.1 ± 0.1	7–11
Endothelin (16-21)	48.9 ± 2.8**	84.9 ± 20.0**	1.1 ± 0.1	4–8
Porcine proendothelin-1	29.1 ± 1.4**/††	109 ± 9***	3.0 ± 0.4	4

These values were obtained from fitting a logistic function to the dose-response data.
All experiments were carried out in the presence of a functional endothelium (except for sodium nitroprusside −EC) and of 10 μM indomethacin. The concentration of methoxamine in the perfusate was 100 μM.
Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ from the corresponding values for acetylcholine; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ from those for endothelin (16-21).

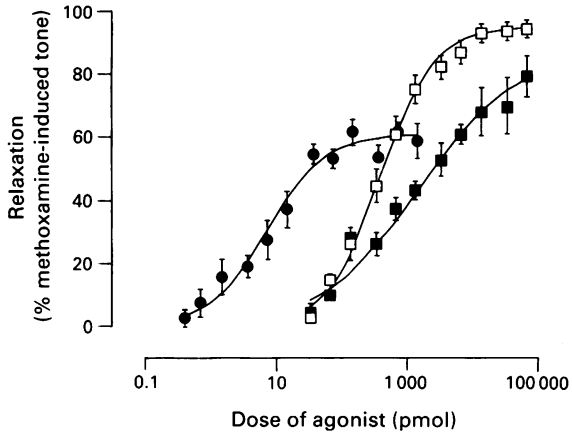

Figure 2 Log dose-response curves for the relaxant responses to acetylcholine (●) in the presence of a functional endothelium and to sodium nitroprusside in the presence (■) and absence (□) of the endothelium. The points show the mean and the vertical bars ± s.e.mean. The lines drawn are those obtained from the curve-fitting procedure.

ability of the vascular smooth muscle to relax. Prior to CHAPS perfusion, 30 nmol noradrenaline increased perfusion pressure by 35.6 ± 4.6 mmHg (n = 11) whereas the same dose, co-administered in a bolus dose with 0.4 nmol acetylcholine, induced a significantly smaller pressor response (15.3 ± 2.0 mmHg, n = 11; $P < 0.001$). After perfusion of the mesentery with 0.3% CHAPS, 30 nmol noradrenaline increased the perfusion pressure by 39.3 ± 4.6 mmHg and 39.7 ± 3.8 mmHg (n = 11 for both) when administered either alone or with 0.4 nmol acetylcholine, respectively. However, after endothelial destruction, sodium nitroprusside, unlike acetylcholine, still caused dose-dependent reductions in methoxamine-induced tone in the mesentery (Figure 2; Table 1). Endothelial cell destruction enhanced the potency of this nitrovasodilator ($P < 0.05$) without altering the maximum degree of vasodilatation obtained (Figures 1 and 2; Table 1).

Relaxant effects of endothelin (16-21) and its amide

Endothelin (16-21) caused dose-related relaxation in the preconstricted mesenteric bed with an intact endothelium (Figure 3; Table 1) without inducing any secondary increases in tone (Figure 1). It was less potent than acetylcholine but more potent than sodium nitroprusside (Table 1). Figure 3 shows that this peptide did not cause vasorelaxation in mesenteries pretreated with CHAPS (n = 4).

Substitution of the free acid to give endothelin (16-21) amide significantly reduced the potency of the hexapeptide as a vasodilator with the result that, despite using a wide dose-range (0.1–100 nmol; n = 3–7), a full dose-response relationship could not be obtained. Nevertheless, it may be seen from Figure 3 that, since its ED_{50} was in excess of 40 nmol, the amide was at least 500 times less potent than the free acid.

In contrast to acetylcholine and sodium nitroprusside, the relaxant responses to both of these peptides were very transient, lasting for a maximum of 15–30 s (Figure 1).

Vasoconstrictor actions of endothelin (16-21) and its amide in the mesenteric arterial bed

Neither endothelin (16-21) (0.5–20 nmol; n = 3) nor endothelin (16-21) amide (0.1–20 nmol; n = 3) had any vasoconstrictor activity in preparations exposed to CHAPS and which were not precontracted with methoxamine.

Effects of [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 in the mesenteric arterial bed

Between 0.1 and 2000 pmol, neither of the alanyl-substituted analogues of endothelin-1 reduced the methoxamine-induced tone in mesenteric beds with an intact endothelium (n = 4 for both). However, [Ala^{3,11}]endothelin-1 caused vasoconstrictor responses, over and above the tone induced by methoxamine, at doses of 100 pmol or more (Figure 1) although [Ala^{1,3,11,15}]endothelin-1 produced no pressor responses over the dose-range studied.

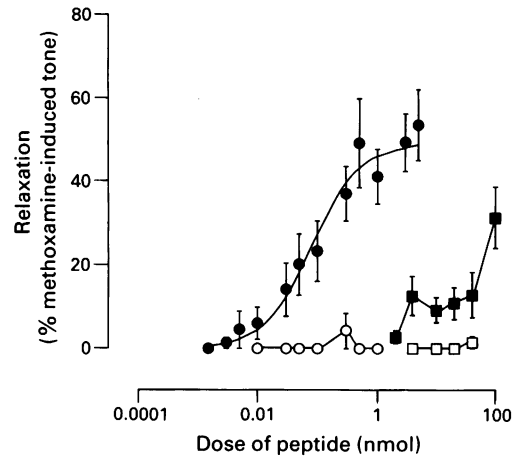

Figure 3 Dose-response relations for the relaxation responses to (●, ○) endothelin (16-21) and (■, □) endothelin (16-21) amide in the presence (●, ■) and absence (○, □) of the endothelium in the methoxamine-preconstricted mesenteric arterial bed. The points are the mean response and vertical bars show ± s.e.mean except where the error is contained within the symbol. The curve shown for endothelin (16-21) is that obtained from the curve-fitting procedure.

Effects in the mesenteric bed of human proendothelin-1, porcine proendothelin-1 and porcine proendothelin-1 (22-39)

Both the human and porcine proendothelins relaxed methoxamine-induced tone in the mesenteric vascular bed (Figure 4). In the case of porcine proendothelin-1 it was possible to obtain a full dose-response curve (Table 1) since it only gave very small secondary vasoconstrictor responses; perfusion pressure was increased by 0.6 ± 0.6, 1.2 ± 1.2, 3.1 ± 1.2, 1.1 ± 1.1 and 6.2 ± 1.6 mmHg respectively after doses of 40, 100, 200, 400 and 1000 pmol. In mesenteric beds which were not preconstricted with methoxamine, porcine proendothelin (1-39) was only a very weak vasoconstrictor agonist in either the presence or absence of the endothelium and subsequently it was not possible to show any modulation of these responses by the endothelium; the responses obtained at the highest doses used were 3.2 ± 0.9 mmHg (n = 4; 4 nmol; with endothelium) and 5.9 ± 1.4 mmHg (n = 3; 10 nmol; without endothelium).

It was not possible to carry out curve-fitting on the vasorelaxant responses given by human proendothelin-1. The maximum response occurred at 250 pmol (Figure 4) and, at this and higher doses, secondary vasoconstriction also occurred. These secondary responses were much higher than those seen with the porcine precursor; for example, although at 250 pmol the response was 1.6 ± 0.7 mmHg (n = 7), the responses rose in a dose-dependent manner to reach 24.7 ± 5.4 mmHg (n = 5) at 5 nmol.

Porcine proendothelin-1 (22-39) had no vasodilator effects between 2 pmol and 4 nmol in endothelium-intact, methoxamine preconstricted, preparations (n = 3) nor did it cause vasoconstriction in de-endothelialized preparations between 400 pmol and 20 nmol (n = 3).

Effects on systemic blood pressure

When administered i.v., the most striking feature of the systemic response to endothelin-3 was a fall in blood pressure which was relatively long lasting, not returning to the basal value for

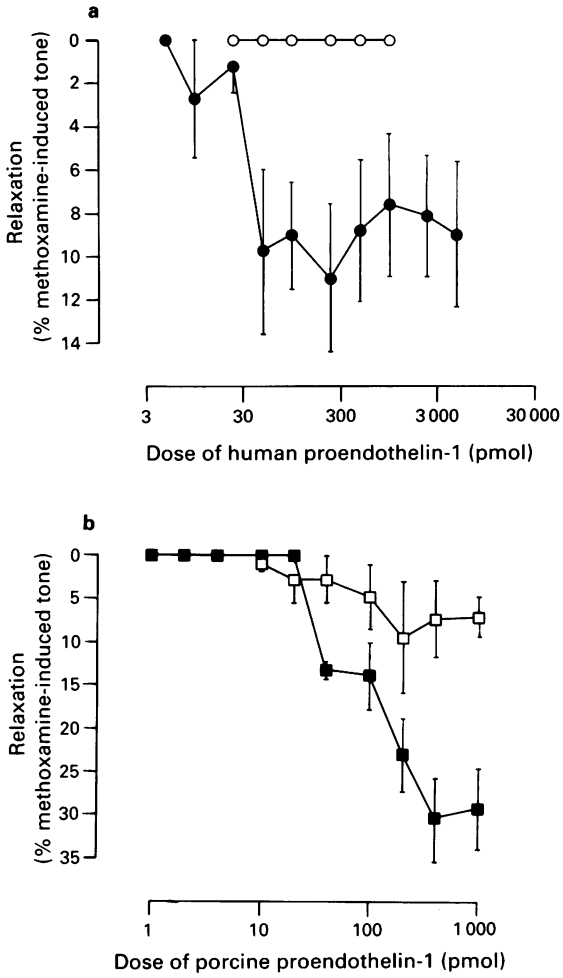

Figure 4 Dose-relaxation response relations for (●, ○) human proendothelin-1 (a) and (■, □) porcine proendothelin-1 (b) in the isolated, preconstricted mesenteric arterial bed of the rat. The closed symbols show the mean responses obtained in the presence, and the open symbols those obtained in the absence, of a functional endothelium. Vertical bars show ± s.e.mean except where the error lies within the symbol.

around 5 min (Figure 5), and which was dose-related (Figure 6). In contrast, both [Ala^{3,11}]endothelin-1 (Figure 7) and [Ala^{1,3,11,15}]endothelin-1 (Figure 8) produced depressor responses which were only transient (lasting for only 30–60 s; Figure 5) and which therefore more closely resembled the responses previously reported for endothelin-1 and the proendothelins (Douglas & Hiley, 1991a) than those to endothelin-3. Table 2 shows that endothelin-3 gave the same maximum systemic depressor response as endothelin-1 whereas both [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 gave maxima which were about 70% less. Also, endothelin-3 did not significantly differ in potency from endothelin-1 whilst the two alanyl-substituted analogues were significantly less potent agonists.

Neither endothelin (16-21) (0.004–100 nmol kg^{-1}; Figure 9) nor porcine endothelin (22-39) (1–20 nmol kg^{-1}) had any significant systemic vasodepressor activity. With 100 nmol kg^{-1} endothelin (16-21), 2 out of 4 animals showed no depressor response, one responded with a decrease of 2 mmHg and the fourth (shown in Figure 5d) gave a depressor response of 20 mmHg; thus 100 nmol kg^{-1} may be a threshold dose for a systemic depressor effect.

Figure 5 shows that the systemic depressor actions of endothelin-3, [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]-endothelin-1 were followed by longer lasting vasopressor responses which reached their maxima in 5–10 min and took approximately 30–45 min to return to the basal resting values. The responses were dose-related and endothelin-3 (Figure 6) and [Ala^{3,11}]endothelin-1 (Figure 7) gave similar maximum pressor effects which were not significantly different from that previously found for endothelin-1 (Table 2). In contrast, [Ala^{1,3,11,15}]endothelin-1 (Figure 8) gave a maximum response which was 20–30% lower. Endothelin-3, [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 were significantly less potent systemic pressor agonists than endothelin-1 (Table 2).

Endothelin-3 was approximately 2–3 times more potent as a systemic vasodepressor agent than it was as a pressor agonist but the alanyl-substituted analogues [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 were equipotent as depressor and pressor agents. These results contrast with those for endothelin-1 which was about 2–3 fold more potent as a systemic pressor agent rather than a depressor agent (Douglas & Hiley, 1991a).

Whereas endothelin (16-21) had no consistent depressor activity, it caused significant increases in systemic arterial blood pressure (Figure 9). Due to limitations in solubility, it was not possible to construct a full dose-response curve but it was clearly the least potent of the peptides examined (Table 2).

Between 1–20 nmol kg^{-1}, porcine endothelin (22-39) had no vasopressor activity.

At the higher doses examined, the changes in mean arterial pressure obtained with some of these peptides were associated with pronounced reductions in heart rate; e.g. 400 pmol kg^{-1} endothelin-3 reduced heart rate by 31 ± 5 beats min^{-1} (n = 7), 2 nmol kg^{-1} [Ala^{3,11}]endothelin-1 reduced it by 30 ± 7 beats min^{-1} (n = 11) and [Ala^{1,3,11,15}]endothelin-1 caused a

Table 2 Comparison of the systemic potency and maximal responses for the initial vasodepressor and subsequent vasoconstrictor effects of some endothelin family peptides following intravenous injection in the anaesthetized rat

	Endothelin-1 n = 6–12	*Endothelin-3* n = 4–7	[*Ala*^{3,11}] *endothelin-1* n = 5–13	[*Ala*^{1,3,11,15}] *endothelin-1* n = 6–10	*Endothelin (16-21)* n = 3–6
Basal parameters					
Heart rate (min^{-1})	390 ± 13	415 ± 18	405 ± 12	425 ± 16	393 ± 14
Mean arterial pressure (mmHg)	110 ± 7	95 ± 10	115 ± 7	118 ± 8	107 ± 10
Systemic depressor response					
R_{max} (mmHg)	35.8 ± 2.5	35.2 ± 5.2	24.3 ± 1.0***	24.2 ± 1.6**	No consistent
ED_{50} (pmol kg^{-1})	141 ± 45	88.3 ± 36.9	267 ± 31*	344 ± 62**	depressor
n_H	0.7 ± 0.1	1.4 ± 0.5	1.8 ± 0.2	1.6 ± 0.2	response
Systemic pressor response					
R_{max} (mmHg)	28.7 ± 2.1	28.9 ± 3.2	31.9 ± 2.2	23.3 ± 1.2*	>22.7
ED_{50} (pmol kg^{-1})	48.0 ± 11.0†	216 ± 60**/†	326 ± 84**	265 ± 47***	>40000
n_H	1.0 ± 0.1	1.3 ± 0.3	0.9 ± 0.1	1.3 ± 0.1	

The values obtained for endothelin-1 are from Douglas & Hiley (1991a) and are reproduced in this table for ease of comparison.
The data shown represent the mean ± s.e.mean of n observations. The values given are those obtained from fitting a logistic function.
Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ from the corresponding value for endothelin-1 and † $P < 0.05$ between the ED_{50} values for the systemic depressor and pressor responses for an individual peptide.

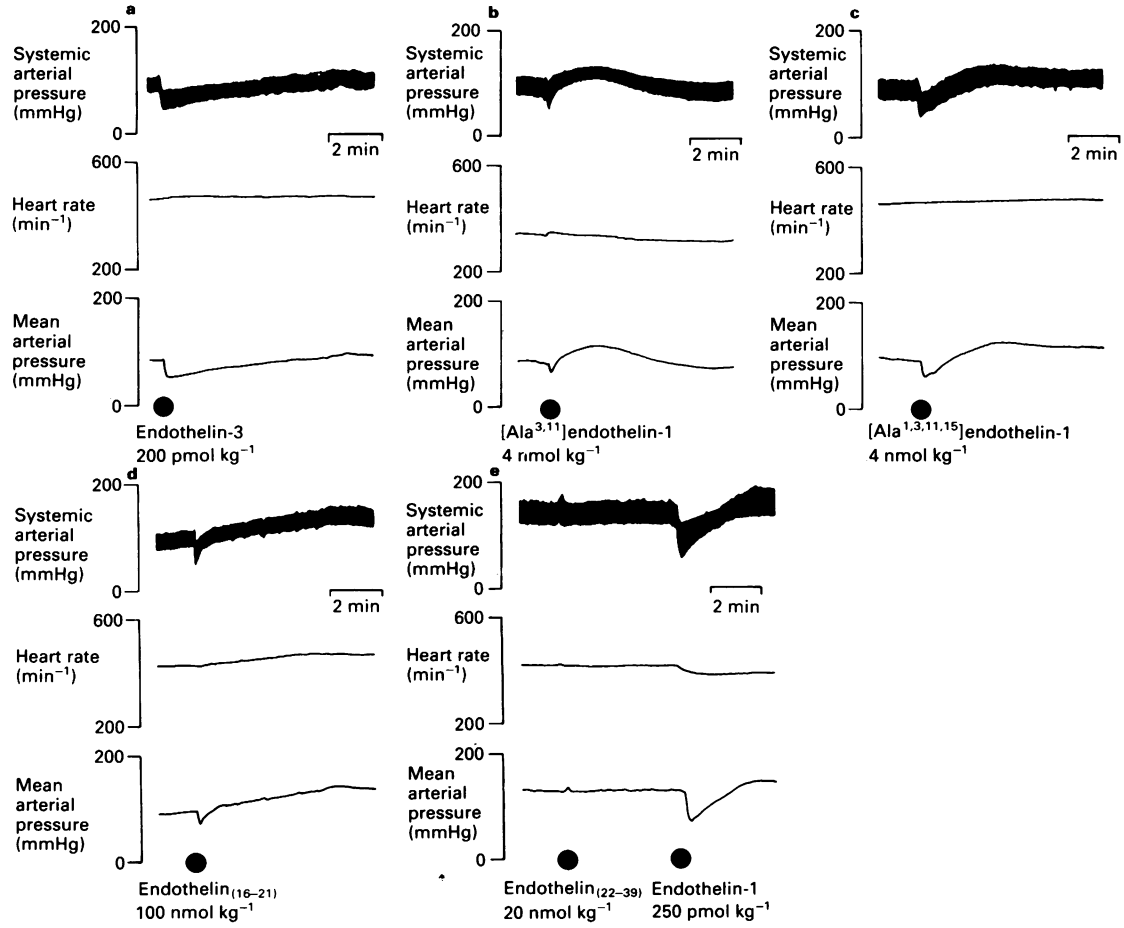

Figure 5 Traces of representative systemic blood pressure and heart rate responses obtained after i.v. administration to anaesthetized rats of (a) 200 pmol kg^{-1} endothelin-3, (b) 4 nmol kg^{-1} [$Ala^{3,11}$]endothelin-1, (c) 4 nmol kg^{-1} [$Ala^{1,3,11,15}$]endothelin-1, (d) 100 nmol kg^{-1} endothelin (16-21) and (e) 20 nmol kg^{-1} endothelin (22-39) followed by 250 pmol kg^{-1} endothelin-1.

bradycardia of 30 ± 5 beats min^{-1} ($n = 9$). The C-terminal hexapeptide endothelin (16-21) had no significant effects on heart rate at the doses examined.

Discussion

This study shows that, whether or not the endothelium is present, the C-terminal hexapeptide endothelin (16-21) does not elicit pressor responses in the isolated superior mesenteric arterial bed of the rat. It has previously been reported to be a full agonist (relative to endothelin-1) in the guinea-pig isolated bronchus and rat isolated vas deferens but to have relatively little activity, if any, in the rat isolated thoracic aorta, guinea-pig ileum, human urinary bladder, renal pelvis or renal artery (Maggi *et al.*, 1989a,b). On the basis of these studies it has been proposed that endothelin (16-21) distinguishes between endothelin receptor subtypes, acting as an agonist in the guinea-pig isolated bronchus but not in the rat thoracic aorta (Maggi *et al.*, 1989a,b). The lack of contractile activity in the rat isolated mesentery accords with the observations made by Maggi *et al.* (1989a,b) in another arterial preparation, the rat thoracic aorta, and suggests that there are no functional receptors for this hexapeptide in mesenteric arterial smooth muscle. However, this does not mean that endothelin (16-21) lacks contractile activity in all vascular smooth muscle preparations since it does contract isolated rat jugular veins and rabbit pulmonary arteries. Therefore, it would appear that both regional and species differences exist in the sensitivity of vascular smooth muscle to this peptide (Maggi *et al.*, 1989a).

Despite lacking contractile activity in the isolated mesentery, endothelin (16-21) does act in this preparation to cause endothelium-dependent vasorelaxation. Indeed, it is a potent agonist, being about one order of magnitude less potent than acetylcholine but around 20 times more potent than the endothelium-independent vasorelaxant, sodium nitroprusside. Amongst the preparations they studied, Maggi *et al.* (1989a) found the most sensitive to endothelin (16-21) to be the guinea-pig isolated bronchus where it was 30–35 times less potent than endothelin-1 as a spasmogen. However, endothelin (16-21) is approximately equipotent with endothelin-1 as a vasorelaxant in the precontracted mesentery since the threshold dose of endothelin-1 for relaxation is 50 pmol (Randall *et al.*, 1989).

Incidentally, it is interesting to note that the potency of sodium nitroprusside was enhanced after endothelium destruction whilst the maximum degree of relaxation remained unaltered. A similar potentiation, not only to sodium nitroprusside but also to glyceryl trinitrate, has been reported in rat aortic rings by Moncada *et al.* (1991). The reason for this supersensitivity is unclear but may reflect changes at the level of the cytosolic guanylate cyclase with which the nitrovasodilators interact. It is also possible that this enhanced potency results from the removal of a diffusion barrier to nitroprusside.

We have previously reported that members of the endothelin/sarafotoxin family are not only potent pressor agents in the isolated mesentery but are also endothelium-dependent vasodilators. Thus, endothelin (16-21) shares the latter of these actions. However, it is not possible to say whether or not this endothelin fragment shares a common receptor with the full 21 amino acid peptides as has been suggested for guinea-pig bronchial smooth muscle by Maggi *et al.* (1989a,b). At this stage, there is no evidence to refute the suggestion that endothelin (16-21) might act at other peptide receptors mediating relaxation, neither is it easy to discount

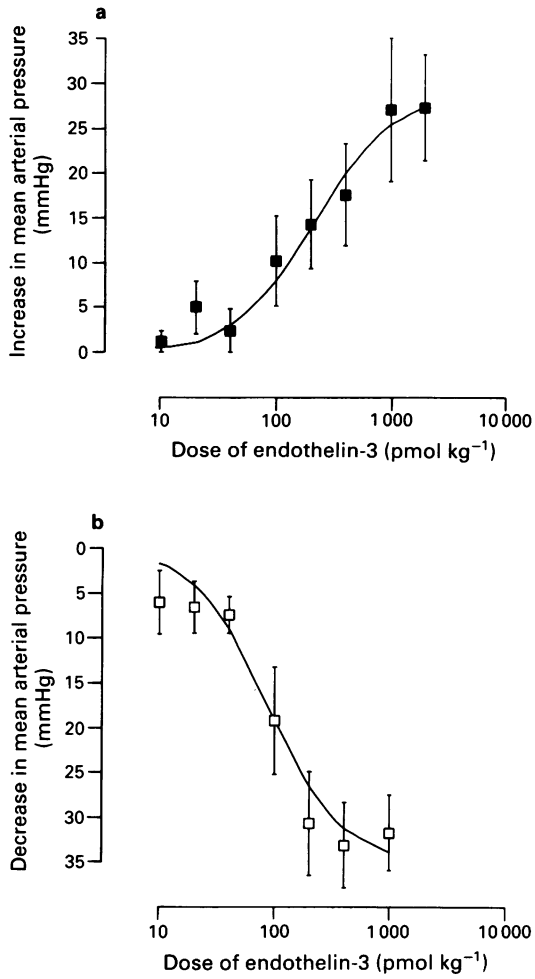

Figure 6 Log dose-response curves for the systemic (a) pressor and (b) depressor responses to endothelin-3 administered i.v. to anaesthetized rats. The points show the mean responses, the curves shown are those obtained from the curve-fitting procedure and the vertical bars show ± s.e.mean.

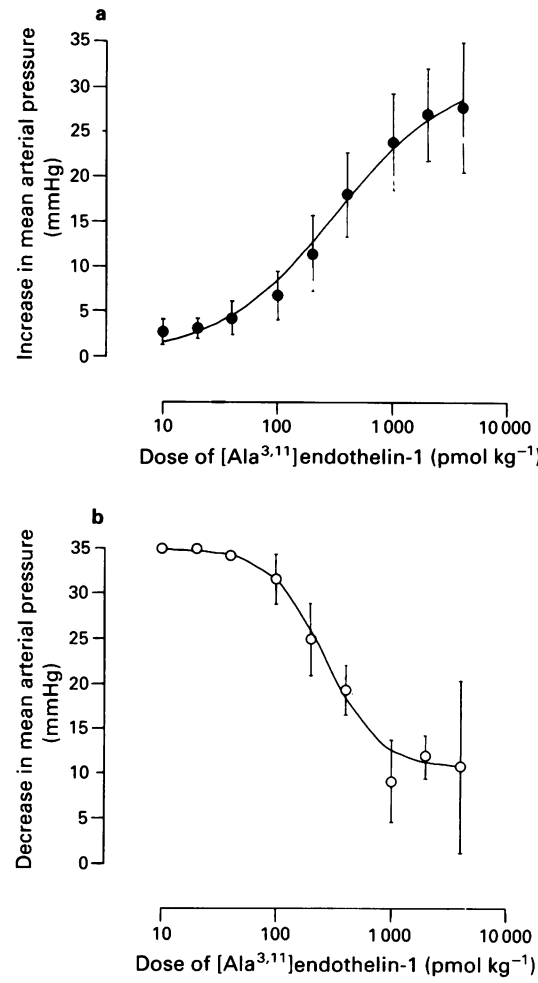

Figure 7 Log dose-response relationships for the systemic (a) pressor and (b) depressor responses to [Ala^{3,11}]endothelin-1 administered i.v. to anaesthetized rats. The points show the mean responses, the curves shown are those obtained from the curve-fitting procedure and the vertical bars show ± s.e.mean.

the possibility that this peptide is an agonist at a previously uncharacterized class of receptors.

It is clear that it is important that the C-terminal Trp residue is in the free acid form in order to see the activity of endothelin (16-21) as an endothelium-dependent vasodilator and a similar requirement has been reported for the vasoconstrictor actions of endothelin-1 (Nakajima *et al.*, 1989; Kimura *et al.*, 1988). Amidation of the C-terminal Trp of endothelin (16-21) lowers vasodilator potency in the isolated mesentery by 2–3 orders of magnitude and this accords with the findings that the amide was much less potent than the free acid in a number of isolated tissues including the guinea-pig bronchus, rabbit pulmonary artery and rat vas deferens (Maggi *et al.*, 1989a,b).

Evidence for the presence of two different endothelin/sarafotoxin receptor subtypes in the mesentery, one type on the vascular smooth muscle and another on the endothelium, has come from examining the rank order of potency of the peptides in inducing vasoconstriction and vasorelaxation. Thus, endothelin-1 is more potent than endothelin-3 in causing constriction whilst endothelin-3 is the more potent of the two in causing endothelium-dependent relaxation (Douglas & Hiley, 1990). Additional evidence for subtypes of endothelin receptor in the isolated mesenteric bed has come from our observations that, unlike the endogenous members of this peptide family such as endothelin-1, the pressor actions of [Ala^{3,11}]endothelin-1 and [Ala^{1,15}]endothelin-1 are not modulated in any way by the endothelium (Hiley *et al.*, 1989; Randall *et al.*, 1989; Douglas & Hiley, 1990). This has been interpreted as showing that both disulphide bridges are required to cause EDRF release from the endothelium. This inference now has direct support from the finding that [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 cannot relax preconstricted mesenteries; both disulphide bonds, whilst not being a prerequisite for smooth muscle receptor activation, are required for agonist activity at the endothelial cell receptor.

Recently, much interest has been focused on the mechanisms underlying the systemic actions of the endothelin/sarafotoxin family peptides in a number of species of animals. We, and many others, have reported that the systemic response in the anaesthetized rat to i.v. administration of endothelin-1 is biphasic; initially, a transient depressor response is observed after which mean arterial pressure is raised for a prolonged period of time (Douglas & Hiley, 1991a). The depressor response has received much attention and is not thought to involve reflex mechanisms since it occurs in rats chemically denervated with mecamylamine (Wright & Fozard, 1989) or a combination of bunazosin, propranolol and atropine (Yanagisawa *et al.*, 1988) and in pithed animals (MacLean *et al.*, 1989). Also, reductions in heart rate or cardiac output do not occur (Le Monnier de Gouville *et al.*, 1989). Indeed, cardiac output is increased during the depressor phase in conscious animals (Gardiner *et al.*, 1990c) and the reduction in blood pressure appears to be the result of a short lived decrease in total peripheral resistance (Gardiner *et al.*, 1990c,e). Thus, i.v. administration of endothelin-1 initially increases carotid and hindquarter vascular conductances and these are followed by more sustained reductions in conductance (the secondary conductance change

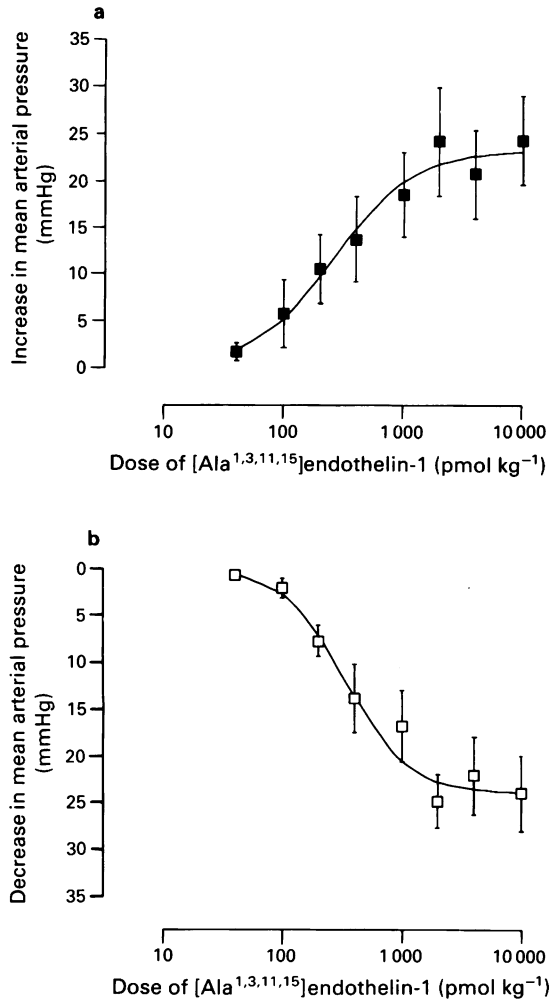

Figure 8 Log dose-response curves for the systemic (a) pressor and (b) depressor responses to [Ala^{1,3,11,15}]endothelin-1 administered i.v. to anaesthetized rats. The points show the mean responses, the curves shown are those obtained from the curve-fitting procedure and the vertical bars show ± s.e.mean.

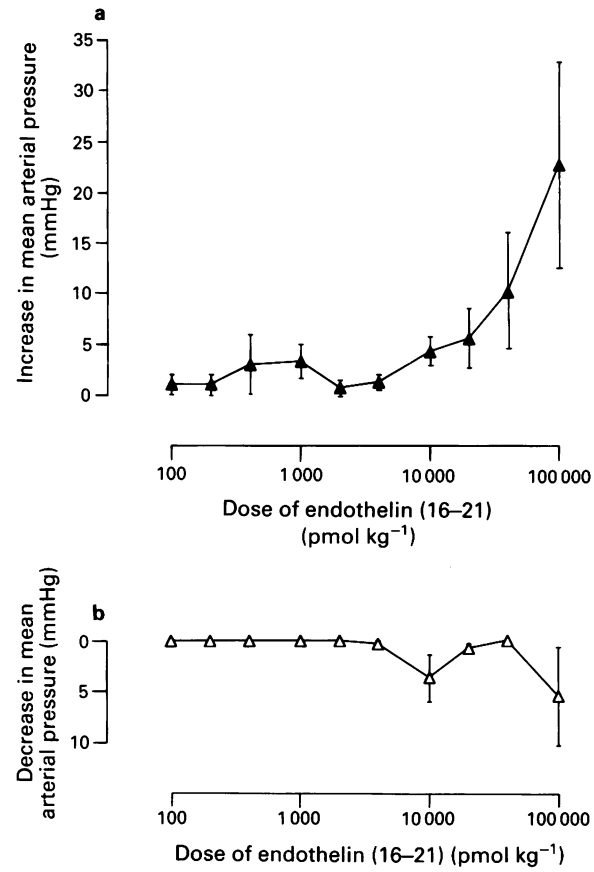

Figure 9 Dose-response relations for the systemic (a) pressor and (b) depressor responses to endothelin (16-21) administered i.v. to anaesthetized rats. The points show the means and the vertical bars show the s.e.mean except where the error lies within the symbol.

being associated with the prolonged systemic hypertension; Wright & Fozard, 1988; Gardiner *et al.*, 1989a). Since these peptides are known to cause the release of EDRF (Randall *et al.*, 1989; Warner *et al.*, 1989; Douglas & Hiley, 1990), it was originally proposed that it is this action which underlies the initial systemic depressor response to i.v. administration of peptide.

This study found that, in the anaesthetized rat, endothelin-3 caused a prolonged depressor response lasting for about 5 min. This contrasts both with the short-lasting depressor response found in the same conditions for endothelin-1 (Douglas & Hiley, 1991a) and for endothelin-3 itself in conscious rats (Gardiner *et al.*, 1990c). The difference between the results with endothelin-3 in conscious and anaesthetized animals may represent a more rapid correction taking place due to reflex actions in the conscious animals. In the anaesthetized rats, the difference between endothelin-3 and endothelin-1 may be due to the fact that endothelin-3 is more potent in causing a systemic depressor response than the secondary increase in blood pressure. The reverse is true for endothelin-1 (Douglas & Hiley, 1991a). Since endothelin-3 is more potent as a vasorelaxant than a vasoconstrictor *in vitro*, and the opposite is true for endothelin-1 (Randall *et al.*, 1989; Douglas & Hiley, 1990), these findings would not conflict with the hypothesis that the initial hypotension observed with these peptides on i.v. administration is a reflection of their ability to cause endothelium-dependent relaxation in peripheral vascular beds. Such a mechanism was proposed by Whittle *et al.* (1989) who were able to attenuate the initial systemic depressor response to endothelin-1 using N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthase. However, the role of EDRF/nitric oxide in mediating the initial hypotensive response observed with these peptides *in vivo* has been questioned by other groups. Gardiner *et al.* (1989b) could not reproduce the findings of Whittle *et al.* (1989); they found that N^G-monomethyl-L-arginine had no effect upon the hypotensive action of endothelin-1. However, N^G-nitro-L-arginine methyl ester, a more potent nitric oxide synthase inhibitor, attenuated some of the regional increases in vascular conductance but not the systemic depressor response, although the latter was attenuated relative to that of nitroglycerin (Gardiner *et al.*, 1990e). In contrast, Hoffman *et al.* (1989) reported that the depressor response to endothelin-1 was unaffected by methylene blue. Thus, the evidence for the hypothesis that the systemic depressor response to the endothelin/sarafotoxin family peptides is the result of a peptide-induced release of EDRF/nitric oxide is mixed. On the other hand, the systemic vasodepressor effect could be the result of the action of eicosanoids, which are released from rat lung by endothelin-1 (de Nucci *et al.*, 1988), but this seems unlikely since indomethacin has no effect on this response (Gardiner *et al.*, 1990d).

If the systemic depressor response were due to the release of EDRF/nitric oxide, it would follow that [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 should only increase mean arterial blood pressure on systemic administration since this study shows that they do not release EDRF, at least in the isolated mesentery. However, these analogues induced biphasic changes in mean arterial blood pressure after i.v. administration. Therefore it would appear that the initial systemic hypotensive response induced by the endothelin/sarafotoxin family peptides does not result from their ability to induce EDRF release in the mesentery. This is particularly surprising in the case of endothelin-3 which is more potent in releasing

EDRF in the isolated mesentery than it is in causing vasoconstriction since, *in vivo*, it increases mesenteric vascular resistance, which suggests sufficient peptide reaches this bed to activate its smooth muscle receptors, but does not cause any decrease in mesenteric conductance (Gardiner *et al.*, 1990b,c). Nevertheless, it has to be acknowledged that the systemic vascular actions of these peptides might involve EDRF release at other sites in order to bring about the systemic depressor response. Whether or not EDRF is involved elsewhere, it seems that the mesenteric vascular bed does not contribute to this response.

It is of interest to note that a monocyclic analogue of endothelin-1, where the inner intramolecular disulphide bridge has been prevented from forming using blocking acetamidomethyl groups at residues Cys^3 and Cys^{11}, has no systemic actions in the anaesthetized cat at a dose of $30\,nmol\,kg^{-1}$ (Minkes & Kadowitz, 1991). The contrast between the lack of activity of this monocyclic analogue and the clear systemic actions of the [$Ala^{3,11}$]endothelin-1 in the anaesthetized rat may result from the presence of blocking groups on Cys^3 and Cys^{11} causing a greater alteration in the peptide conformation than is seen by the pseudoisosteric substitution of Cys residues with Ala residues in positions 3 and 11; indeed, a similar effect has been noted in a ligand binding study with [^{125}I]endothelin-1 (Hiley *et al.*, 1990).

Although endothelin (16-21) is a potent endothelium-dependent relaxant in the isolated mesentery, even in doses of up to $100\,nmol\,kg^{-1}$ no reproducible depressor response was observed on systemic administration. Paradoxically, the only clear systemic effects observed with the hexapeptide were pressor responses and, for these, it was about 3 orders of magnitude less potent than endothelin-1. This finding agrees with that of Giuliani *et al.* (1991) and supports the hypothesis that the systemic depressor response observed to the endothelin/sarafotoxin family peptides does not involve the release of EDRF.

Despite lacking contractile activity in the isolated mesentery at doses of up to 20 nmol, endothelin (16-21) produced quite marked increases in mean arterial pressure. However, Minkes & Kadowitz (1991) have recently reported that it is inactive on the systemic blood pressure over a similar dose-range in the anaesthetized cat. The reason for this disparity is unclear but may reflect species differences in the sensitivity of vascular smooth muscle to this peptide. However, since it contracts rat jugular vein preparations *in vitro* (Maggi *et al.*, 1989a) an action on venous tissue may explain why it causes systemic pressor responses. Certainly, the systemic pressor response would not be expected to be due to any change in mesenteric arteriolar resistance since the hexapeptide had no contractile effects in the isolated mesentery.

[$Ala^{1,3,11,15}$]endothelin-1 has previously been shown to have no vasoconstrictor activity in the isolated mesentery (Randall *et al.*, 1989) and here we have shown that it lacks vasodilator activity in the same preparation. However, on i.v. administration, it induced a hypotensive response which was followed by a prolonged pressor response. On the basis of the findings that [$Ala^{1,3,11,15}$]endothelin-1 lacks activity in the isolated mesentery, one would expect this peptide to have no effect on mean arterial pressure when given systemically. However, the isolated mesenteric bed contains only arteriolar smooth muscle and studies performed in the *in situ* blood perfused mesentery, which also contains the venous side of the resistance bed, have shown that [$Ala^{1,3,11,15}$]endothelin-1 is a weak partial agonist (Randall *et al.*, 1989). Since rabbit and dog mesenteric veins are approximately an order of magnitude more sensitive to endothelin-1 than the corresponding mesenteric arteries (De Nucci *et al.*, 1988; Cocks *et al.*, 1989), and since the duration of the contractile response observed to endothelin-1 is more prolonged in the venous side of the rat isolated mesenteric bed (Warner, 1990), it is not surprising that the contractile actions of this weak agonist are difficult to observe in the isolated mesentery. However, when administered systemically, [$Ala^{1,3,11,15}$]endothelin-1 is able to gain access to act on the veins. It is also unwise to assume that the mesenteric bed is typical of all vascular beds with respect to the way in which it responds to these peptides. For example, during the initial period following i.v. administration of endothelin-1, the predominant effect observed in the mesentery is an increase in vascular resistance whereas the hindquarters show an increase in vascular conductance (Wright & Fozard, 1988; Gardiner *et al.*, 1989a). It is just such a regional variation, and the absence of the venous side of the circulation in isolated arterial beds, which requires caution to be exercised when trying to draw comparisons between *in vitro* observations and responses recorded in the intact cardiovascular system.

Regional differences in vascular sensitivity may help explain the differences in the relative potency of [$Ala^{1,3,11,15}$]endothelin-1 to that of endothelin-1 in isolated vascular preparations and following systemic administration. However, there is a good correlation between the relative potencies of [$Ala^{3,11}$]endothelin-1 and endothelin-1 *in vitro* and *in vivo*; [$Ala^{3,11}$]endothelin-1 is approximately 6 times less potent than endothelin-1 as a vasoconstrictor in the isolated mesenteric preparation (Randall *et al.*, 1989) and about 7 times less potent as a systemic pressor agent. These similar differences in functional potency correspond well with [$Ala^{3,11}$]endothelin-1 having an apparent K_i approximately 9 times higher than the parent peptide in the kidney (Jones *et al.*, 1989).

We have previously reported that human proendothelin-1 is a vasoconstrictor in the isolated mesenteric arterial bed, and that both human and porcine proendothelin are active not only in the *in situ* blood-perfused mesenteric bed but also when administered systemically (Douglas & Hiley, 1990; Douglas & Hiley, 1991a). Here we found that porcine proendothelin-1 was a weak vasoconstrictor *in vitro* and the responses obtained were too small for it to be shown unambiguously whether or not they were modulated by the endothelium. However, both proendothelins caused endothelium-dependent relaxation in the isolated mesentery and this explains why the vasoconstrictor actions of human proendothelin-1 are augmented following endothelium removal (Douglas & Hiley, 1990). Indeed, porcine proendothelin-1 is equipotent with endothelin (16-21) as a vasodilator in the isolated mesentery.

Endothelin-1 gives a significantly smaller maximum pressor response than porcine proendothelin-1 on i.v. administration (Douglas & Hiley, 1991a). On the basis of this, it would appear that either porcine proendothelin-1 does not require conversion *in vivo* to release endothelin-1 to be vasoactive or that the peptide fragment that was produced together with endothelin-1 following cleavage at residues Trp^{21}–Val^{22} was vasoactive itself. However, since this study has shown that endothelin (22-39) has no vasoactivity either *in vitro* or *in vivo*, it would appear that porcine proendothelin-1 is vasoactive in itself and does not require cleavage at Trp^{21}–Val^{22} in order to possess full biological activity.

In summary, the endothelins need both disulphide bonds to induce endothelium-dependent relaxation in the mesenteric vascular bed and this response is mediated by different receptors from those which mediate contraction of the mesenteric vascular smooth muscle. Vasorelaxation caused by endothelin (16-21) and its amide suggests that there is a receptor on the endothelium similar to one reported in the guinea-pig trachea. In the case of endothelin (16-21) it appears that its systemic pressor response does not arise from mesenteric arteriolar constriction and, finally, the ability of endothelin peptides to cause systemic vasodepressor responses is not simply related to the ability to cause endothelium-dependent relaxation.

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Neuropeptide Y (NPY) metabolism by endopeptidase-2 hinders characterization of NPY receptors in rat kidney

[1]*J.S. Price, †A.J. Kenny, **N.S. Huskisson & *M.J. Brown

*Clinical Pharmacology Unit, University of Cambridge Clinical School, Level 2, F&G Block, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ; †MRC Membrane Peptidase Research Group, Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT and **Microchemical Facility, Institute of Animal Physiology and Genetics Research, Babraham Hall, Babraham, Cambridge CB2 4AT

1 Despite the observation of pharmacological responses to neuropeptide Y (NPY) in mammalian kidneys, there are species differences in the ease with which specific NPY binding sites can be demonstrated; we have investigated whether this can be explained by differential metabolism of NPY by a membrane-bound peptidase.

2 NPY receptors were identified on cell membranes isolated from the rabbit kidney ($K_D = 97 \pm 16$ pM, $B_{max} = 290 \pm 30$ fmol mg^{-1} protein), and this preparation did not degrade [^{125}I]-NPY. However, a similar preparation of cell membranes from the rat kidney exhibited a much lower apparent receptor affinity (IC_{50} approximately 30 nM); these membranes rapidly degraded [^{125}I]-NPY to fragments which did not bind NPY receptors in either tissue.

3 [^{125}I]-NPY binding sites were revealed in the rat kidney when degradation was inhibited by insulin B chain. Chelating agents also inhibited degradation, but interfered with receptor binding. Binding sites could not be demonstrated in sections of rat kidney, even in the presence of insulin B chain.

4 The difference in degradative activity between rat and rabbit renal cell membranes, inhibition of degradation by chelating agents and insulin B chain, and insensitivity to phosphoramidon suggest that the enzyme responsible was endopeptidase-2, and this was confirmed by comparing the hydrolysis of [^{125}I]-NPY by purified enzyme with rat renal tissue. Activity of this enzyme explains the difficulties encountered demonstrating receptors in the rat kidney.

5 Renal cell membranes from the mouse digested [^{125}I]-NPY in a similar manner and this may be due to the closely related enzyme, meprin. NPY degradation has not previously been reported. The results suggest that NPY should be added to the list of peptides sensitive to these enzymes.

Keywords: Neuropeptide Y; metabolism; endopeptidase-2; neuropeptide Y receptor

Introduction

Neuropeptide Y (NPY), a 36 amino acid peptide first isolated from pig brain (Tatemoto, 1982), has been shown to colocalize with noradrenaline in the storage granules of peripheral sympathetic nerve termini (Lundberg *et al.*, 1982; Ekblad *et al.*, 1984; Freid *et al.*, 1985). Potent pharmacological pre- and postsynaptic effects have been described, leading to the suggestion that NPY is a neurotransmitter, neuromodulator or hormone; NPY contracts vascular smooth muscle directly, potentiates the response to noradrenaline and inhibits the electrically-evoked release of noradrenaline and adenosine 5'-triphosphate (ATP) (Lundberg *et al.*, 1982; Ekblad *et al.*, 1984; Edvinsson *et al.*, 1984a,b; Lundberg & Stjarne, 1984; Stjarne *et al.*, 1986). NPY exerts its effects through specific membrane bound receptors, subclassified as Y_1 and Y_2 on the basis of biological activity and their affinity for NPY fragments; these receptors are linked to classical transmembrane signalling mechanisms including G protein and calcium channel activation (Wahlstedt *et al.*, 1986; Pernow *et al.*, 1987b; Kassis *et al.*, 1987; Sheikh *et al.*, 1988; Reynolds & Yokota, 1988; Lundberg *et al.*, 1988). In the rabbit kidney, NPY is a renal vasoconstrictor (Allen *et al.*, 1986), while in the rat kidney, NPY induces vasoconstriction and natriuresis (Allen *et al.*, 1985a). It seems likely that these renal effects are also mediated by specific NPY receptors, which may respond *in vivo* to NPY released locally from storage sites in the juxtaglomerular apparatus (Ballesta *et al.*, 1984). However, Leys and her colleagues were unable to demonstrate [^{125}I]-NPY binding in rat kidney tissue sections, unlike the rabbit kidney where specific binding sites were readily seen (Leys *et al.*, 1987).

We also have experienced difficulty demonstrating [^{125}I]-NPY binding by autoradiography in the rat kidney, in contrast to the rabbit kidney (unpublished observations). In the course of our studies we have examined [^{125}I]-NPY binding in cell membrane preparations from each species, and set out to relate the apparent differences in receptor populations to the presence of different peptidase activities. The kidney is a rich source of peptidases, especially those located in the brush border membrane (for review see Kenny *et al.*, 1987). The inactivation of NPY has not been previously reported, but is likely to require the action of an endopeptidase, since it is amidated at the C-terminus and has penultimate proline residue at the N-terminus. For many peptides the initial attack by pig renal brush border membranes has been shown to require the action of endopeptidase-24,11 (Stephenson & Kenny, 1987). This peptidase is specifically inhibited by phosphoramidon, the use of which revealed that rat kidney brush border membranes contain a second, phosphoramidon-insensitive enzyme, endopeptidase-2 (Kenny *et al.*, 1981); this enzyme has been isolated and characterized in detail (Kenny & Ingram, 1987; Stephenson & Kenny, 1988; Barnes *et al.*, 1989). It is closely related to meprin, a peptidase first recognized in mouse kidneys (Beynon *et al.*, 1981). Thus neuropeptide binding studies on renal membranes may be complicated by significant degradation of the labelled peptide by either of these peptidases. Moreover, any observed differences in ligand binding to membranes from rat and rabbit

[1] Author for correspondence.

kidneys might relate to the presence of the second endopeptidase in the rat.

In the present study we have examined the binding of [^{125}]-NPY to cell membranes from rat and rabbit kidney and have demonstrated a role for endopeptidase-2 in rat membranes in degrading this peptide.

Methods

Materials were obtained from Sigma unless otherwise indicated.

Preparation of cell membranes

Fresh kidneys from adult New Zealand White rabbits, 10–20 week old male Wistar rats and 10–12 week old female Balb/c mice were weighed, minced and homogenized with three 30 s bursts at maximum speed with a Polytron homogenizer, in 10 volumes (w/v) ice-cold Tris/HCl 50 mM containing sucrose 0.32 M, PMSF 100 μM and EDTA 5 mM, pH 7.4. After centrifugation at 15,000 g for 20 min at 4°C to remove tissue fragments and whole cells, the supernatant was centrifuged at 50,000 g for 60 min at 4°C. The pellet was resuspended in Tris/HCl 50 mM, containing sucrose 0.32 M and EDTA 5 mM, pH 7.4 and centrifuged again at 50,000 g. The pellet was resuspended in Tris/HCl 50 mM, pH 7.4. The protein content of this 'cell membrane preparation' was measured by the modified Bradford technique (materials and methods from Bio-Rad) before it was stored at −70°C for up to 1 week before study.

Binding of [^{125}I]-neuropeptide Y to cell membranes

Cell membranes were incubated at a final protein concentration of 0.01 or 0.1 mg ml^{-1} in Tris/HCl 50 mM containing $MgCl_2$ 10 mM and bovine serum albumin (BSA) 0.5%, pH 7.4, with 20 pM [^{125}I]-NPY (Bolton-Hunter labelled, Amersham International) and various concentrations of porcine NPY from 0 to 1 μM for 60 min at 23°C; the volume was 250 μl. Termination of incubation was by filtration through Whatman GF/C filters that had been presoaked for 60 min in 0.3% polyethyleneimine (PEI): this reduced non-specific binding of [^{125}I]-NPY by over 80%. The filters were washed with 9 ml ice-cold Tris/HCl 50 mM containing BSA 0.2%, pH 7.4, and trapped radioactivity was measured in a Beckman gamma counter. Binding of [^{125}I]-NPY to rabbit renal cell membranes was also measured in the presence of vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP), substance P or substance K (neurokinin A), all 1 μM, in NPY. In certain studies, insulin B chain 0.6 mM was added to inhibit competitively endopeptidase-2; phosphoramidon 10 μM (Boehringer), thiorphan 10 μM, EDTA 1 to 10 mM, 1,10-phenanthroline 1 mM, PMSF 100 μM, leupeptin 500 μM, E-64 200 μM, antipain 160 μM, pepstatin A 1 mM, bacitracin 0.1%, bestatin 10 mM, amastatin 40 μM, and Des-^{2}Pro-bradykinin 50 μM (Bachem) were added alone or in combination to inhibit other membrane-bound peptidases.

K_D and B_{max} were calculated from the following relationships: $K_D = IC_{50}$-[[^{125}I]-NPY], where IC_{50} is the concentration of NPY required to inhibit specific [^{125}I]-NPY binding by 50%, and $B_{max} = B \times IC_{50}/$[^{125}I]-NPY] where B is the membrane bound [^{125}I]-NPY concentration in fmol mg^{-1}, calculated from the specific activity; [^{125}I]-NPY and NPY are assumed to have the same receptor binding properties. This method of data analysis avoids the problems associated with Scatchard analysis when there is high non-specific binding of the ligand (DeBlasi *et al.*, 1989).

Radioactive fragments of [^{125}I]-NPY collected from high performance liquid chromatography (h.p.l.c.) after digestion (see below) were substituted for [^{125}I]-NPY in binding assays to determine their ability to bind the rabbit renal NPY receptor under standard conditions. In these studies an equivalent amount of radioactivity to 20 pM [^{125}I]-NPY was added and incubation proceeded as above.

Purification of endopeptidase-2

Endopeptidase-2 was purified after release from rat renal microvillar membranes with papain by ion-exchange and gel filtration chromatography as previously described (Kenny & Ingram, 1987).

Degradation of [^{125}I]-neuropeptide

Cell membranes from rabbit, rat and mouse kidney at final protein concentrations of 0.01–0.1 mg ml^{-1} were incubated with 20 pM [^{125}I]-NPY in Tris/HCl 50 mM, pH 7.4 for up to 60 min at 23°C; the volume was 250 μl. Incubations with rat renal cell membranes were also performed at 4°C and 37°C, and at 23°C after heating the membranes to 65°C for 10 min. Incubation was terminated by centrifugation at 50,000 g for 10 min at 4°C. The supernatant was removed and 200 μl injected onto a C18 column for analysis by reverse phase h.p.l.c. (Waters system). The radioactivity of each sample was measured before injection to enable confirmation of successful elution from h.p.l.c. Radioactivity was eluted with a linear gradient of 10–80% acetonitrile in trifluoroacetic acid, 0.1%, over 50 min; the flow rate was 1 ml min^{-1} and there were 10 and 20 min equilibration phases at the beginning and end of each run respectively. The radioactive content of each 1 ml eluted fraction was measured in a Beckman gamma counter.

In a further series of experiments, insulin B chain 0.6 mM, a substrate for endopeptidase-2, was added (with phosphoramidon 10 μM, amastatin 40 μM, Des-^{2}Pro-bradykinin 50 μM and PMSF 100 μM to inhibit insulin B chain degradation by endopeptidase-24,11 and other enzymes) to determine whether it would competitively inhibit [^{125}I]-NPY degradation; the effects of the other peptidase inhibitors added in [^{125}I]-NPY binding experiments (see above) were studied in the same way.

[^{125}I]-NPY, 20 pM, was also incubated in 250 μl of the same buffer with 10 μm sections of rat kidney, cut on a Bright cryostat and freeze-thaw mounted onto gelatine-coated glass microscope slides (immersed in 5 mg ml^{-1} porcine gelatine in chrom alum 0.05% and allowed to dry), for 60 min at 23°C. The supernatant was carefully aspirated and analysed by h.p.l.c. as above. Insulin B chain 0.6 mM, phosphoramidon 10 μM, PMSF 100 μM, Des-^{2}Pro-bradykinin 50 μM and amastatin 40 μM were included in some experiments. The protein content of 10 identical, unmounted sections of rat kidney was measured by the modified Bradford assay as before.

[^{125}I]-NPY, 20 pM, was also incubated with 100 ng endopeptidase-2 in a final volume of 250 μl Tris/HCl 50 mM, pH 7.4, for up to 60 min at 23°C. Termination of incubation was by boiling for 5 min, and the solution was analysed by reverse phase h.p.l.c. as above.

Radioimmunoassay of fragments of [^{125}I]-neuropeptide Y

Radioactive material was collected after separation by h.p.l.c., concentrated by overnight evaporation of acetonitrile under vacuum, and resuspended in 100–250 μl Tris/HCl 50 mM, pH 7.4. [^{125}I]-NPY, 5–20 fmol, or an equivalent amount of radioactive fragments from h.p.l.c. separation was incubated at 23°C for 60 min with 1/10,000 (final concentration) rabbit anti-NPY antiserum and varying amounts of porcine NPY from 0 to 10,000 fmol in sodium phosphate buffer 50 mM containing Nonidet-P40 0.01%, BSA 0.05% and sodium azide 0.01%, pH 7.4; the volume was 1 ml. Incubation was terminated by adding 200 μl activated charcoal (2.5 mg ml^{-1} porcine gelatine, Na_2HPO_4 67 mM, KH_2PO_4 6 mM, EDTA 10 mM, sodium azide 0.05%, 20 mg ml^{-1} charcoal and 2 mg ml^{-1} dextran T70) and centrifuging the mixture at 3000 g for 15 min at 4°C. The supernatant (containing antibody-bound [^{125}I]-NPY)

was aspirated and its radioactive content measured in a Beckman gamma counter.

Nature of fragments of [^{125}I]-neuropeptide Y bound to rat renal cell membranes

The nature of radioactivity bound to rat renal cell membranes was analysed by terminating the binding assay (see above) by centrifugation at 50,000 *g* for 10 min at 4°C and washing and resuspending the pellet in an 'elution buffer' (Tris/HCl 50 mM, Triton X-100 0.1%, bacitracin 0.1%, EDTA 10 mM, leupeptin 1 mM, pepstatin A 1 mM, phosphoramidon 10 μM, PMSF 100 μM, pH 4). After incubation at 4°C for 15 min, the membranes were centrifuged at 50,000 *g* for 10 min at 4°C and the supernatant analysed by h.p.l.c. (see below); successful elution was confirmed by measuring radioactivity remaining in the membrane pellet.

Data analysis

Results were analysed by non-parametric rank sums testing (Mann Whitney).

Results

Binding of [^{125}I]-neuropeptide Y to cell membranes

[^{125}I]-NPY bound to rabbit renal cell membranes was displaced by porcine NPY but not ANP VIP, substance P or substance K (Figure 1a). Equilibrium binding was achieved rapidly under the conditions described (Figure 1b). The K_D was 97 ± 16 pM, and B_{max} was 290 ± 30 fmol mg^{-1} protein. [^{125}I]-NPY was also displaced from binding to rat renal cell membranes, but only when the protein content of the assay was reduced to 10 μg ml^{-1} (Figure 1a). The IC_{50} for this binding was approximately 30 nM; binding was increased at this protein concentration in the presence of insulin B chain 0.6 mM (Table 1). This concentration of insulin B chain did not alter the displaceable binding of [^{125}I]-NPY to rabbit renal cell membranes (Table 1); addition of other peptidase inhibitors did not alter binding except for the chelating agents EDTA and 1,10-phenanthroline, which inhibited binding to rat and rabbit renal cell membranes (Table 2); the effects on binding to rat membranes were greater than to rabbit membranes.

Fragments of [^{125}I]-NPY did not bind to rabbit renal cell membranes to any significant degree: only 2 to 4% of added radioactivity bound in the absence and presence of NPY (1 μM), compared with 50 ± 4 and 23 ± 5% (n = 3) respectively for undigested [^{125}I]-NPY.

Degradation of [^{125}I]-neuropeptide Y

The supernatant fraction after pelleting rat renal cell membranes incubated with [^{125}I]-NPY contained radioactivity that eluted from h.p.l.c., with 3 distinct major peaks and 2 smaller peaks (Figure 2a); all of the radioactivity introduced onto the C18 column eluted under the conditions chosen. Peaks 1 and 2 had retention times of 31–34 and 35–36 min respectively, and peak 3 (retention time 60–64 min) corresponded to undigested [^{125}I]-NPY (Figure 2g). Disappearance from the supernatant of the peak of radioactivity corresponding to [^{125}I]-NPY occurred more quickly at higher concentrations of membrane bound protein (Figure 3). [^{125}I]-NPY incubated with endopeptidase-2 generated a similar radioactive profile of h.p.l.c. (Figure 2d), as did radioactivity in the supernatant of mouse renal cell membranes (Figure 2b), but that incubated with rabbit renal cell membranes eluted with an identical profile to intact [^{125}I]-NPY (Figure 2c); the small peak observed at 3 min is thought to represent free ^{125}iodine. Degradation by endopeptidase-2 proceeded rapidly, with 60% hydrolysis after 5 min under the conditions studied. At 10 μg ml^{-1} membrane protein, degradation proceeded more rapidly at 37°C (24 ± 6% recovery of [^{125}I]-NPY after 10 min compared with 43 ± 5% at 23°C, $P < 0.05$), and was

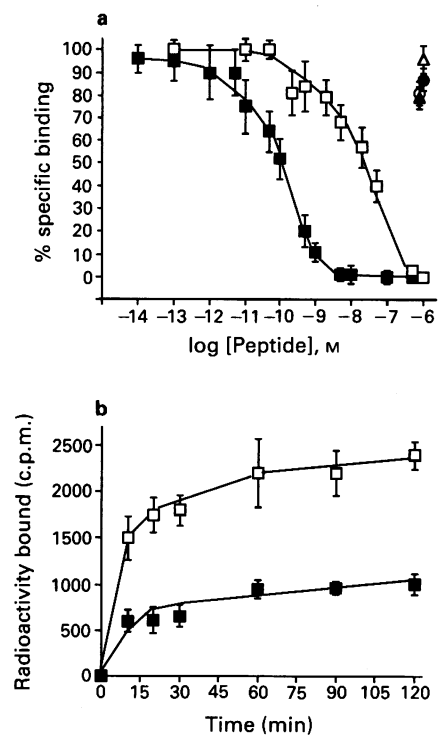

Figure 1 (a) Binding of [^{125}I]-neuropeptide Y ([^{125}I]-NPY) to rabbit renal cell membranes in the presence of NPY (■), vasoactive intestinal polypeptide (△), atrial natriuretic peptide (●), substance P (○) and substance K (▲), and to rat renal cell membranes (□). Incubations with 10 μg ml^{-1} membrane protein were for 60 min at 23°C. Results are mean (n = 6) with s.e.mean shown by vertical bars. (b) Time-dependency of binding of [^{125}I]-NPY to rabbit renal cell membranes at 23°C in the presence (■) and absence (□) of NPY (1 μM). Results are mean (n = 3), with s.e.mean shown by vertical bars.

Table 1. Percentage binding of [^{125}I]-neuropeptide Y ([^{125}I]-NPY) to rat and rabbit renal cell membranes (10 μg ml^{-1} protein) at 23°C for 60 min: effect of insulin B chain (0.6 mM)

	% of added [^{125}I]-NPY bound	*% of bound [^{125}I]-NPY that was displaceable*
Rat kidney (n = 6)		
Control	24 ± 4	7 ± 4
Insulin B chain	53 ± 6*	17 ± 5*
Rabbit kidney (n = 3)		
Control	45 ± 6	64 ± 7
Insulin B chain	41 ± 6	61 ± 5

Results are mean ± s.e.mean.
* = significant differences from control, $P < 0.05$.

Table 2 Effect of peptidase inhibitors on percentage [^{125}I]-neuropeptide Y ([^{125}I]-NPY) bound to rat and rabbit renal cell membranes (10 μg ml^{-1} protein, 60 min incubations at 23°C) in the presence and absence of NPY (1 μM)

	Rat kidney		*Rabbit kidney*	
	NPY: 0	1 μM	0	1 μM
Peptidase inhibitor:				
No inhibitor	18.5 ± 3	11.5 ± 3	50 ± 4	23 ± 5
EDTA 1 mM	6.5 ± 1	7 ± 2	52 ± 5	30 ± 6
EDTA 5 mM	3 ± 2	2.5 ± 1	33 ± 7	25 ± 3
1,10-phenanthroline 1 mM	4 ± 2.5	4 ± 2	34 ± 4	24.5 ± 3

Results are mean ± s.e.mean, $n = 3$.

reduced by incubation at 4°C (68 ± 12% recovery after 30 min compared with 31 ± 3% at 23°C, $P < 0.05$). Preheating the cell membranes to 65°C for 10 min before assay reduced degradation; 70 ± 4% [^{125}I]-NPY was recovered after 10 min incubation at 23°C ($P < 0.05$) ($n = 4$–13 for this series of assays). Peptidase inhibitors did not reduce degradation by rat renal cell membranes (Table 3), but the degradable peptide insulin B chain reduced the rate of degradation by about 50% (Table 3, $P < 0.05$).

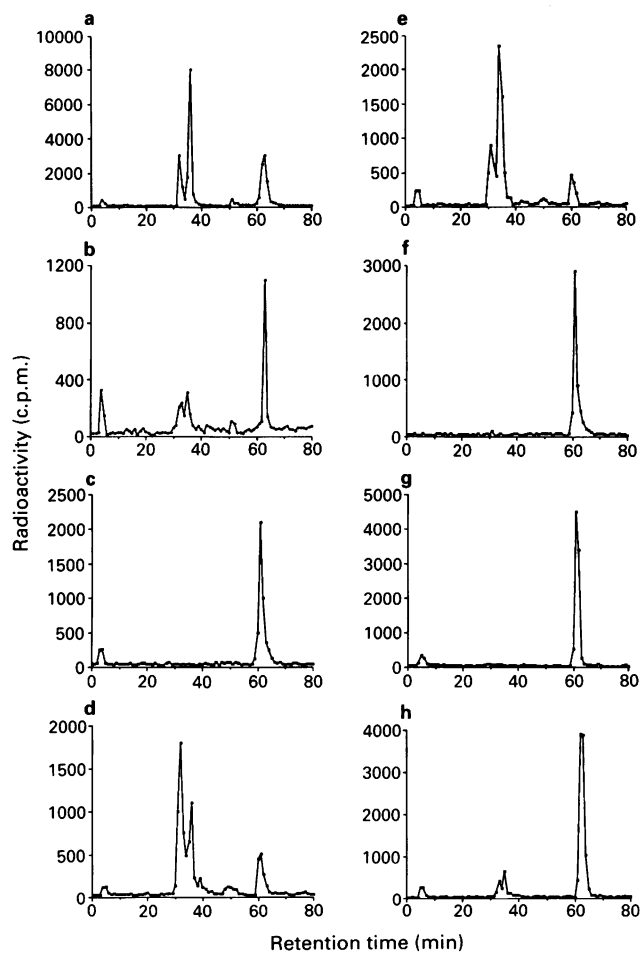

Figure 2 Radioactivity (counts per minute, c.p.m.) contained in sequential 1 ml fractions eluted from h.p.l.c. after applying the supernatants from (a) rat (10 μg ml^{-1} protein), (b) mouse (10 μg ml^{-1} protein) or (c) rabbit (100 μg ml^{-1} protein) renal cell membranes, (d) 100 ng endopeptidase-2 or (e) 10 μM sections of rat kidney, incubated with [^{125}I]-neuropeptide Y ([^{125}I]-NPY) for 60 min at 23°C; (f) shows the h.p.l.c. profile of membrane-bound radioactivity, which was eluted from rat renal cell membranes at the end of incubation, (g) shows [^{125}I]-NPY incubated with buffer alone for 60 min at 23°C and (h) is the h.p.l.c. profile of radioactivity in the supernatant of rat renal cell membranes incubated as above but in the presence of insulin B chain 0.6 mM.

[^{125}I]-NPY was also degraded by tissue sections of rat kidney mounted on glass slides (Figure 2e), but not by sections of rabbit kidney. The protein content of each section was 580 ± 21 μg. In this situation insulin B chain, in the presence or absence of other peptide inhibitors, had no effect.

Radioimmunoassay of fragments of [^{125}I]-neuropeptide Y

The only radioactive material in the supernatant of rat renal cell membranes that bound anti-NPY was that which coeluted from h.p.l.c. with [^{125}I]-NPY, namely 'peak 3' (Figure 4). The other fragments bound to anti-NPY at background levels only.

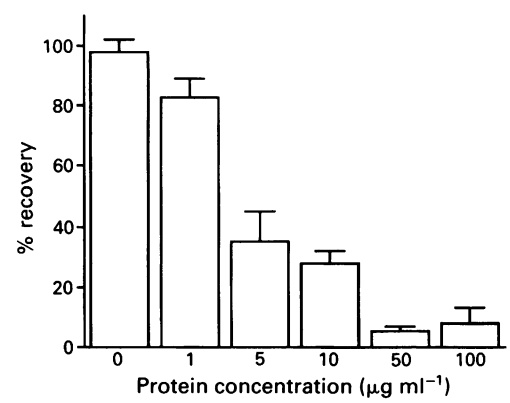

Figure 3 Percentage recovery of [^{125}I]-neuropeptide Y from the supernatant of rat renal cell membranes, incubated for 60 min at 23°C. Results are mean ($n = 3$) with s.e.mean shown by vertical bars.

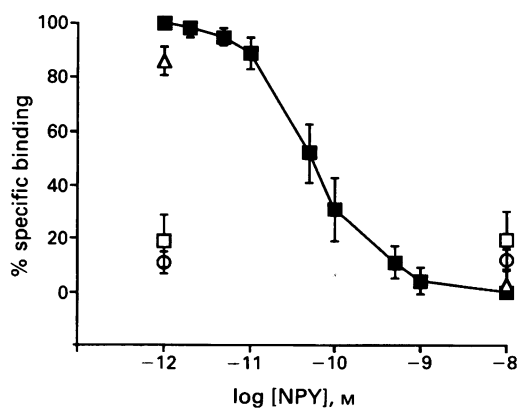

Figure 4 Radioimmunoassay of neuropeptide Y (NPY) using intact and hydrolysis products of [^{125}I]-NPY [■ = untreated [^{125}I]-NPY, ○ = 'peak 1' (retention time 31–34 min), □ = 'peak two' (retention time 35–36 min), △ = 'peak 3' (retention time 60–64 min); see Methods and Figure 2 for details]. Results are mean of 4 values from 2 assays; vertical bars show s.e.mean.

Table 3 Effect of peptidase inhibitors on degradation of [^{125}I]-neuropeptide Y ([^{125}I]-NPY) incubated with rat renal cell membranes for 60 min at 23°C

Peptidase inhibitor	% [^{125}I]-*NPY undigested*
No inhibitor	38.5 ± 11, $n = 4$
Insulin B chain 0.6 mM	89 ± 11, $n = 4$
1,10-phenanthroline 1 mM	88.5
EDTA 5 mM	78
EDTA 1 mM	65
Bestatin 10 mM, bacitracin 0.1%, leupeptin 500 μM, pepstatin A 1 μM, phosphoramidon 10 μM and PMSF 100 μM	51
E-64 200 μM, thiorphan 10 μM, antipain 160 μM and amastatin 400 μM.	45
Amastatin 400 μM	40.5
Des-^{2}Pro-bradykinin 50 μM	36
Thiorphan 10 μM	30.5
Antipain 160 μM	26
E-64 200 μM	24

Results are expressed as the % of radioactivity applied to h.p.l.c. that co-eluted with undigested [^{125}I]-NPY.

Nature of fragments of [^{125}I]-neuropeptide Y bound to rat renal cell membranes

Less than 10% of radioactivity remained bound to rat renal cell membranes after treatment with 'elution buffer'. Eluted radioactivity exhibited the h.p.l.c. profile of undigested [^{125}I]-NPY (Figure 2g).

Discussion

The methods used in this study demonstrated specific NPY binding sites in the rabbit kidney, and these membranes did not degrade [^{125}I]-NPY. Demonstration of receptors on rat renal cell membranes was hampered however by hydrolysis of [^{125}I]-NPY to several fragments by membrane-associated peptidases; none of the radioactive fragments was able to bind the NPY receptor in rat or rabbit kidney, and this did not appear to be due to any susceptibility of the fragments to the conditions used for h.p.l.c. or concentration process. As equilibrium conditions could not be achieved, binding data have not been calculated for the rat kidney. NPY degradation during the assay is suggested by the apparently high IC_{50} for that tissue: the estimate obtained is not indicative of the true receptor affinity, as the concentration of displacing ligand present at the termination of incubation was much lower than the amount added. It seems unlikely that such a high IC_{50} for the rat renal NPY receptor could be explained by a much higher affinity for [^{125}I]-NPY, or lower affinity for NPY, than the rabbit receptor, though neither possibility can be excluded without the means to prevent ligand degradation. If the affinities of the rabbit and rat renal NPY receptors were the same, the large increase in measured IC_{50} for the latter must be attributed to a more rapid rate of degradation of NPY than [^{125}I]-NPY: NPY might be a preferred substrate for endopeptidase-2 in view of the iodinated Bolton-Hunter moiety in the radioactive form. Equilibrium conditions for [^{125}I]-NPY binding in the rat kidney are unlikely to be achieved without the use of a specific inhibitor for the peptidase(s). Unfortunately chelating agents, capable of inhibiting metallo-endopeptidases, also inhibited [^{125}I]-NPY binding, possibly by chelating other cations which have been reported to increase NPY-receptor binding (Unden *et al.*, 1984). Since these experiments were completed we have observed that actinonin (3-[[1-[[2-(hydroxymethyl)-1 pyrolidinyl]carbonyl] - 2 methylpropyl]carbamoyl]octano - hydroxamate) is a potent, though non-specific inhibitor of endopeptidase-2 (Kenny & Ingram, unpublished observation) and the use of this reagent might permit equilibrium ligand binding conditions to be achieved with rat membranes. Some success was obtained with insulin B chain as a competing substrate. When present at 38 times its K_m for endopeptidase-2 (Kenny & Ingram, 1987) it inhibited degradation sufficiently to increase displaceable binding only when the amount of protein in the binding assay was reduced to 10 μg ml^{-1}. Even then, inhibition was not complete, and this concentration was ineffective as an inhibitor when [^{125}I]-NPY was incubated with sections of rat kidney. The extent to which [^{125}I]-NPY degradation and binding in the cell membrane preparation and tissue sections differed was unexpected. It is possible that the sections contained additional peptidases, not present in the membrane fraction, which degraded [^{125}I]-NPY or insulin B chain. More importantly however, the protein content in the membrane binding assay was only 2.5 μg, but the protein content of rat renal tissue sections was more than 230 times greater. Apart from the presence of intra- and intercellular protein as well as membrane associated protein, there will probably have been much more endopeptidase-2 activity in kidney sections. Complete degradation of [^{125}I]-NPY alone could account for the failure to demonstrate NPY receptors in rat kidney sections, as the radioactive fragments did not bind. Using the V_{max} for the digestion of insulin B chain by endopeptidase-2 (0.25 μmol min^{-1} mg^{-1}), calculated by Kenny & Ingram (1987), and an estimate (based on results given in Figure 3) that the rat renal cell membranes contained 40 ng endopeptidase-2 per μg protein, it can be anticipated that, effectively, all of the insulin B chain added to glass slide mounted tissue sections (150 nmol in 250 μl buffer) would be digested at the end of 60 min, even allowing for a drop in digestion rate as the concentration of insulin B chain fell. This contrasts with an expected fall in insulin B concentration of approximately 1% during incubation with 2.5 μg membrane protein from homogenized rat kidneys. Thus insulin B chain is unlikely to be an effective competitive inhibitor of [^{125}I]-NPY hydrolysis by endopeptidase-2 in sections of rat kidney.

The mechanism of clearance of NPY from the circulation, or its degradation has not previously been reported. Endopeptidase-2 is found in rat, but not rabbit kidneys and is known to be sensitive to cation chelating agents but resistant to other protease inhibitors. Furthermore there was considerable similarity between the h.p.l.c. characteristics of radioactive fragments of [^{125}I]-NPY produced by endopeptidase-2 and rat renal cell membranes. It appears from our data that NPY is a substrate for endopeptidase-2, and this may be a mechanism of physiological importance in the rat. In mice, meprin may perform a clearance function. In man, the digestion of NPY by the intestinal enzyme PABA-peptide hydrolase (Sterchi *et al.*, 1982) is worthy of study: PABA-peptide hydrolase has many similarities to rat endopeptidase-2 (Barnes *et al.*, 1989), and NPY has demonstrable pharmacological effects on human vascular tone *in vivo* (Allen *et al.*, 1985b; Pernow *et al.*, 1987a).

Species and organ differences in NPY metabolism may thus hinder the study of NPY receptors. Degradation of [^{125}I]-

NPY accounts for the difficulty demonstrating receptors in the rat kidney noted in the present study and previously. It was not possible to overcome degradation in a satisfactory way that would permit analysis of equilibrium binding data, as no specific inhibitor of endopeptidase-2 is yet available. Whether NPY is the substrate for other peptidases, or whether enzymes with a similar potential as endopeptidase-2 and meprin are more widespread throughout mammalian tissue remains to be seen. If identified, such degrading enzymes may become the target for inhibitors with therapeutic uses, for example to potentiate inotropic support in shock, where there is experimental evidence for a beneficial effect of NPY (Evoquez *et al.*, 1987). Further work is now required to establish the importance of these enzymes in the metabolism of NPY, to perform kinetic analysis using the native peptide and to identify any products of degradation which may exhibit biological activity.

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Methylxanthine-induced attenuation of pecking in chickens

[1]M.R. Zarrindast & T. Nasir

Department of Pharmacology, Medical Faculty, University of Tehran, Tehran, Iran

1 Apomorphine induced dose-dependent pecking in chickens.

2 The response was decreased by theophylline or caffeine in a dose-dependent manner.

3 Administration of theophylline or caffeine alone did not exert any effect on pecking behaviour.

4 Dipyridamole administration neither induced pecking nor altered the pecking induced by apomorphine.

5 Administration of 5-N-ethylcarboxamide-adenosine to animals caused variable effects on pecking induced by apomorphine. The drug did not induce pecking by itself.

6 It is concluded that the inhibitory response to methylxanthines may be mediated through blockade of adenosine receptors. However, to prove this, more experiments are needed.

Keywords: Apomorphine; pecking; theophylline; caffeine; adenosine receptors; 5-N-ethylcarboxamide-adenosines.

Introduction

Available evidence suggests that adenosine is a neurotransmitter in the central nervous system (Snyder, 1985). The extracellular adenosine and adenine nucleotide are capable of altering the cellular functions in most organ systems (Stiles, 1986). The effects of adenosine in brain seem to be mediated through two separate receptors termed A_1 and A_2 sites. A_1 receptors inhibit adenylate cyclase and A_2 receptors stimulate adenylate cyclase (Van Calker *et al.*, 1979; Londos *et al.*, 1980). Whereas A_1 receptor sites are widely distributed in brain, A_2 receptors are localized in dopamine-rich brain areas such as striatum, nucleus accumbens and olfactory tubercle (Bruns *et al.*, 1986).

Methylxanthines have a number of pharmacological properties. The behavioural stimulant effects of xanthines such as caffeine and theophylline, appear to involve blockade of adenosine receptors (Daly, 1982), however, there is evidence that caffeine and theophylline analogues function as adenosine receptor antagonist and also as phosphodiesterase inhibitors (Choi *et al.*, 1988). Early studies indicated that methylxanthines could induce and potentiate turning in rats with unilateral striatal lesions (Fuxe & Ungerstedt, 1974; Arbuthnott *et al.*, 1974; Fredholm *et al.*, 1976). Our previous work showed that the yawning behaviour induced by physostigmine or apomorphine (Zarrindast & Poursoltan, 1989), morphine antinociception (Zarrindast & Moghaddampour, 1989) and licking induced by apomorphine (unpublished data) could be reduced by theophylline pretreatment. In the present study the effects of methylxanthines on pecking induced by apomorphine have been investigated.

Methods

Chickens of either sex weighing 90–150 g (7–17 days old) were used in these experiments. Groups of 5 animals were housed in large cages under a 12 h light/dark cycle. Food and water were freely accessible except during the time of experiments. To perform the experiments, chicken were placed individually in a glass cylinder (28 cm diameter, 30 cm height) and allowed to habituate 30 min before drug injection. Pecking counts were recorded by a direct observer for a period of 30 min after drug administration. All drugs were administered intraperitoneally (i.p.) except for apomorphine which was injected subcutaneously (s.c.). The analysis of data was performed by analysis of variance followed by Student's t test and differences with $P < 0.05$ were considered significant.

[1] Author for correspondence.

Drugs

The following drugs were used: apomorphine hydrochloride (Apo: McFarlan, Smith Ltd, England), theophylline, NECA(5'-N-ethylcarboxamide-adenosine) and dipyridamole crystalline (Sigma, England) and caffeine (E. Merck, Germany). All drugs were dissolved in distilled water except dipyridamole which was suspended in carboxymethylcellulose (1%).

Results

Subcutaneous (s.c.) injection of different doses of Apo induced a dose-related increase of pecking behaviour in chickens (see Figure 1). The maximum response was obtained with 0.5 mg kg^{-1} of the drug.

Effects of methylxanthines on apomorphine-induced pecking

The effects of caffeine on pecking induced by Apo are shown in Figure 2. Intraperitoneal (i.p.) administration of different doses of caffeine (25 and 50 mg kg^{-1}) 60 min before Apo (0.25 mg kg^{-1}, s.c.) decreased the pecking induced by Apo. The inhibitory effect of caffeine was dose-dependent. The response to the same effect of Apo was also reduced in animals pretreated with different doses (10 and 20 mg kg^{-1}, i.p., 60 min) of theophylline (Figure 3). The effect of theophylline was exerted in a dose-dependent manner. Results in Figure 4 demonstrate that a single injection of either caffeine (25 mg kg^{-1}, i.p.) or theophylline (10 mg kg^{-1}, i.p.) 60 min before Apo administration decreased the pecking induced by the different doses of Apo (0.125, 0.25 and 0.5 kg^{-1}, s.c.).

Effect of dipyridamole or 5'-N-ethylcarboxamide-adenosine on pecking induced by apomorphine

Pretreatment of animals with different doses of dipyridamole (30, 60 and 90 mg kg^{-1}, i.p., 60 min) did not alter the Apo (0.25 mg kg^{-1}, s.c.)-induced pecking response (Figure 5). Administration of the same doses of dipyridamole alone, did not induce pecking (not shown). Pretreatment of animals with NECA (0.01 mg kg^{-1}, i.p., 60 min) increased the Apo-induced pecking, while a large dose of drug (1 mg kg^{-1}, i.p., 60 min) decreased the response to Apo (Figure 6). Administration of different doses of NECA alone (0.005, 0.01, 0.1 and 1 mg kg^{-}) did not induce pecking (data not shown).

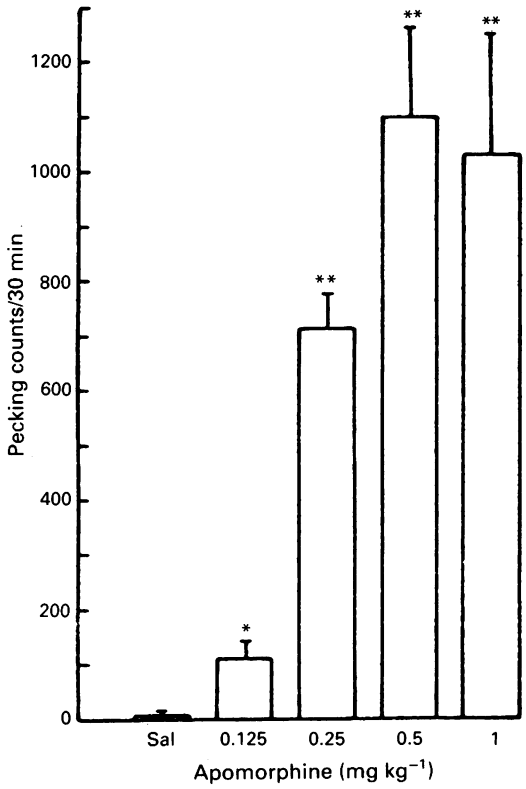

Figure 1 Effect of apomorphine (Apo) on pecking behaviour in chickens. Animals were injected subcutaneously (s.c.) with saline (Sal, 2.5 ml kg^{-1}) or different doses of Apo (0.125, 0.25, 0.5 or 1 mg kg^{-1}). Each column shows the mean of total pecking counts for a period of 30 min after drug injection ($n = 7$); vertical bars show s.e.mean. *$P < 0.01$; **$P < 0.001$ different from saline-treated animals.

Discussion

The involvement of dopaminergic mechanisms in turning, locomotion, stereotyped behaviour (Waddington & O'Boyle, 1989) and pecking (Amsler, 1923; Deshpande *et al.*, 1961; Cheng & Long, 1974; Brunelli *et al.*, 1975) has been shown previously. Evidence also supports a role for adenosine in the

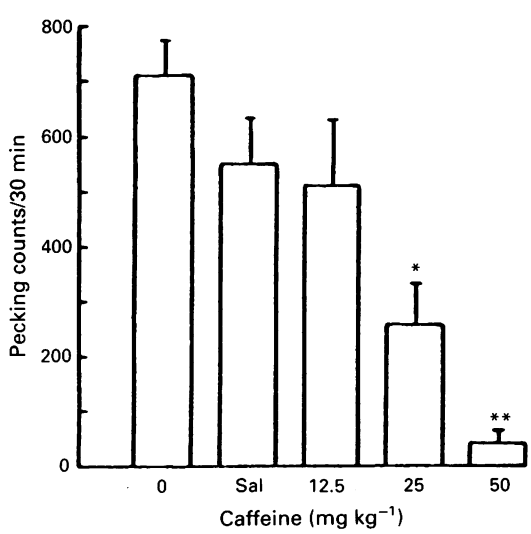

Figure 2 Effect of caffeine on apomorphine (Apo)-induced pecking in chickens. Animals were injected (s.c.) with Apo (0.25 mg kg^{-1}) alone or in combination with either injection (i.p.) of saline (Sal, 2.5 ml kg^{-1}) or different doses of caffeine (12.5, 25 or 50 mg kg^{-1}) 60 min before Apo administration. Pecking response was recorded for 30 min after Apo injection. Each column shows the mean response of at least 7 animals; vertical bars show s.e.mean. *$P < 0.02$; **$P < 0.001$ different from saline + Apo-treated groups.

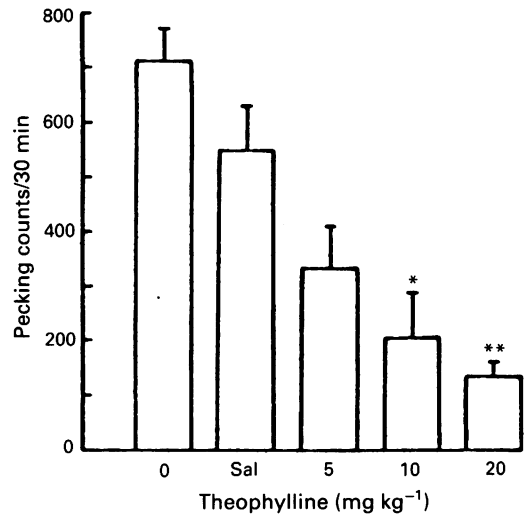

Figure 3 Effect of theophylline on apomorphine (Apo)-induced pecking in chickens. Animals were injected with Apo (0.25 mg kg^{-1}, s.c.) alone or were pretreated with saline (Sal, 2.5 ml kg^{-1}, i.p.) or different doses of theophylline (5, 10 or 20 mg kg^{-1}, i.p.) 60 min before Apo injection. Pecking was recorded for a period of 30 min after Apo administration. Each column shows the mean response of at least 7 animals; vertical bars show s.e.mean. *$P < 0.01$; **$P < 0.001$ different from saline + Apo-treated groups.

mammalian CNS functions (Jarvis & Williams, 1987). Previous studies also show that xanthines induce and potentiate turning in rats with unilateral lesions (Jarvis & Williams, 1987) and increase locomotion or schedule-controlled responding in rodents or monkeys (Snyder *et al.*, 1981; Carney, 1982; Katims *et al.*, 1983; Glowa & Spealman, 1984).

The psychomotor stimulant effects induced by caffeine or theophylline have been attributed to adenosine antagonism.

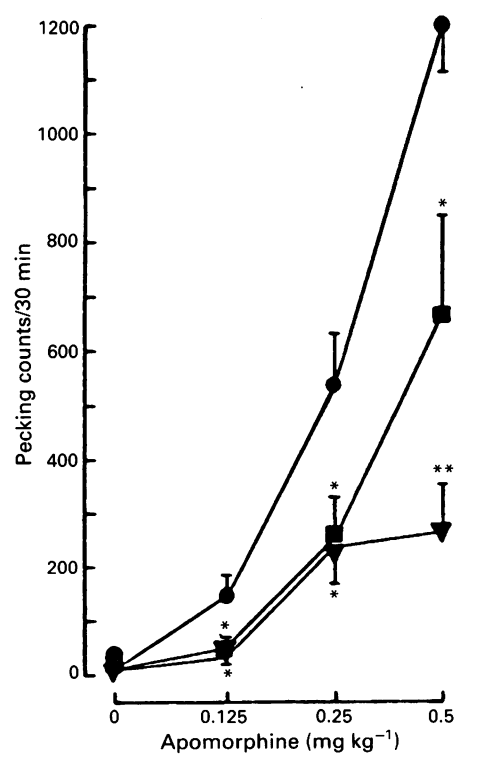

Figure 4 Effects of methylxanthines on pecking induced by apomorphine (Apo). Chickens were pretreated with saline (●, 2.5 ml kg^{-1}, i.p.), caffeine (■, 25 mg kg^{-1}, i.p.) or theophylline (▼, 10 mg kg^{-1}, i.p.) 60 min before different doses of Apo (0.125, 0.25 or 0.5 mg kg^{-1}, s.c.) administration. Pecking was recorded for 30 min. Each point is the mean of at least 7 animals; vertical bars show s.e. mean. *$P < 0.01$; **$P < 0.001$ different from saline + Apo-treated animals.

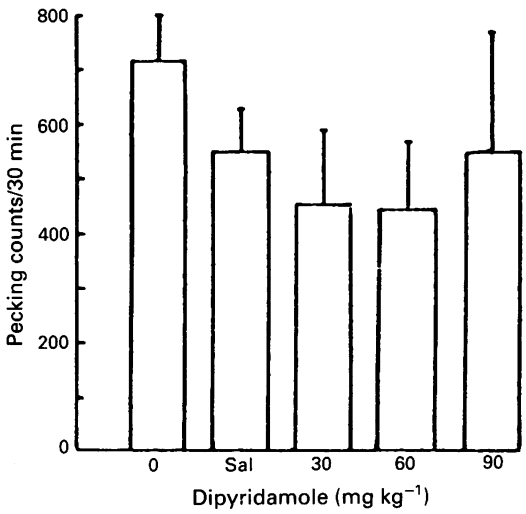

Figure 5 Effect of dipyridamole on pecking induced by apomorphine (Apo) in chickens. Animals were injected with Apo (0.125 mg kg^{-1}, s.c.) alone, saline (Sal, 2.5 ml kg^{-1}, i.p.) or different doses of dipyridamole (30, 60 or 90 mg kg^{-1}, i.p.) 60 min prior to Apo administration. Pecking was recorded for 30 min. Each column is the mean of at least 7 experiments; vertical bars show s.e.mean.

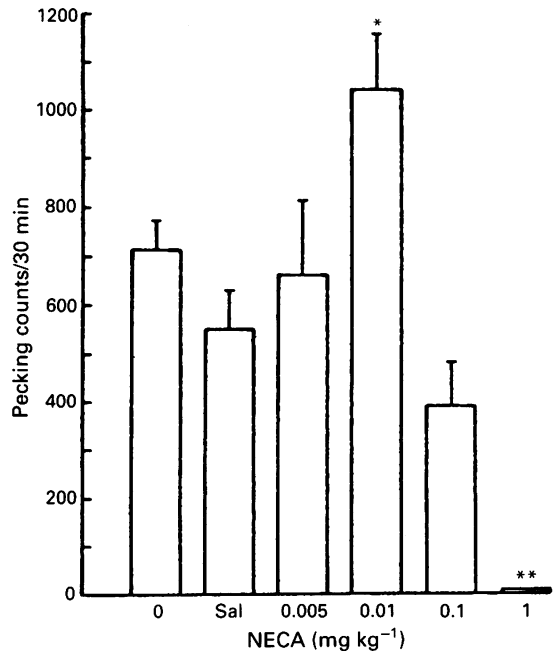

Figure 6 Effect of 5-N-ethylcarboxamide adenosine (NECA) on apomorphine (Apo)-induced pecking in chickens. Animals were administered Apo (0.25 mg kg^{-1}, s.c.) alone, saline (Sal, 2.5 ml kg^{-1}, i.p.) or NECA (0.005, 0.01, 0.1 or 1 mg kg^{-1}, i.p.) 60 min before Apo injection. Pecking was recorded for a period of 30 min. Each column is the mean of at least 7 experiments; vertical bars show s.e.mean. *$P < 0.01$; **$P < 0.001$ different from saline + Apo-treated animals.

However, some data showed that 3-isobutyl-1-methylxanthine (IBMX), a potent adenosine receptor antagonist, caused behavioural depression rather than stimulation (Snyder *et al.*, 1981). The present study was undertaken to investigate the interactions of adenosine mechanisms with dopamine-mediated pecking behaviour.

The results demonstrate that caffeine and theophylline, both of which are adenosine antagonists and phosphodiesterase inhibitors (Choi *et al.*, 1988), caused a dose-dependent decrease in pecking induced by Apo. It is unlikely that the decrease in pecking can be explained by inhibition of phosphodiesterase because it has been proposed that dopamine receptor hypersensitivity may be associated with a decreased phosphodiesterase activity (Von Voigtlander *et al.*, 1975). If inhibition of phosphodiesterase had occurred, the methylxanthines used in the present work would have increased rather than decreased the pecking.

The possibility has also been raised that methylxanthines, caffeine or theophylline exert their actions on central dopamine mechanism over and above those that can be explained by phosphodiesterase inhibition in effector cells (Fredholm *et al.*, 1976). However, in the dose-range employed in this study, the methylxanthines are generally considered to act as adenosine antagonists; blockade of adenosine receptors is the most likely mechanism for the inhibition of Apo-induced pecking.

The subtypes of adenosine recognition sites termed A_1 and A_2 have been distinguished biochemically as having opposite influences on adenylate cyclase activity (Van Calker *et al.*, 1979; Londos *et al.*, 1980). Methylxanthines are not selective for the two subtypes of adenosine receptor (Bruns *et al.*, 1986). Therefore it may be difficult to differentiate the involvement of A_1 or A_2 receptor sites in pecking or other stereotyped behaviours. Considering that the A_2 receptors which are linked to adenylate cyclase increase adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Van Calker *et al.*, 1979; Londos *et al.*, 1980) and the D_1 dopamine receptor activation which may be involved in pecking (Zarrindast & Amin, 1991) also increase the cyclic AMP levels (Kebabian & Calne, 1979), the possibility should be considered that reduction of Apo-induced pecking by caffeine or theophylline may be mediated through inhibition of A_2 receptor sites. Dipyridamole, a phosphodiesterase inhibitor and an adenosine uptake blocker, potentiated the turning induced by L-DOPA in rats with unilateral denervation of dopaminergic neurones (Fredholm *et al.*, 1976). In these experiments, the drug was not able to alter pecking induced by Apo. The data may further indicate that phosphodiesterase blockade or increase in levels of adenosine are not involved in the inhibition of Apo-induced pecking.

It has been reported that NECA (an adenosine agonist) decreases locomotion (Heffner *et al.*, 1989). In the present study, depending upon the dose used, NECA administration exerted opposite effects on Apo-induced pecking. A small dose of the drug (0.01 mg kg^{-1}) increased the Apo response, while a large dose of the drug (1 mg kg^{-1}) inhibited the Apo-induced pecking. NECA has affinity for both A_1 and A_2 receptors (Bruns *et al.*, 1989). High doses of the adenosine agonists may induce ataxia (Heffner *et al.*, 1989) and the A_1 activation may also cause sedation (Stiles, 1986). Therefore it is possible that the inhibition of Apo-induced pecking is mediated either through activation of the A_1 receptors, or is the result of sedation and ataxia caused by higher dose of NECA, whereas small doses of the drug increase the effect of Apo through A_2 activation.

To clarify the exact mechanism of the inhibitory action of caffeine and theophylline on the response to Apo, more studies are required.

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Effects of okadaic acid and ATPγS on cell length and Ca^{2+}-channel currents recorded in single smooth muscle cells of the guinea-pig taenia caeci

[1]R.J. Lang, I.Z. Ozolins & [2]R.J. Paul

Department of Physiology, Monash University, Clayton, Victoria, 3168, Australia

1 The effects of inhibiting phosphatase activity on Ca^{2+}-channel currents and cell shortening in single cells of the guinea-pig taenia caeci were investigated by whole-cell voltage clamp and video recording techniques.

2 Ca^{2+}-channel currents were isolated by use of pipette solutions containing Cs, tetraethylammonium and adenosine triphosphate (ATP) (3 mM). Ca^{2+} or Ba^{2+} (7.5 mM) in the bathing solution acted as the charge carrier during inward current flow.

3 Ca^{2+}-channel currents in 7.5 mM Ba^{2+} (I_{Ba}) were recorded at potentials positive to −40 mV, were maximal near 0 mV and reversed near +60 mV. Both the inward and outward flow of current was blocked by 100 μM Cd^{2+}.

4 Addition of the ATP analogue, adenosine 5′-*O*(3-thiotriphosphate) (ATPγS) (1 mM) to the pipette solution (containing 3 mM ATP) caused cell shortening to 23 ± 2% (n = 5) of their initial length within 5 min. Control cells (containing 4 mM ATP) did not contract during recording periods up to 60 min in duration.

5 I_{Ba}, recorded 1–2 min after membrane rupture, was 134 ± 19 (n = 13) pA, compared with 209 ± 25 (n = 5) pA in control cells, otherwise there were no significant time-dependent effects of ATPγS. In particular, ATPγS did not prevent the decrease in amplitude, nor the acceleration of inactivation when Ca^{2+} (7.5 mM) replaced Ba^{2+} as the permeating ion.

6 Okadaic acid (OA) (50 μM), a chemical inhibitor of phosphatase activity, produced similar effects when applied intracellularly. When OA (25 μM) was applied extracellularly the rate of rundown of I_{Ba} was slowed.

7 Isoprenaline (1 μM) alone had no effect on I_{Ba}, but induced a small increase in I_{Ba} in the presence of OA (25 μM).

8 Thus, our results indicate that (1) the contractions in ATPγS and OA may well arise from the activation of a kinase which phosphorylates myosin at low concentrations of Ca^{2+}, and (2) changes in the state of phosphorylation of Ca^{2+} channels, or associated proteins, in the taenia caeci modulate their function, but probably not via mechanisms involving cyclic AMP-dependent protein kinases.

Keywords: Taenia caeci; ATPγS; okadaic acid; Ca^{2+} channels; whole-cell voltage clamp; single smooth muscle cells; phosphatase inhibition

Introduction

Mechanisms involving phosphorylation/dephosphorylation of cell proteins play a major role in the regulation of contractility in smooth muscle. Phosphorylation of the 20 kD protein, myosin light chain, is thought to be obligatory for initiating the interaction between actin and myosin, whereas, dephosphorylation has been suggested to lead to a crossbridge with a slow detachment rate, the so-called 'latch bridge'. Calcium sensitivity is conferred by the calcium-calmodulin dependence of the myosin light chain kinase (for review, see Paul, 1989). Inhibition of phosphoprotein phosphatases, however, induces contraction in various smooth muscles (Shibata *et al.*, 1982; Bialojan *et al.*, 1988) which are not necessarily dependent on Ca^{2+} or calmodulin (Ishihara *et al.*, 1989; Obara *et al.*, 1989).

The presence of Ca^{2+}-selective ion channels and ion pumps in the plasmalemma, on the other hand, is a major factor in the regulation of the intracellular Ca^{2+} necessary to activate the myosin light chain kinase. By analogy to cardiac myocytes (Kameyama *et al.*, 1985), though with less evidence, phosphorylation/dephosphorylation mechanisms may well modulate Ca^{2+} channels and pumps in smooth muscle. In the rat aorta, contractions induced upon phosphatase inhibition were associated with an increase in the cytosolic concentration of Ca^{2+}, prevented by blocking Ca^{2+} entry and enhanced by the Ca^{2+} channel agonist, Bay K 8644 (0.1 μM) (Ishihara *et al.*, 1989). Such a Ca^{2+} entry can occur via a direct modulation of the voltage-activated Ca^{2+} channels or via the opening of Ca^{2+} channels upon depolarization as other membrane channels are being modulated (Kume *et al.*, 1989).

In the present experiments we have studied the concerted effects of reducing protein dephosphorylation on both contractility and whole-cell Ca^{2+} channel currents in single cells of the guinea-pig taenia caeci. Intrinsic phosphatase activity was either inhibited with okadaic acid ($C_{44}H_{66}O_{13}$), a toxin isolated from marine sponges of the genus *Halichondria* (Kodama *et al.*, 1986) or slowed by phosphorylating intracellular phosphoproteins with thiophosphate groups from adenosine 5′-*O*(3-thiotriphosphate) (ATPγS) which are resistant to enzymatic hydrolysis (Gratecos & Fischer, 1974). Some of these results have been presented previously in brief (Paul & Lang, 1989).

Methods

Dispersal and electrical recording from single cells

Single smooth muscle cells were isolated enzymatically from portions of taenia caeci in a physiological salt solution (PSS) plus collagenase (0.6 mg ml^{-1}), trypsin inhibitor (0.5 mg ml^{-1}), bovine serum albumin (2 mg ml^{-1}) and added Ca^{2+} (30 μM) (at

[1] Author for correspondence.
[2] Department of Physiology & Biophysics, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0576, U.S.A.

37°C) for 60 min. Strips were then transferred to PSS with no added enzymes; single cells were dispersed by trituration. Ca^{2+} (0.4 mM) was added to the cells in suspension which were then plated on glass coverslips and stored at 4°C.

Whole-cell currents were recorded by standard patch-clamp techniques (Hamill *et al.*, 1981) and low-resistance pipettes (2–7 MΩ), as described recently (Lang & Paul, 1991). The initial formation of the cell-attached patch and rupture of the underlying cell membrane to create the whole-cell voltage clamp mode were performed in Ca^{2+}-containing (1.5 mM Ca^{2+}) saline (see below). Cells which did not contract upon membrane rupture were used in these experiments. It was usually possible to introduce the Ba^{2+}-containing (7.5 mM) saline and make the first recording of I_{Ba} within 1–2 min (Lang, 1990).

Contractions of single cells

Single cells were monitored continuously during experiments on a TV monitor and recorded with a video cassette recorder. Cell lengths were measured by computer analysis, after digitization of the video signals. A line was drawn along the long axis of cells and the total length calculated. Cell lengths were usually measured every 15–30 s. This computer analysis was kindly provided by Dr T.O. Neild, Dept. of Physiology, Monash University.

Solutions

The dispersal solution contained (in mM): NaCl 126, HEPES 6, glucose 11, KCl 6, $MgCl_2$ 1 at pH 7.4. To record Ca^{2+} or Ba^{2+} currents through Ca^{2+} channels the intracellular solution in the patch pipette contained (in mM): CsCl 131, HEPES 6, glucose 11, tetraethylammonium (TEA) 5, Na_2ATP 3, EGTA 3, $MgCl_2$ 3 at pH 7.4. Extracellular solutions contained (in mM): NaCl 126, HEPES 6, glucose 11, CsCl 6, TEA 5, $MgCl_2$ 1 and $CaCl_2$ or $BaCl_2$ 7.5; pH 7.4.

Results

The fundamental properties of the whole-cell Ca^{2+} channel currents recorded in single cells from the guinea-pig taenia caeci bathed in 7.5 mM Ba^{2+}, have been described recently (Ganitkevich *et al.*, 1986; Lang & Paul, 1991) and are summarized in Figure 1. All cells were held at −80 mV. Inward Ba^{2+} currents (I_{Ba}) were recorded at potentials positive to −40 mV, were maximal between −10 and +10 mV and reversed into an outward current at potentials positive to +60 mV (Figure 1b). Both the inward and outward flow of current was blocked by 100 μM Cd (Figure 1a,b).

The amplitude of I_{Ba} at any potential did not remain constant throughout an experiment. I_{Ba} tended to grow in amplitude during the first 5–10 min after impalement and then gradually decreased over the next 10–30 min. The initial increase in amplitude is thought to reflect a time-dependent blockade of residual K currents and/or entry of ATP as the cells were perfused with the Cs-, ATP-containing pipette solution (Langton *et al.*, 1989; Lang & Paul, 1991). The later decline in amplitude is thought to arise from Ca^{2+} channel rundown, the rate of which was variable between cells (Lang & Paul, 1991). Rundown was established in each experiment by use of stimulation protocols similar to that illustrated in Figure 1c. Trains of 4–8 depolarizations, to 0 mV every 30 s, were separated by rest periods of 2–3 min. The amplitude of the first I_{Ba} in successive trains of stimulation gives a measure of Ca^{2+} channel rundown, the decline of I_{Ba} within a train of stimulation reflects use-dependence.

Effects of adenosine 5'-O(3-thiotriphosphate)

In the present experiments dephosphorylation of smooth muscle proteins was slowed by the intracellular infusion of

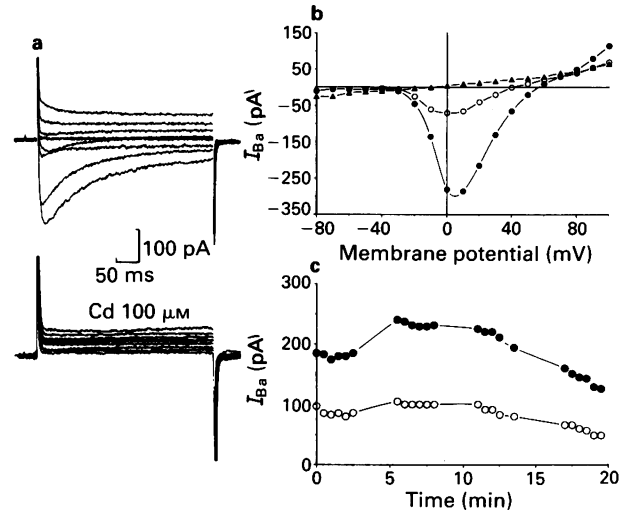

Figure 1 Characterization of membrane currents in 7.5 mM Ba^{2+} saline (I_{Ba}) recorded in single Cs-filled cells of the guinea-pig taenia caeci under voltage clamp. (a) Current responses to depolarizing steps (400 ms) in potential which increased 20 mV successively; holding potential −80 mV. Threshold for I_{Ba} was positive to −40 mV, I_{Ba} was maximal near 0 mV and had a reversal potential near +60 mV. Cd^{2+} (100 μM) blocked both the inward and outward current flow through these Ca^{2+} channels. (b) Plot of the current-voltage relationship for I_{Ba} where the peak amplitude of I_{Ba} ($I_{(peak)}$) (●) and the amplitude of I_{Ba} at the end of these 400 ms depolarizations ($I_{(400)}$) in the absence (○) and presence of Cd^{2+} (▲) are plotted against potential. (c) Typical plot of $I_{(peak)}$ (●) and $I_{(400)}$ (○) of I_{Ba} against time after rupture of the membrane underlying the patch pipette.

ATPγS or okadaic acid. ATPγS donates thiophosphate groups to cell proteins, which are resistant to enzymatic hydrolysis (Gratecos & Fischer, 1974). Dephosphorylation of these proteins therefore proceeds more slowly than proteins loaded with phosphate groups donated by ATP.

Electrical recordings were usually made from cells approximately 150 μm in length. When 1 mM ATPγS was added to the pipette solution (which contains 3 mM ATP) there was a considerable shortening of these cells within 5 min of rupture of the membrane underlying the patch pipette (Figure 2). As cells shortened, large blebs often protruded from the middle regions of the cell. In Figure 2 the length of four cells perfused with 1 mM ATPγS: 3 mM ATP has been expressed as a fraction of the cell length before membrane rupture and plotted against time. The averaged length for these cells was 133 ± 4 μm. After 5 min these cells had shortened to 23 ± 2% ($n = 4$) of their initial length. Control cells perfused with a pipette solution containing 4 mM ATP did not show any shortening with time (Figure 2, open squares).

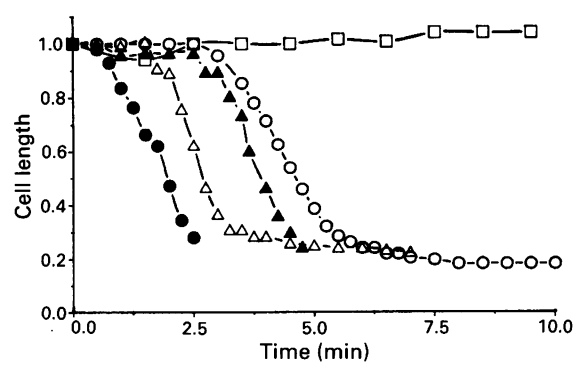

Figure 2 Effects of infusing adenosine 5'-*O*(3-thiotriphosphate) (ATPγS, 1 mM) on length of single cells of the taenia caeci. Cell length expressed as a fraction of its initial length and plotted against time for four cells perfused with 1 mM ATPγS: 3 mM ATP (●, △, ▲, ○) and one cell perfused with 4 mM ATP (□).

In a number of cells the high resistance seal between the patch pipette and the cell membrane was maintained during these contractions. Addition of ATPγS (1 mM) to the pipette solution decreased the initial amplitude of I_{Ba} recorded as soon as was practical after membrane rupture. The averaged peak amplitude ($I_{(peak)}$) of I_{Ba} in cells filled with 1 mM ATPγS: 3 mM ATP was 134 ± 19 ($n = 13$) pA, significantly smaller than control cells containing 4 mM ATP (209 ± 24 ($n = 5$) pA; $P \leq 0.05$) (Lang & Paul, 1991).

The presence of ATPγS did not prevent the reduction of the Ca^{2+}-channel current amplitude, nor the acceleration of its decay when Ca^{2+} replaced Ba^{2+} as the permeant ion. In control cells, Ca^{2+}-channel currents, at 0 mV from a holding potential −80 mV, decreased 46 ± 6% ($n = 4$) when the Ba^{2+} (7.5 mM) in the bathing solution was replaced with Ca^{2+} (7.5 mM) (Lang & Paul, 1991). The acceleration of the decay of the current in Ca^{2+} (I_{Ca}) could be seen clearly by multiplying the Ca^{2+} current appropriately (×1.75) to superimpose the amplitude of the Ba^{2+} current (Figure 3a). In cells filled with ATPγS, I_{Ba} was decreased 53 ± 10% ($n = 4$) when Ca^{2+} (7.5 mM) replaced Ba^{2+}. In Figure 3c the background membrane and capacitive currents (in 100 μM Cd) of the ATPγS-filled cell (Figure 3b) have been subtracted electrically from I_{Ba} and I_{Ca} (Figure 3c). In this cell it was necessary to multiply the amplitude of I_{Ca} 1.6 times to superimpose the amplitude of I_{Ba} (Figure 3c, middle trace). Their time course of decay were fitted, by least squares, with two exponentials of the form:

$$F(t) = A_0 + A_1 \exp(t/T_1) + A_2 \exp(t/T_2)$$

where A_1, A_2 and T_1, T_2 are respectively the amplitudes (at time zero) and time constants of the contributing exponentials (Figure 3d).

In Table 1 it can be seen that cells perfused with either ATP (4 mM) or 1 mM ATPγS: 3 mM ATP showed an acceleration of Ca^{2+}-channel inactivation when Ca^{2+} replaced Ba^{2+}. In *Helix* neurones I_{Ca} was decreased in amplitude and its rate of inactivation slowed by the intracellular application of ATPγS, which prompted the suggestion that a Ca^{2+}-activated phosphatase may have been involved in the mechanism of Ca^{2+} channel inactivation (Chad & Eckert, 1986). One would anticipate that a reduction in the rate of Ca^{2+}-activated dephosphorylation as would occur with ATPγS in the present experiments would have slowed the decay of I_{Ca} to a rate similar to that seen when Ba^{2+} acted as the charge carrier. As ATPγS did not prevent the acceleration of inactivation when Ba^{2+} was substituted by Ca^{2+}, it is unlikely that a Ca^{2+}-activated phosphatase underlies Ca^{2+}-dependent inactivation of Ca^{2+} channels in the taenia caeci.

It was also possible to monitor the effects of ATPγS on the amplitude of I_{Ba} against time, by use of the stimulation protocol described above (Figure 1c). In Figure 4, $I_{(peak)}$ of I_{Ba} has been plotted against time for 5 cells perfused with 1 mM ATPγS: 3 mM ATP (Figure 4a) and 3 cells filled with 4 mM ATP (Figure 4b). To assess the effects of ATPγS on the amplitude of I_{Ba} the data in Figure 4a,b were grouped into 2–3 min blocks (0–2.5, 3.0–5.5, 6.0–8.5, 9.0–12.0 min respectively). The data were such that each experiment contributed two values to each time block. A two-way analysis of variance, corrected for repeated measures, was used to test for differences between (i) the ATPγS and control groups, (ii) the various time blocks and (iii) the experiments within each treatment group. During the 12 min after membrane rupture, $I_{(peak)}$ of I_{Ba} in cells infused with 1 mM ATPγS: 3 mM ATP was significantly smaller ($P < 0.04$) than in the control cells (4 mM ATP). The increase and subsequent decline of I_{Ba} with time did not prove to be significant in either the ATPγS-filled or the control cells

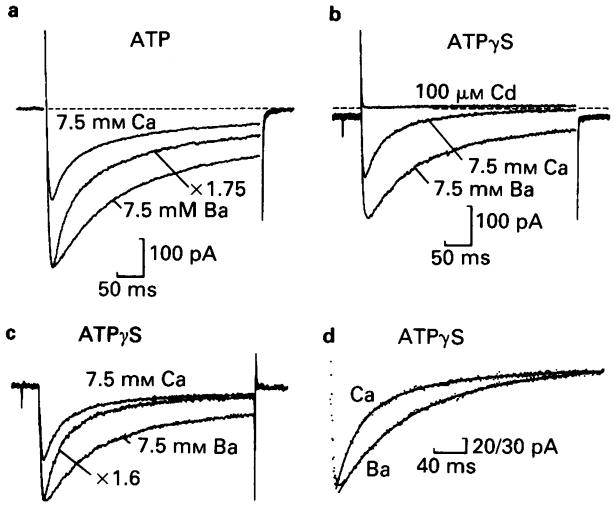

Figure 3 Effects of adenosine 5′-*O*(3-thiotriphosphate) (ATPγS) on Ca^{2+} channel currents in 7.5 mM Ca^{2+} and 7.5 mM Ba^{2+} saline. (a) Superimposed I_{Ba} and I_{Ca} at 0 mV recorded from a single cell perfused with 4 mM ATP; holding potential −80 mV. When I_{Ca} was multiplied ×1.75 and superimposed on I_{Ba} it can be seen that inactivation of I_{Ca} was more rapid. (b) Superimposed I_{Ba} and I_{Ca} at 0 mV from a single cell perfused with 1 mM ATPγS: 3 mM ATP and when 100 μM Cd was added; holding potential −80 mV. (c) Superimposed I_{Ba} and I_{Ca} from the same cell in (b), the background membrane and capacitive currents in Cd^{2+} subtracted previously. I_{Ca} was multiplied ×1.6 to match the amplitude of I_{Ba} (middle trace). (d) Inactivation of I_{Ba} (lower trace) and I_{Ca} (upper trace) were fitted, by a least squares method, with two exponentials such that

$$F(t) = A_0 + A_1 \exp(t/T_1) + A_2 \exp(t/T_2)$$

with time constants T_1 and T_2. In 7.5 mM Ba^{2+}, T_1 and T_2 were 64.5 and 227 ms respectively; in 7.5 mM Ca^{2+}, they were 23.6 and 113 ms.

Table 1 Comparison of the rates of inactivation of Ca^{2+}-channel currents in adenosine 5′-*O*(3-thiotriphosphate) (ATPγS)-filled cells with control cells, bathed in either Ca^{2+}- or Ba^{2+}-containing (7.5 mM) saline

	ATPγS: ATP (1 mM: 3 mM)				*ATP* (4 mM)				
7.5 mM	A_1 (pA)	T_1 (ms)	A_2 (pA)	T_2 (ms)	A_1 (pA)	T_1 (ms)	A_2 (pA)	T_2 (ms)	(N)
Ca^{2+}	81 ±38	25 ±2*	47 ±22*	137 ±19	90 ±22	27 ±4	56 ±18	139 ±22	(3)
Ba^{2+}	62 ±24	46 ±7*	130 ±38*	545 ±435	79 ±17	39 ±15	138 ±46	163 ±30	(3)

Currents were recorded at 0 mV from a holding potential of −80 mV. Decays of I_{Ca} and I_{Ba} were fitted, by least squares, with two exponentials of the form:

$$F(t) = A_0 + A_1 \exp(t/T_1) + A_2 \exp(t/T_2)$$

where A_1, A_2 are the amplitudes (at time zero) and T_1, T_2 the time constants of the contributing exponentials. * denotes significant difference ($P < 0.05$) between means within a particular column.

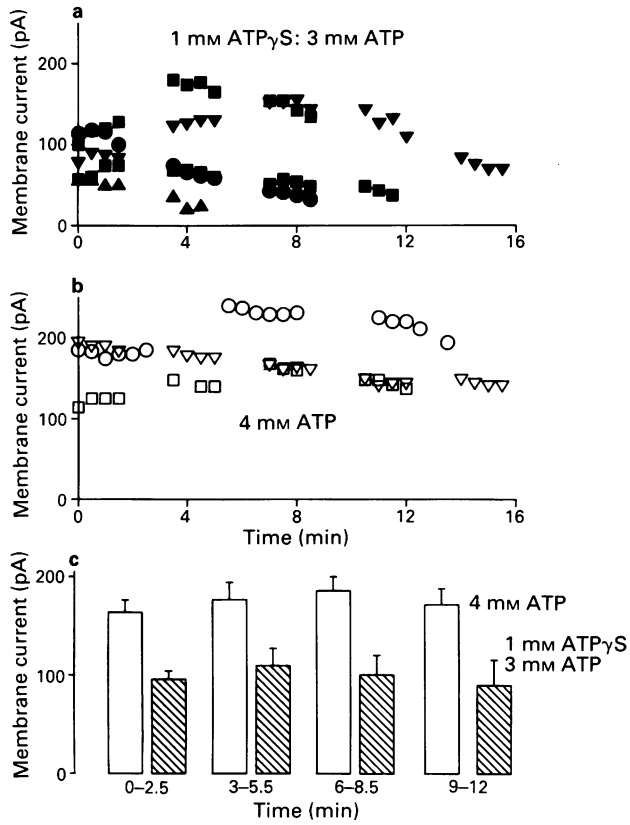

Figure 4 Plot of $I_{(peak)}$ of I_{Ba} against time in 5 cells containing 1 mM adenosine 5'-*O*(thiotriphosphate) (ATPγS): 3 mM ATP (a) and 3 cells containing 4 mM ATP (b). (c) Statistical analysis of variance of pooled data from (a) and (b) (see text) reveals that I_{Ba} in 1 mM ATPγS: 3 mM ATP filled cells (hatched columns) was significantly smaller than I_{Ba} in control cells (4 mM ATP) (open columns) and that there were no significant time-dependent effects of ATPγS.

($P > 0.67$ and $P > 0.82$, respectively). Thus, except for the initial decrease in the amplitude of I_{Ba}, no particular time-dependent effect on I_{Ba} could be ascribed to the intracellular presence of 1 mM ATPγS.

Effects of okadaic acid

Okadaic acid (OA) is a potent inhibitor of a number of phosphatases isolated from the brain and smooth muscle. In cardiac muscle OA (10–40 μM) increases contractile force associated with an increase in the duration of the action potential and underlying Ca^{2+} current (Kodama *et al.*, 1986; Hescheler *et al.*, 1988). In smooth muscle, OA has both excitatory and inhibitory actions. OA (0.1–10 μM) inhibits contractions induced by high concentrations of K^+ in the rabbit aorta and in the taenia coli and vas deferens of the guinea-pig. Contractions to noradrenaline (rabbit aorta; guinea-pig vas deferens), carbachol (bovine trachea) and ATP (guinea-pig vas deferens) are all blocked by OA (Karaki *et al.*, 1989; Tansey *et al.*, 1990; Shibata *et al.*, 1991). In 'skinned' smooth muscle strips, OA (10–50 μM) can also induce contraction in the presence, or absence, of Ca^{2+} and calmodulin (Bialojan *et al.*, 1988; Karaki *et al.*, 1989).

When OA (50 μM) was added to the pipette solution, single cells of the taenia caeci showed a time-dependent contraction to 29 ± 5% ($n = 4$) of their initial length within 5 min of membrane rupture (Figure 5a). Note, however, that when these contractions started the rate of contraction was greater with OA than with ATPγS (compare Figure 2 with Figure 5a). Two of the cells illustrated (Figure 5a open and solid circles), contracted away from the recording electrode, making concurrent recording of I_{Ba} impossible. It was possible, however, to maintain electrical contact between the electrode and the cell upon

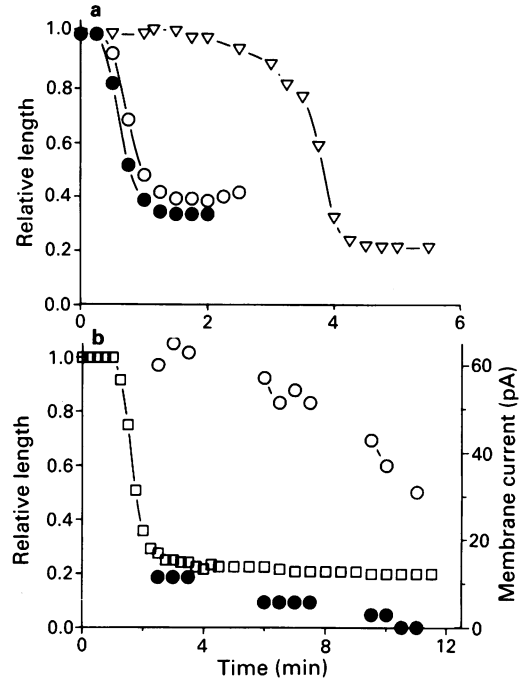

Figure 5 Effect of introducing okadaic acid (OA) into single smooth muscle cells of the taenia caeci. (a) Plots of relative cell length against time of three cells perfused with 50 μM OA: 3 mM ATP. (b) Plot of relative cell length (open squares) and $I_{(peak)}$ (open circles) and $I_{(400)}$ (solid circles) of I_{Ba} against time in a single cell perfused with 50 μM OA: 3 mM ATP.

OA infusion in four cells. In the cell illustrated in Figure 5b, the relative cell length, I_{peak} of I_{Ba} and the current after 400 ms ($I_{(400)}$) (at 0 mV) have been plotted against time. This cell contracted to approximately 20% of its initial length within 3 min. The amplitude of I_{Ba} in this cell, however, peaked at 65 pA after 3.5 min and then showed the same slow decline with time seen in control cells. Note that the mean initial amplitude of I_{Ba} (93 ± 10 pA ($n = 4$)) after OA infusion was of similar amplitude to cells infused with ATPγS and smaller than control cells (see above).

In cardiac cells, the effects of threshold concentrations of isoprenaline, which is known to increase I_{Ca} via adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent phosphorylation of Ca^{2+} channel proteins, were potentiated by the extracellular application of OA (Hescheler *et al.*, 1988). Okadaic acid was therefore applied extracellularly to single cells of taenia caeci, after use-dependence and the rate of rundown of I_{Ba} had been established previously. In Figure 6a, OA (25 μM) did not induce cell contraction (open squares), but slowed the rate of rundown of I_{Ba}, as can be seen by the change of slope of the plots of $I_{(peak)}$ (open circles) and $I_{(400)}$ (solid circles) against time. Isoprenaline (Iso) (1 μM) produced a small increase (≈10%) in I_{Ba} only in the presence of OA (25 μM) when Ba^{2+} (Figure 6a,b) or Ca^{2+} (Figure 6c) acted as the charge carrier.

Discussion

The intracellular perfusion of either ATPγS (1 mM) or OA (50 μM) caused contraction of single cells of the taenia caeci, as has been reported in intact, or chemically permeabilized, smooth muscle strips (Ozaki *et al.*, 1987; Bialojan *et al.*, 1988; Karaki *et al.*, 1989). In the present experiments, however, cells were perfused with 3 mM EGTA and bathed in 7.5 mM Ba^{2+}. These contractions may therefore arise from (i) the intrinsic activity of the Ca^{2+}-, calmodulin-dependent myosin light chain kinase at low levels of Ca^{2+}, (ii) the activation of this kinase by Ba^{2+}, or (iii) the activation of a not yet character-

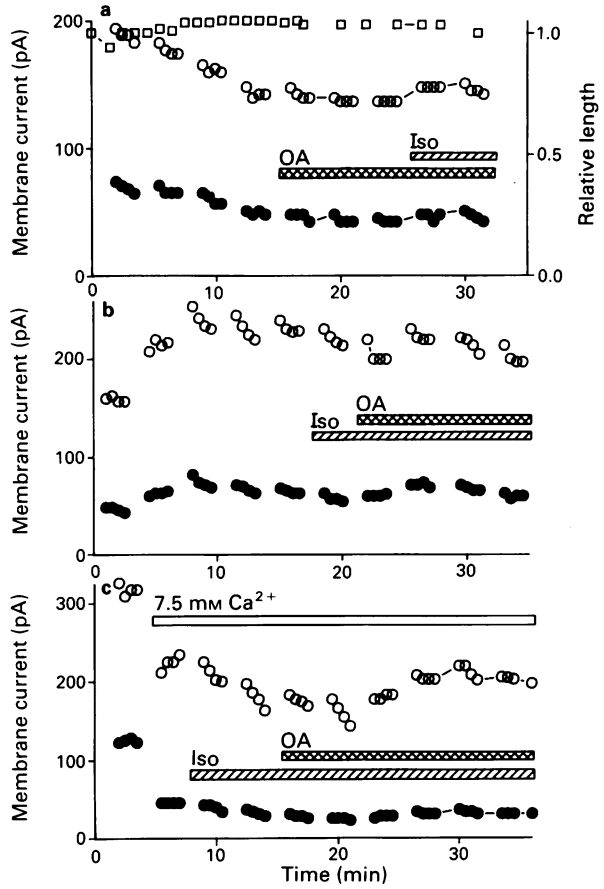

Figure 6 Effects of externally applied okadaic acid (OA, 25 μM) on cell length and I_{Ba} plotted against time. (a) OA (25 μM) applied externally had little affect on cell length (open squares); but reduced the time-dependent rundown of $I_{(peak)}$ (open circles) and $I_{(400)}$ (solid circles) of I_{Ba}. Isoprenaline (Iso 1 μM) included only a small increase of I_{Ba} in the presence of OA (25 μM) when Ba^{2+} (b) or Ca^{2+} (c) acted as the charge carrier.

ized kinase which phosphorylates myosin in the absence of Ca^{2+}.

These effects of ATPγS and OA in the taenia caeci are similar to those induced by another toxin, calyculin-A, also isolated from a marine sponge (Ishihara *et al.*, 1989). Okadaic acid and calyculin-A have both been shown recently to (i) produce considerable phosphorylation of the 20 kDa myosin light chain, (ii) not act as a calmodulin antagonist and (iii) not affect directly the Ca-, calmodulin-dependent myosin light chain kinase or protein kinase C. Both substances have also been shown to be potent inhibitors of type-1 and type-2A phosphatases (Ishihara *et al.*, 1989; Obara *et al.*, 1989). It seems likely therefore that contraction induced by both these substances involves a phosphorylation of the myosin light chain via the direct activation of and/or the unmasking of a myosin light chain kinase upon the inhibition of the rate of dephosphorylation. The more rapid contraction of these single cells of the taenia caeci perfused with OA than with ATPγS may also suggest that ATPγS may be a relatively poor substrate for the protein kinases causing contraction (in the absence of Ca^{2+}) and that the intrinsic activity of this kinase may be relatively high (Ishihara *et al.*, 1989).

The extracellular application of OA (25 μM) produced a slowing of Ca^{2+}-channel rundown (Figure 6a). The loss of high energy phosphate groups from intracellular proteins and a rise in internal levels of Ca^{2+} are both thought to play an important role in the time-dependent loss of Ca^{2+} channel function in single neuronal and cardiac cells recorded with patch pipettes (Doroshenko *et al.*, 1982; Irisawa *et al.*, 1983; Lee & Tsein, 1984; Chad & Eckert, 1986; Belles *et al.*, 1988). The internal application of ATP (0.3–10 mM), EGTA (0.1–10 mM), but not cyclic AMP (< 300 μM), increased the amplitude and slowed the rundown of Ca^{2+} channel currents in the rabbit small intestine and portal vein (Ohya *et al.*, 1987; 1988), guinea-pig mesenteric artery and bladder (Klöckner & Isenberg, 1985; Ohya & Sperelakis, 1989) and dog colon (Langton *et al.*, 1989). The effects of extracellularly applied OA were quite different from those obtained with OA in the patch pipette (see below), suggesting a different site of action. This may be related to the fact that, though OA is membrane permeable, it is likely that only low internal concentrations of OA are being established when it is applied extracellularly. This is supported by the lack of contraction under these conditions.

The major effect of intracellular application of OA (50 μM) on ATPγS (1 mM) was to decrease the initial amplitude of I_{Ba} (Figures 3, 4 and 5). These results are similar to the recently reported effects of OA and calyculin-A on the slow waves recorded intracellularly in the intact colon and gastric muscles, and on I_{Ca} recorded in the single cells isolated from these tissues (Ward *et al.*, 1991). In both tissues, calyculin-A and OA led to a reduction of I_{Ca} and the duration of the plateau phase of the slow waves. In smooth muscle, therefore, blockade of intrinsic phosphatase activity, by analogy with heart cells (Kameyama *et al.*, 1985; Hescheler *et al.*, 1988), decreases the number of functional Ca^{2+} channels. It may well be that a novel mechanism of controlling Ca^{2+} channels is present in smooth muscle, whereby a phosphorylation of the Ca^{2+} channel protein, or associated proteins, inhibits their function (Ward *et al.*, 1991). Such an action of OA may well underlie the decrease in the contractions to raised concentrations of K^+, or excitatory-receptor activation, reported recently in the taenia coli and vas deferens of the guinea-pig, rat aorta and bovine trachea (Karaki *et al.*, 1989; Tansey *et al.*, 1990; Shibata *et al.*, 1991).

These results, however, contrast with effects of ATPγS and OA on I_{Ca} in single heart cells. When applied internally, ATPγS (3 mM) led to a slow small increase in I_{Ca} over 5 min; OA (50 μM) doubled I_{Ca} within 2 min (Kameyama *et al.*, 1985; Hescheler *et al.*, 1988). ATPγS and OA also potentiated and prolonged the increase of I_{Ca} induced upon β-adrenoceptor activation, due to a cyclic AMP-dependent phosphorylation of the Ca^{2+} channel proteins (Kameyama *et al.*, 1985; 1986; Hescheler *et al.*, 1987; 1988). In the present experiments, isoprenaline (1 μM) applied alone had no affect on I_{Ba}, but did produce a small increase in I_{Ba} in the presence of OA (25 μM) (Figure 6). Therefore, cyclic AMP-dependent kinases do not appear to play a major role in controlling Ca^{2+} channels function in the taenia caeci (Klöckner & Isenberg, 1985; Ward *et al.*, 1991).

In summary, inhibition of phosphatase action elicited a massive contraction in isolated single smooth cells of the guinea-pig taenia caeci. Mechanisms involving phosphorylation, most likely of the myosin light chain, are sufficient for initiation of contractile activity. In contrast, Ca^{2+} channel currents are unlikely to be a major site for increasing contractility via phosphorylation/dephosphorylation as they were decreased upon inhibition of phosphatase activity.

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Vasodilator response of coronary smooth muscle to the sympathetic co-transmitters noradrenaline and adenosine 5′-triphosphate

[1]Laura Corr & Geoffrey Burnstock

Department of Anatomy and Developmental Biology, and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT

1 Vasodilator and vasoconstrictor responses to noradrenaline (NA), adenosine and adenosine 5′-triphosphate (ATP) were examined in isolated ring segments of the left anterior descending coronary artery of the rabbit in the absence of endothelium.

2 NA caused dose-dependent relaxation of potassium-constricted vessels in the absence of β-adrenoceptor blockade, with a pD_2 of 5.96 ± 0.21. This was inhibited by 1 μM propranolol. Constrictor responses of vessels at baseline tension were only seen at concentrations greater than 1 mM, and reached a maximum of 6% of the contraction to 30 mM KCl.

3 ATP caused relaxation of the potassium-constricted ring segments in a dose-dependent manner, although a transient constriction often preceded the relaxation. Adenosine was equipotent with ATP in producing relaxation; this was significantly inhibited by the P_1-purinoceptor antagonist, 8-phenyltheophylline (8-PT). The responses to ATP were little affected by 8-PT, indicating that ATP was not acting through breakdown to adenosine. At basal tone, ATP produced transient vasoconstriction only at concentrations greater than 0.1 mM, reaching a maximum of 38% of the contraction to 30 mM KCl.

4 We conclude that in the rabbit coronary artery both NA and ATP produce vasodilatation by a direct action on the smooth muscle.

Keywords: Noradrenaline; adenosine 5′-triphosphate; adenosine; coronary artery

Introduction

Noradrenaline (NA) and adenosine 5′-triphosphate (ATP) are now well established as co-transmitters in sympathetic nerves supplying many blood vessels. They have been shown to have synergistic activity on the smooth muscle of many blood vessels causing vasoconstriction via α_1-adrenoceptors and P_{2X}-purinoceptors respectively (Burnstock, 1988). Vasodilatation to ATP may be observed in some of these blood vessels but this has generally been shown to be mediated via receptors located on the endothelium (De Mey & Vanhoutte, 1981; Furchgott, 1981; Furchgott, 1983; Hardebo *et al.*, 1983). These endothelial receptors are unlikely to be activated by neurally-released ATP but may mediate responses to ATP from sources such as aggregating platelets and endothelial cells.

Coronary arteries are supplied with both α- and β-adrenoceptors (Yurchak *et al.*, 1964; Lioy, 1967; Andersson *et al.*, 1972; Mark *et al.*, 1972); the contribution of each seems to vary both between species and between vessels of different calibre in the same species (Zuberbuhler & Bohr, 1965; Vatner, 1985). In the rabbit, the epicardial coronary artery is reported to respond to NA almost exclusively by vasodilatation, with minimal vasoconstriction at doses up to 0.1 mM, even in the presence of β-adrenoceptor blocking agents (Han & Abel, 1987).

The aim of this study was to examine the smooth muscle responses of the rabbit coronary artery to NA and ATP. To avoid vasodilatation via endothelial purinoceptors, the endothelium was removed from the preparations.

Methods

Pharmacology

Male New Zealand White rabbits (2.6–3.8 kg) were killed by barbiturate anaesthesia and exsanguination. The heart was rapidly removed and the circumflex branch of the left coronary artery was carefully dissected free of adherent myocardium. Ring segments of 4 mm in length were cut and the endothelium was removed where indicated by drawing a fine silk thread through the lumen of the vessel. Confirmation of the presence or absence of the endothelium was ascertained in each preparation by pharmacological assay of the response to a single dose of 0.1 μM acetylcholine, and in some cases by histological examination of segments for the presence or absence of endothelial cell nuclei.

Each ring was mounted horizontally under isometric tension in 6 ml water-jacketed organ baths by inserting two tungsten wires into the lumen according to the method of Bevan & Osher (1972). The tissues were bathed in a modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.3, $NaHPO_4$ 1.35, glucose 7.8, $MgSO_4$ 0.61 and $CaCl_2$ 1.89 (modified from Bülbring, 1953). This solution was gassed with 95% oxygen/5% carbon dioxide and maintained at 37°C. The ring segments were then allowed to equilibrate for at least 1 h, during which time the tension was adjusted to maintain a resting tension of 1 g. Subsequently, the preparations were repeatedly exposed to 30 mM KCl until successive contractions did not differ significantly from each other (usually three to four contractions). Following washout of initial doses of KCl, the resting tension relaxed to around 0.75 g, which allowed optimal responses for this vessel. This resting tension was then stable throughout the experiment. Isometric tension generated by the vascular smooth muscle was recorded with a force displacement transducer (Model UFI, Devices, Welwyn Garden City) and a Grass ink-writing oscilloscope (Model 79).

The vasoconstrictor response to 30 mM KCl in ring segments with endothelium removed was compared with the response in segments in which the endothelium had been left intact to ensure that the smooth muscle had not been damaged during the process. As a further control, relaxant responses to sodium nitroprusside, a direct-acting dilator of vascular smooth muscle, were also investigated in the presence and absence of endothelium.

At basal tone, ATP caused a very transient response and rapidly desensitized the preparations; it was therefore added

[1] Author for correspondence.

as single doses at 40 min intervals and the preparations were washed repeatedly between each application to avoid desensitization. The contractile response to a given concentration of ATP was taken as the peak of the transient response obtained. For relaxant responses, the tone of the ring preparations was raised with 30 mM KCl, which produced a stable and sustained contraction. At raised tone, the vasodilator responses did not show desensitization and agonists were added to the bath in a cumulative fashion. ATP and NA both produced stable, dose-dependent relaxation of the preparations, although a transient contraction sometimes preceded relaxation to ATP; in these preparations, the relaxant response to ATP was measured after the initial contraction had disappeared and the subsequent sustained relaxant response had reached a plateau (usually 4–8 min). Where indicated, the antagonists 8-phenyltheophylline (8-PT) or propranolol were added to the organ bath 20–30 min before agonist responses were re-examined.

Drugs

The following drugs were used: adenosine, adenosine 5'-triphosphate (ATP), acetylcholine chloride (ACh), (−)-noradrenaline bitartrate (NA), 8-phenyltheophylline (8-PT), propranolol hydrochloride, sodium nitroprusside. All drugs were obtained from the Sigma Chemical Company and all, except 8-PT, were prepared freshly on the day of each experiment. NA was dissolved in ascorbic acid (100 μM) to inhibit its oxidation; 8-PT was prepared as a stock solution by dissolving it in 80% v/v methanol, 20% 1 M NaOH, and diluted as required using distilled water. The remainder of the drugs used were all prepared in distilled water.

Statistics

Vasodilator responses were calculated as percentage relaxation of the tone induced by KCl. Vasoconstrictor responses to KCl were expressed as grams of tension generated, and the vasoconstrictor responses to other agonists were expressed as a percentage of the KCl-induced contraction. The mean responses for each concentration of agonist were calculated for a number of animals, denoted as n in the figure legends, and the results expressed as mean ± s.e.mean. Student's t test (paired or unpaired as appropriate) was used for the statistical analysis and a probability of less than 0.05 was considered significant.

Results

Raised tone preparations

In all ring segments, KCl (30 mM) produced reproducible, sustained contractions, with no significant difference in the mean tension generated in the presence and absence of endothelium (2.02 ± 0.20 g tension with endothelium, 1.78 ± 0.12 g tension without endothelium). Acetylcholine (0.1 μM) produced relaxation of the ring segments in the presence of intact endothelium, which was abolished or converted to a contraction in preparations with the endothelium removed. Sodium nitroprusside, a direct-acting dilator of vascular smooth muscle, produced greater than 90% relaxation of all ring segments. The maximum relaxation was not reduced by removal of the endothelium and there was a small, significant increase in the potency of sodium nitroprusside in preparations with the endothelium removed (Table 1).

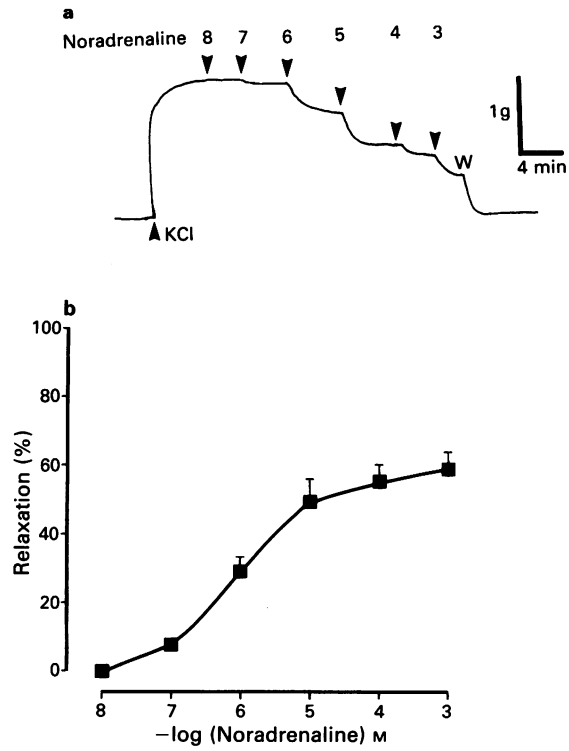

Figure 1 (a) Effect of noradrenaline 10^{-8}–10^{-3} M, added in a cumulative fashion, on a ring segment of rabbit coronary artery with endothelium removed. The tone was raised by the addition of 30 mM KCl to the bath as indicated; W = washout. (b) Log cumulative relaxation-response curves of the rabbit coronary artery to noradrenaline (10 nM–1 mM). Results are expressed as percent relaxation of the KCl-induced tone; mean ± s.e.mean (vertical bars) of data from six rabbits.

NA (10 nM–1 mM) caused reproducible, concentration-dependent, sustained relaxation in ring segments without endothelium (Figure 1a, b), although responses at concentrations above 100 μM were variable. The pD_2 for NA was 5.96 ± 0.21. In separate experiments, the relaxation to NA was shown to be markedly inhibited by propranolol (1 μM) (pD_2 for NA 5.86 ± 0.23 vs. 3.86 ± 0.14 after propranolol; n = 7). The contraction to 30 mM KCl was not

Table 1 Responses of isolated ring preparations of the rabbit coronary artery with (+E) and without (−E) endothelium

Agonist		*Response*	(n)	$-log\ [EC_{40}]$	*Maximum relaxation* (%)
NA	−E	Relaxation	(6)	5.53 (0.23)	65.0 (3.0)
ATP	−E	Contraction, then relaxation	(7)	4.07 (0.28)	Not reached
Adenosine	−E	Relaxation	(6)	4.33 (0.09)	Not reached
SNP	+E	Relaxation	(6)	7.96 (0.11)*	93.5 (1.5)^{NS}
	−E	Relaxation	(6)	8.04 (0.06)	94.3 (1.2)

EC_{40} is defined as the concentration required to produce a 40% relaxation of the KCl-induced tone and is used because responses to ATP and adenosine did not reach a maximum. Values given are mean (standard error of the mean); n = number of rabbits. *$P < 0.05$, NS = no significant difference between preparations with and without endothelium. Abbreviations: adenosine 5'-triphosphate (ATP); noradrenaline (NA); sodium nitroprusside (SNP).

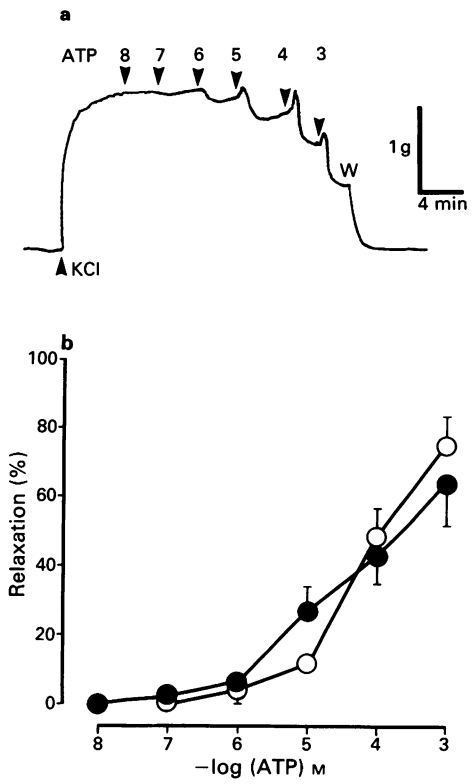

Figure 2 (a) Effect of 10^{-8}–10^{-3} M adenosine 5'-triphosphate (ATP), added in a cumulative fashion, on a ring segment of the rabbit coronary artery denuded of endothelium. The tone was raised by 30 mM KCl. In this preparation, a particularly prominent transient contraction precedes the relaxation at higher concentrations of ATP. W = washout. (b) Log cumulative relaxation-response curves to ATP in the absence (●) and presence (○) of 8-phenyltheophylline (8-PT). There was no significant difference in the response before and after 8-PT. Results are expressed as percentage relaxation of the KCl-induced tone, and are the mean ± s.e.mean (vertical bars) of the data from seven rabbits.

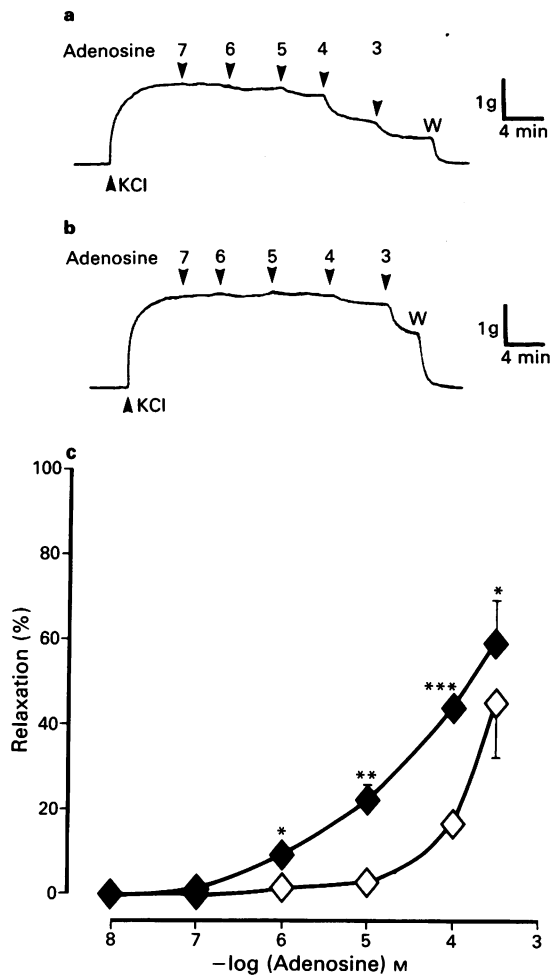

Figure 3 Effect of adenosine 10^{-8}–10^{-4} M, added in a cumulative fashion, on ring segments of the rabbit coronary artery with endothelium removed (a) in the absence, and (b) in the presence of 10^{-5} M 8-phenyltheophylline (8-PT). The tone of the preparations was raised with 30 mM KCl. W = washout. (c) Log cumulative relaxation-response curve of the coronary artery to 10 nM–0.3 mM adenosine (◆). The responses were significantly inhibited by 10 μM 8-PT (◇), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Results are expressed as a percentage of the KCl induced tone, and are the mean ± s.e.mean (vertical bars) of data from six rabbits for all concentrations except 0.3 mM, where results are the mean of preparations from two rabbits.

significantly affected by β-adrenoceptor blockade (1.53 ± 0.09 g tension vs. 1.43 ± 0.08 g tension after propranolol; $n = 7$). In order to compare the potency of NA with adenosine and ATP (which did not reach their maximum responses), the EC_{40}, defined as the concentration required to produce a 40% relaxation of the KCl-induced tone, is shown in Table 1.

Despite the absence of endothelium, ATP (10 nM–1 mM) also caused concentration-dependent relaxations of the coronary vascular smooth muscle (Figure 2b). Maximum relaxation was not reached and no true EC_{50} could be calculated, but the EC_{40} as defined above, was 4.07 ± 0.28 (Table 1). The EC_{40}, as defined above, was 4.07 ± 0.28 (Table 1). The relaxation in response to high concentrations was often preceded by a small, transient constriction which lasted 30–60 s; Figure 2a shows a preparation in which these transient contractions were prominent. The contractile responses appeared to show desensitization and were diminished at high concentrations of ATP.

Adenosine (10 nM–1 mM) induced concentration-dependent relaxations which were significantly inhibited by the potent, competitive, P_1-purinoceptor antagonist 8-PT (10 μM; Figure 3a–c). The responses did not reach a maximum over the range of concentrations examined; the EC_{40}, as defined above, is given in Table 1.

Comparison of the −log [EC_{40}] for adenosine and ATP shows that they were equipotent in causing direct relaxation of the smooth muscle in the rabbit coronary artery, while NA was significantly more potent. To determine whether ATP was acting via breakdown to adenosine, concentration-response curves to ATP were also constructed in the presence of 10 μM 8-PT, a concentration that significantly antagonized the relaxation to adenosine. There was no shift in the concentration-response curve (Figure 2b), indicating that ATP was likely to be acting directly on P_2-purinoceptors located on the smooth muscle and was not acting via the P_1-purinoceptor following breakdown to adenosine 5'-phosphate or adenosine.

Basal tone preparations

At basal tone, none of the agonists used caused contraction at doses less than 0.3 mM. At higher concentrations, NA produced a weak vasoconstriction in approximately 50% of the ring preparations examined, which reached a maximum of 6% of the contraction to KCl (Figure 4b, c). The non-cumulative addition of ATP also produced a transient vasoconstriction at high concentrations, which reached a maximum of 38 ± 10% of the response to KCl at a concentration of 3 mM (Figure 4a, c). Adenosine did not cause a contraction at any dose examined up to 3 mM. Since the contractile responses to the agonists did not approach a maximum even at these concentrations, no direct comparison of the EC_{50} values could be made.

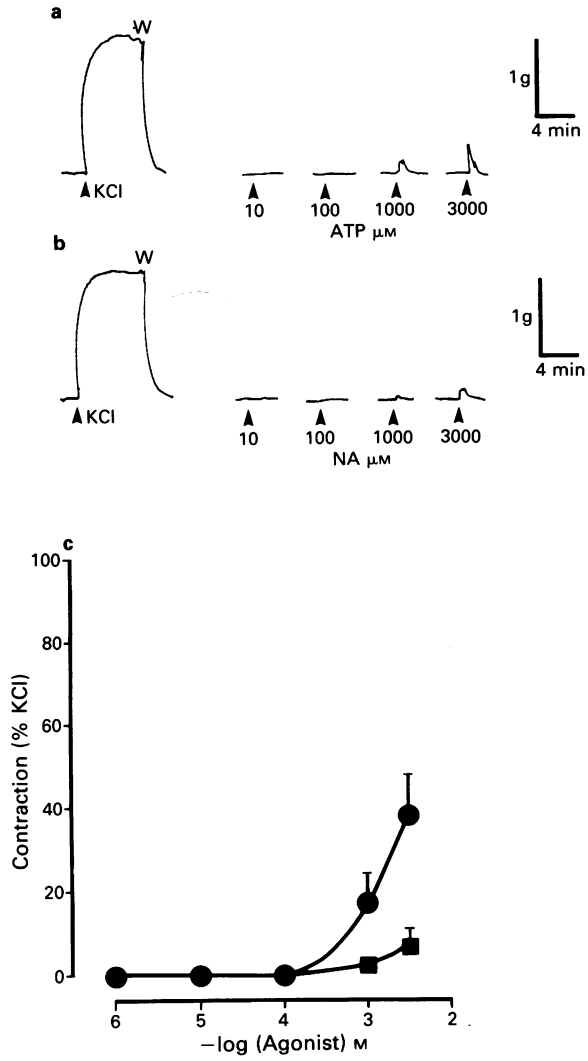

Figure 4 Response of the rabbit circumflex coronary artery at basal tone to (a) adenosine 5'-triphosphate (ATP), and (b) noradrenaline (NA), in ring segments with endothelium removed; 30 mM KCl was used to contract rings as a control, W = washout of KCl. ATP and NA were added as single doses and were washed out as soon as maximal contraction was reached, or after 2 min if there was no response, to avoid desensitization of the preparations. (c) Non-cumulative log contraction-response curves to NA (■) and ATP (●) (both 10 μM–30 mM) in the absence of endothelium. Results are expressed as a percentage of the contraction to 30 mM KCl, and are the means ± s.e.mean (vertical bars) of data from at least six rabbits.

Discussion

This study demonstrates that the predominant response of the rabbit epicardial coronary artery to exogenous NA in the absence of α-adrenoceptor blocking agents is relaxation of the smooth muscle. The relaxation is markedly inhibited by β-adrenoceptor blockade, which suggests that there is a substantially greater population of β- than α-adrenoceptors in this vessel; the weak vasoconstriction seen in some preparations with very high concentrations of NA may represent stimulation of a small population of α-adrenoceptors. In preparations dissected expressly to preserve the delicate adventitia of the vessel, we have found a moderately dense network of adrenergic nerves running in the adventitial-medial border (Corr, unpublished data) but in the present study the contractile responses to KCl, which would depolarize these perivascular nerves, were not enhanced by β-blockade. It is improbable that these observations suggest that the nerves do not functionally innervate the vessel and it is much more likely that they were damaged during removal of the adherent myocardium in preparation for this study.

ACh is known to produce endothelium-dependent relaxation of the dog coronary artery (Angus & Cocks, 1984) but not coronary arteries from other species such as the pig and sheep (Graser *et al.*, 1986; Miller *et al.*, 1984). In the rabbit, we found that relaxation to ACh occurred at low concentrations of ACh and this provided a marker for the presence or absence of the endothelium. Relaxation in response to sodium nitroprusside was not impaired in arterial segments without endothelium, indicating that the method of removal of the endothelium did not impair the vasodilator responses of the preparations. In fact, although the maximum relaxation was unchanged, the potency of sodium nitroprusside in this study was slightly but significantly augmented by removal of the endothelium. Sodium nitroprusside is believed to act by giving rise to nitric oxide which directly dilates the smooth muscle (Murad *et al.*, 1979; Vanhoutte & Rimele, 1983). Similar potentiation of the response to sodium nitroprusside by removal of the endothelium has been reported in the rat thoracic aorta (Shirasaki *et al.*, 1986) and the rabbit mesenteric artery (Warland, 1987) and may indicate release of an endothelium-derived contracting factor stimulated by this agent.

ATP was potent in causing relaxation of the rabbit coronary artery even in the absence of endothelium. The relaxant responses reached a stable plateau after a few minutes but were often preceded by transient contractions, evident in the raised tone preparations at concentrations of ATP around 10–100 μM. In preparations at basal tone, ATP added in a non-cumulative fashion produced a transient net contractile response only at concentrations greater than 100 μM so it is possible that ATP is less effective as a vasoconstrictor at low pre-existing levels of vascular tone. However, the contractions to 10–100 μM ATP at raised tone were not sufficiently powerful to produce constriction beyond the baseline tone induced by KCl and were diminished at higher concentrations of ATP, which suggests that they showed desensitization with the cumulative addition of ATP. ATP was added in a non-cumulative fashion at basal tone, so the effect of the pre-existing level of vascular tone on the response to ATP cannot be determined. It is likely that ATP is acting on two different populations of purinoceptors, one of which mediates vasoconstriction and shows desensitization while the other mediates the more sustained relaxant responses.

We examined the possibility that the relaxant responses were in fact mediated by breakdown of ATP to adenosine, since Kennedy & Burnstock (1985) have reported that ATP produces vasodilatation via P_1-purinoceptors located on the smooth muscle in the rabbit isolated central ear artery. Using the selective P_1-purinoceptor antagonist 8-PT, we showed that the relaxation response to ATP was unaffected although the responses to adenosine were significantly impaired, indicating that in the rabbit coronary artery ATP does not act on the P_1-purinoceptor but acts directly via vasodilator P_2-purinoceptors on the smooth muscle.

Many workers have suggested that ATP is a co-transmitter with NA in sympathetic nerves supplying blood vessels (Su, 1975; Head *et al.*, 1977; Katsuragi & Su, 1981; Muramatsu *et al.*, 1981; Sedaa *et al.*, 1986; Burnstock, 1988). Sympathetic nerve stimulation has been shown to produce contraction mediated by both NA and ATP in the rat tail artery (Sneddon & Burnstock, 1984; Vidal *et al.*, 1986), the mesenteric artery (Ishikawa, 1985; von Kugelgen & Starke, 1985; Muramatsu, 1986), the rabbit ear artery (Kennedy *et al.*, 1986), the rabbit saphenous artery (Burnstock & Warland, 1987) and the dog basilar artery (Muramatsu & Kigoshi, 1987). Exogenous ATP can produce relaxation in many of these vessels but this has been shown to be mediated via P_2-purinoceptors located on the endothelium (De Mey & Vanhoutte, 1981; Furchgott, 1981; Furchgott, 1983; Hardebo *et al.*, 1983). After removal of

the endothelium, the response to ATP is converted to contraction (Kennedy *et al.*, 1985). An important consequence of this pattern of responses to ATP is that conditions associated with disruption or damage to the endothelium, for example atherosclerosis or trauma, may result in a contractile response to ATP released from sources such as aggregating platelets. In the rabbit coronary artery in this study, exogenous NA and ATP both produce a predominantly vasodilator response which appears to be mediated by receptors located directly on the smooth muscle. As a result of this unusual pattern of receptors, ATP would not produce prolonged vasoconstriction of the coronary artery or impaired myocardial blood flow even following endothelial disruption. Furthermore, in view of their synergistic actions, it is intriguing to speculate that NA and ATP may also be vasodilator co-transmitters in nerves supplying the coronary arteries.

A vascular smooth muscle relaxant response to ATP is unusual but it is not unique to this vessel. The vasodilator response to ATP in the rabbit mesenteric artery (Mathieson & Burnstock, 1985) and the rabbit portal vein (Reilly *et al.*, 1987) is mediated via P_{2Y}-purinoceptors located directly on the smooth muscle not on the endothelium, although NA in these arteries produces a vasoconstrictor response. Ramme and coworkers suggest that in the resistance vessels in the smaller branches of the rabbit mesenteric artery, ATP is the sole neurotransmitter acting postjunctionally upon stimulation of the sympathetic nerves and that the NA released concomitantly acts entirely on prejunctional α_2-adrenoceptors to modulate transmitter release (Ramme *et al.*, 1987). Furthermore, there is now substantial evidence that ATP is a sensory neurotransmitter in some primary afferent nerve fibres (Jahr & Jessel, 1983; Salt & Hill, 1983; Fyffe & Perl, 1984) and may be released antidromically upon stimulation of sensory nerves (Holton & Holton, 1954; Holton, 1959; Burnstock, 1987). Thus, exogenous ATP in the rabbit mesenteric artery may be acting upon receptors which would be stimulated physiologically by sensory rather than sympathetic nerves. The rabbit portal vein is innervated by sympathetic nerves but is also supplied with non-sympathetic (purinergic) nerves which release ATP and mediate vasodilatation (Burnstock *et al.*, 1979; Reilly *et al.*, 1987; Su, 1987). These non-adrenergic nerves are the source of approximately 50% of the purines released by electrical field stimulation (Levitt & Westfall, 1982) and may account for the presence of the P_{2Y}-purinoceptor on the smooth muscle in this vessel. However, no such non-adrenergic innervation has been described for the rabbit epicardial coronary arteries.

ATP and NA have been shown to be co-transmitters producing direct constrictor effects on the smooth muscle of many blood vessels. Our study shows that in the rabbit coronary artery they both mediate vasodilatation by direct action on the vascular smooth muscle. This may have important functional consequences in conditions associated with endothelial disruption and is consistent with the general hypothesis that ATP and NA may also be sympathetic co-transmitters in this vessel.

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Effects of calcium modulators on vagally-mediated constriction in the guinea-pig isolated trachea

[1]Dorothy J. McCaig & Susan Aitken

School of Pharmacy, The Robert Gordon Institute of Technology, Schoolhill, Aberdeen AB9 1FR

1 The effects of calcium modulators on tracheal constriction evoked by vagal stimulation were examined in the isolated, innervated trachea of the guinea-pig. Responses were assessed in the Krebs-filled trachea as changes in intraluminal pressure (ILP), increases and decreases reflecting constriction and dilatation, respectively.

2 Preparations had a positive resting ILP, indicating significant spontaneous tone. Verapamil and nifedipine reduced baseline ILP, whilst Bay K 8644 had mixed effects.

3 Verapamil and nifedipine attenuated vagal responses in a concentration-dependent manner. At lower concentrations attenuation was due entirely to postjunctional effects but at higher concentrations prejunctional effects may have contributed to attenuation.

4 Verapamil and nifedipine attenuated vagal responses in the absence or presence of flurbiprofen, indicating that their effects are largely independent of the generation of cyclo-oxygenase products. Nifedipine, however, was less effective in reducing responses to low frequency vagal stimulation (up to 5 Hz) when flurbiprofen was present.

5 Bay K 8644 augmented vagal responses, the degree varying widely between preparations.

6 It was concluded that influx of Ca^{2+} through voltage-operated Ca^{2+} channels contributes significantly to vagally-mediated tracheal constriction in normal trachea and in trachea where endogenous release of cyclo-oxygenase products is inhibited.

Keywords: Trachea; vagus nerve; acetylcholine; Bay K 8644; nifedipine; flurbiprofen; verapamil

Introduction

Airway hyperreactivity is a hallmark of bronchial asthma manifest as a greatly increased sensitivity to a wide range of bronchoconstricting stimuli (Empey, 1982). It has been suggested that excessive permeability of airway smooth muscle cells to Ca^{2+} ions might underly airway hyperreactivity (Middleton, 1980). Increased Ca^{2+} influx from the extracellular fluid could occur if, for example, the number or frequency of opening of voltage-operated Ca^{2+}-channels (VOCs) in the smooth muscle cell membrane were increased. Evidence in support of this concept has been obtained recently in passively sensitized human airway smooth muscle (Black *et al.*, 1989). In the airway smooth muscle of the guinea-pig, and to some extent the dog and ox, there is spontaneous influx of Ca^{2+} ions through VOCs which can be recorded electrophysiologically as regular or irregular depolarizations, often termed slow waves (Small & Foster, 1988). The trachea isolated from albumin-sensitized guinea-pigs, which serve as an animal model of bronchial asthma, exhibits changes consistent with increased Ca^{2+} influx, including increased incidence of slow waves and augmented depolarization and constriction in response to vagal stimulation (McCaig, 1987). The long-term aim of this work is to examine the possibility that altered Ca^{2+}-channel behaviour contributes to vagal hyperresponsiveness and to airway hyperreactivity in general in albumin-sensitized guinea-pigs. There is limited information available, however, on the effects of drugs that modulate VOC behaviour on vagal responses in normal airways. As a first step, therefore, we have examined the effects of two VOC blockers, verapamil and the dihydropyridine, nifedipine, on vagally-mediated constriction in the guinea-pig isolated trachea. The effects of another dihydropyridine, Bay K 8644, which promotes VOC opening (Freedman & Miller, 1984), were studied for comparison. Finally, since the coupling of Ca^{2+} influx to the development of tone is influenced by products of the cyclo-oxygenase pathway of arachidonic acid metabolism (McCaig & Rodger, 1988), the effects of verapamil and nifedipine were compared in the absence and presence of the cyclo-oxygenase inhibitor, flurbiprofen.

[1] Author for correspondence.

Methods

Preparation

Guinea-pigs (male, 300–550 g) were killed by a blow to the head. The trachea, with, or without the right vagus nerve and recurrent laryngeal branch as appropriate, was excised and mounted horizontally in a chamber through which Krebs solution at 37°C flowed at a rate of 5 ml min^{-1} (Blackman & McCaig, 1983). The composition of the Krebs solution was (mM): Na^+ 127, K^+ 5.9, Ca^{2+} 1.2, Mg^{2+} 1.2, Cl^- 121, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25, glucose 11; the solution was equilibrated both in the chamber and the reservoir with a gas mixture of 95% O_2 and 5% CO_2.

The trachea was filled with Krebs solution then the caudal end was closed and the rostral end attached to a pressure transducer (Statham) for continuous monitoring of intraluminal pressure (ILP) on a pen recorder (electromed). Increases and decreases in ILP directly reflect tracheal constriction and dilatation, respectively. The vagus nerve was stimulated pre-ganglionically through a suction electrode with rectangular pulses of amplitude 40 V and duration 1 ms for 5 s at frequencies of 1–50 Hz.

Procedure

After a 20 min equilibration period a frequency-response curve was obtained to vagal stimulation or the response to applied acetylcholine (ACh, 10^{-4} M) was assessed. Tissues were then exposed to one of the test drugs. In some experiments the vagus nerve was stimulated at 20 Hz for 5 s every 90 or 120 s during exposure to the test drug in order to follow the time course of the response. After 20–30 min, a second frequency-

response curve or response to ACh was obtained. Some tissues were then exposed to up to 2 higher concentrations of the test drug and responses monitored as before. In some experiments flurbiprofen, 10^{-6} M, was added to the reservoir of Krebs solution to prevent the generation of cyclo-oxygenase products. The stability of the tissues over time was assessed by obtaining responses repeatedly in the absence of drug. The dihydropyridines were dissolved in ethanol which produced a final bath concentration of 0.1% ethanol. The effects of this concentration of ethanol alone were examined.

Drugs

The following drugs were used: acetylcholine chloride (ACh, Sigma), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer), flurbiprofen (Boots), nifedipine (Bayer) and verapamil hydrochloride (Sigma).

Acetylcholine, verapamil and flurbiprofen were dissolved in distilled water. Nifedipine and Bay K 8644 were dissolved in ethanol and the experiments were performed in a darkened room with the chamber covered in aluminium foil to minimize photolysis of the drugs. Flurbiprofen and verapamil were added to the reservoir of Krebs solution. All other drugs were added directly to the chamber with the perfusion pump off.

Analysis of data

Results are expressed as the mean ± s.e.mean. Statistical significance was assessed by two-way analysis of variance and *t* tests for independent or paired data, as appropriate. The level of significance was set at $P < 0.05$.

Results

Effects of drugs on resting intraluminal pressure

In the absence of flurbiprofen tracheal preparations had significant spontaneous resting tone, as reflected by a positive intraluminal pressure (ILP of $+3.4 \pm 0.4\,cmH_2O$, $n = 8$). Verapamil evoked a small decrease in ILP which developed over 10–15 min and ranged from $-0.6 \pm 0.3\,cmH_2O$ at 10^{-7} M to $-1.1 \pm 0.6\,cmH_2O$ at 10^{-5} M. Nifedipine also elicited a gradual but more substantial fall in ILP, ranging from $-1.0 \pm 0.8\,cmH_2O$ at 10^{-7} M to $-4 \pm 1\,cmH_2O$ at 10^{-5} M. Bay K 8644, 10^{-6} M, reduced ILP by less than $0.5\,cmH_2O$ in two tissues and increased it slightly in four others ($+1.8 \pm 0.6\,cmH_2O$) over a 5 min period, the increase subsiding thereafter in the continued presence of the drug. In the presence of flurbiprofen, 10^{-6} M, spontaneous tone was abolished as reported previously (McCaig, 1989).

Effect of flurbiprofen on responses to vagal stimulation

Preganglionic stimulation of the vagus nerve evoked frequency-dependent increases in ILP, reflecting tracheal constriction (Figure 1). When frequency-response curves were generated repeatedly at 30–40 min intervals in the absence of any drug (time-match controls) response amplitude tended to decrease slightly, but responses at 20 and 50 Hz only were significantly reduced in the fourth such frequency-response curve.

In the presence of flurbiprofen, 10^{-6} M, the frequency-response curve was shifted to the left (Figure 1). Responses at lower frequencies of stimulation (up to 5 Hz) were increased in amplitude to a greater degree than those at higher frequencies, the maximum response was reached at 20 Hz (cf. 50 Hz in the absence of flurbiprofen) and responses declined thereafter such that at 50 Hz the response was significantly smaller in the presence of flurbiprofen. In addition there was no diminution of response amplitude over time in flurbiprofen-treated tissues.

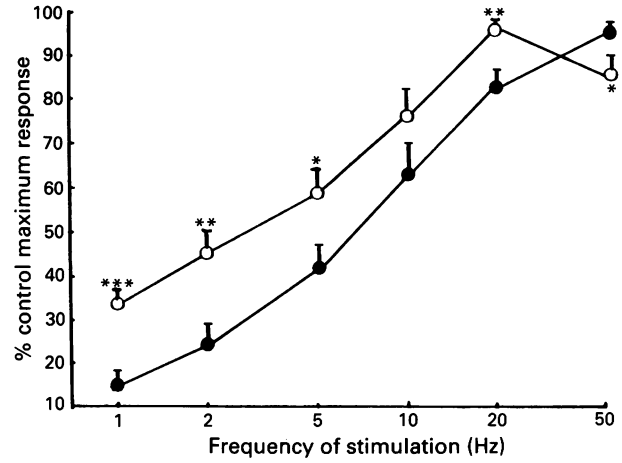

Figure 1 Relationship between vagal responses (expressed as percentage control maximum increase in intraluminal pressure, ordinate scale) and frequency of stimulation (abscissa scale) in guinea-pig isolated trachea in the absence (●) ($n = 19$) and presence (○) ($n = 11$) of flurbiprofen, 10^{-6} M. Values are the mean and bars represent s.e.mean. Asterisks denote values significantly different from corresponding value in the absence of flurbiprofen at $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Effect of verapamil on constrictor responses

Verapamil, 10^{-7} M, in the absence of flurbiprofen, had no significant effect on the amplitude of responses to vagal stimulation (Figure 2a). At a concentration of 10^{-6} M verapamil, vagal responses were attenuated (Figures 2a and 3a) at stimulation frequencies of 5 Hz and above. At lower frequencies of stimulation, however, the effect was variable and, despite increasing the number of experiments to 9, the changes were not significant. Verapamil, 10^{-5} M, reduced response amplitude significantly at all frequencies of stimulation by 60–80% (Figure 2a). Attenuation was evident after 5 min, reached a peak after 30 min contact with the drug and was significantly greater at higher concentrations of verapamil. In 4 tissues, for example, exposed to 10^{-7}, 10^{-6} and 10^{-5} M verapamil successively, responses at 10 Hz were attenuated by $14 \pm 5\%$, $39 \pm 8\%$ and $60 \pm 3\%$, respectively (values all significantly different at $P < 0.05$).

In the presence of flurbiprofen, verapamil also attenuated vagal responses, significant reductions in amplitude occurring at all concentrations tested and at all frequencies of stimulation (except 50 Hz at 10^{-7} M, verapamil, Figure 2b). Attenuation was concentration-dependent (e.g. at 20 Hz, 10^{-7} M: $-17 \pm 6\%$; 10^{-6} M: $-36 \pm 5\%$; 10^{-5} M: $-52 \pm 5\%$, values all significantly different at $P < 0.05$). It was noted that responses at lower frequencies of stimulation (up to 5 Hz) were more profoundly inhibited than those at higher frequencies (> 10 Hz), at any concentration of verapamil in flurbiprofen-treated tissues (e.g. 10^{-6} M verapamil at 2 Hz, $-66 \pm 8\%$; at 20 Hz $-36 \pm 5\%$; values significantly different at $P < 0.05$). Comparison of Figures 2(a) and (b) suggested that verapamil might be more effective in reducing vagal responses in the presence of flurbiprofen, particularly at lower concentrations of verapamil and lower frequencies of vagal stimulation. Statistical analysis, however, indicated that the effects of verapamil at any concentration were not significantly different in flurbiprofen-treated tissues.

Application of ACh, 10^{-4} M, elicited increases in ILP amounting to approximately 80% of the ACh maximum, which were highly reproducible and did not deteriorate over time. All experiments with ACh were performed in the absence of flurbiprofen. Verapamil significantly attenuated this response at each concentration tested (10^{-7} to 10^{-5} M, Figure 4), reductions ranging from 36 to 44%. Attenuation was not significantly greater at higher concentrations of verapamil. At

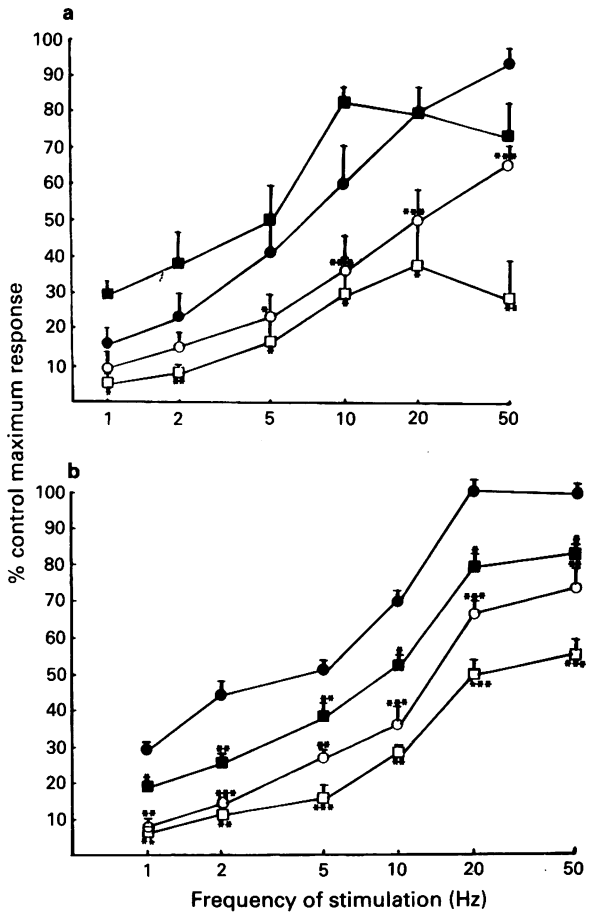

Figure 2 Relationship between vagal responses (expressed as percentage control maximum increase in intraluminal pressure, ordinate scale) and frequency of stimulation (abscissa scale) in guinea-pig isolated trachea before (●) and 20–30 min after addition of verapamil, 10^{-7} M (■), 10^{-6} M (○) and 10^{-5} M (□) in the absence (a) and presence (b) of flurbiprofen, 10^{-6} M. Values are the mean of 4 to 9 observations and bars represent s.e.mean. Asterisks denote values significantly different from corresponding control values at $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

10^{-5} M verapamil, responses to ACh were significantly less attenuated than vagal responses (e.g. ACh, $-44 \pm 7\%$; vagal stimulation at 50 Hz, $-68 \pm 9\%$; values significantly different at $P < 0.05$).

Effect of nifedipine on constrictor responses

In control experiments it was established that ethanol, 0.1%, had no effect on the frequency-response curve to vagal stimulation (results not shown). Nifedipine, 10^{-7} M, in the absence of flurbiprofen did not significantly attenuate vagal responses either (except at 5 Hz) but at higher concentrations, 10^{-6} M and 10^{-5} M, responses were significantly reduced in a concentration-dependent manner at all frequencies of stimulation (range 10^{-6} M: 35–68%; 10^{-5} M: 66–97%; see Figures 3b and 5a). Attentuation increased with concentration of nifedipine (e.g. at 20 Hz, $-9 \pm 20\%$; $-49 \pm 13\%$ and $-71 \pm 10\%$ at 10^{-7} M, 10^{-6} M and 10^{-5} M, respectively, all values significantly different at $P < 0.05$). Responses at lower frequencies of stimulation were reduced more markedly by nifedipine (e.g. at 10^{-5} M responses at 2 Hz were reduced by $93 \pm 3\%$ and at 20 Hz by $71 \pm 10\%$, difference significant at $P < 0.025$). Attenuation of responses began after approximately 3 min and peaked after 16 min contact with nifedipine.

In the presence of flurbiprofen, 10^{-6} M, attenuation of vagal responses occurred also but was less marked, significant mainly at higher frequencies of stimulation and not clearly concentration-dependent (Figure 5b). When the effects of

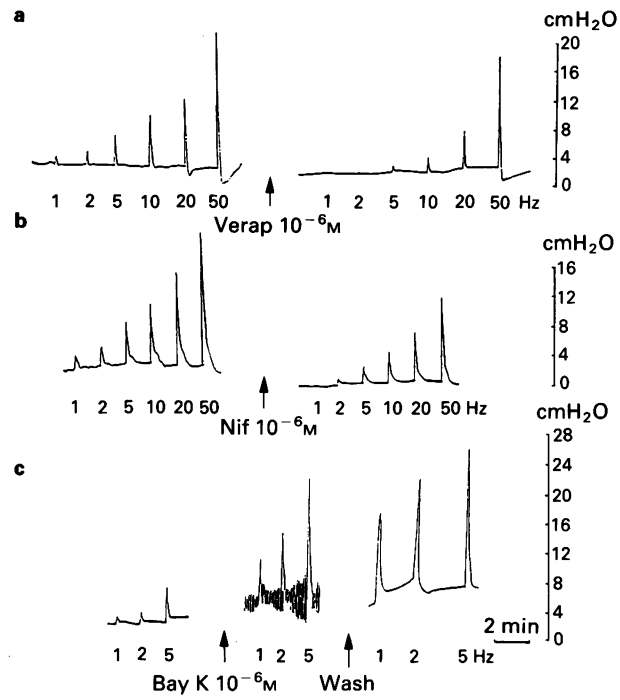

Figure 3 Effect of drugs on vagally-induced increases in intraluminal pressure in guinea-pig isolated trachea. (a) Responses before and 20–30 min after exposure to verapamil (Verap) 10^{-6} M; (b) responses before and 20–30 min after exposure to nifedipine (Nif), 10^{-6} M; (c) responses before, 20–25 min after addition of Bay K 8644, 10^{-6} M, and 3–8 min after start of washout. Traces for each drug are taken from a single trachea. Response amplitude was reduced in the presence of the test drug in (a) and (b) and increased in (c), at each frequency of stimulation.

nifedipine, 10^{-6} M or 10^{-5} M, were compared in the absence and presence of flurbiprofen (Figure 5a and b) it was evident that at low frequencies of stimulation attenuation was considerably less marked in the presence of flurbiprofen (10^{-6} M). At 5 Hz, for example, in the presence of nifedipine, 10^{-6} M, responses were reduced by $63 \pm 12\%$ without flurbiprofen but only by $16 \pm 13\%$ with flurbiprofen present (values significantly different at $P < 0.05$).

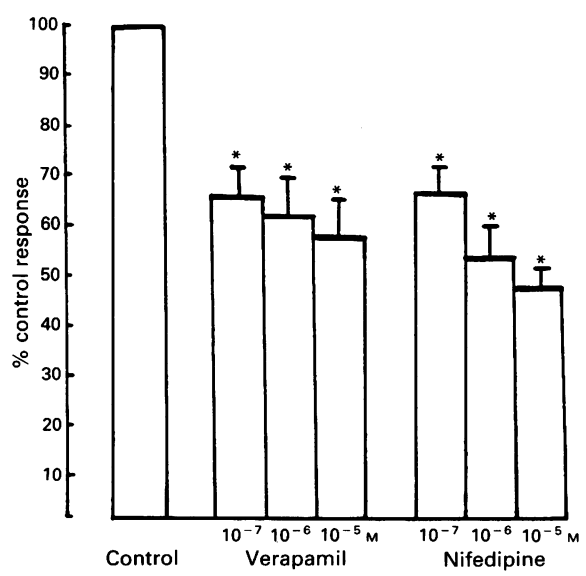

Figure 4 Histogram showing the effect of drugs on the response to applied acetylcholine (ACh), 10^{-4} M, in guinea-pig isolated trachea. Responses are expressed as percentage of the control response to ACh, in the absence of drug. Values are the mean of 8 observations and bars represent s.e.mean. Asterisks denote values significantly different from the control response to ACh at $*P < 0.001$.

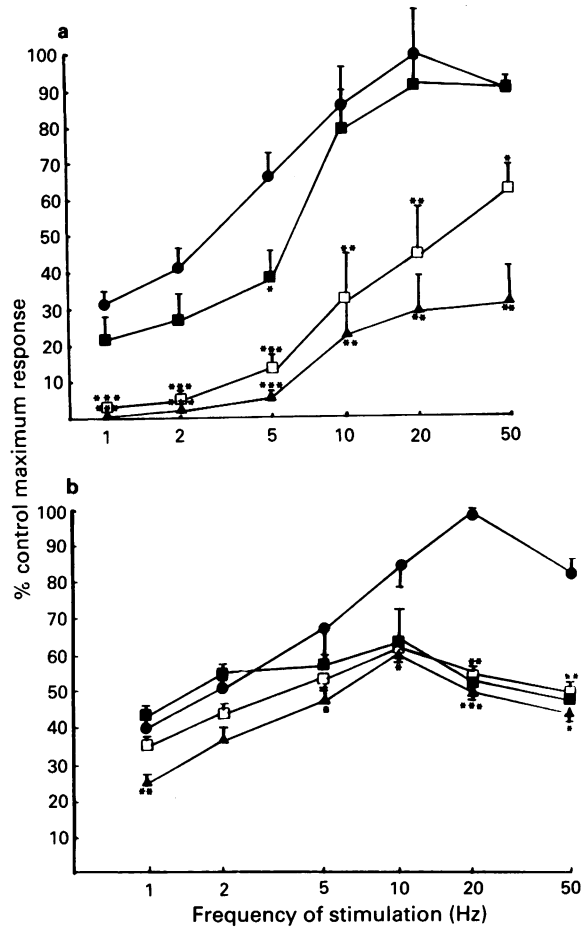

Figure 5 Relationship between vagal responses (expressed as percentage control maximum increase in intraluminal pressure, ordinate scale) and frequency of stimulation (abscissa scale) in guinea-pig isolated trachea before (●) and 20–30 min after the addition of nifedipine 10^{-7} M (■); 10^{-6} M (□) and 10^{-5} M (▲) in the absence (a) and presence (b) of flurbiprofen, 10^{-6} M. Values are the mean of 4 to 9 observations and bars represent s.e.mean. Asterisks denote values significantly different from corresponding controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

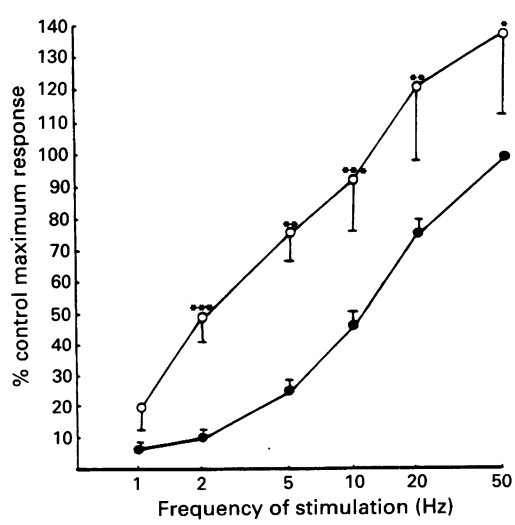

Figure 6 Relationship between vagal responses (expressed as percentage control maximum increase in intraluminal pressure, ordinate scale) and frequency of stimulation (abscissa scale) in guinea-pig isolated trachea before (●) and 20–30 min after (○) the addition of Bay K 8644, 10^{-6} M. Values are the mean of 5 observations and bars represent s.e.mean. Asterisks denote values significantly different from corresponding controls: $*P < 0.05$, $**P < 0.025$; $***P < 0.01$.

Nifedipine, 10^{-7} to 10^{-5} M, also significantly reduced the amplitude of the response to applied ACh (10^{-4} M, Figure 4). The degree of attenuation increased with concentration of nifedipine from $-34 \pm 5\%$ at 10^{-7} M to $-53 \pm 4\%$, at 10^{-5} M (values significantly different at $P < 0.05$). At 10^{-5} M nifedipine, responses to ACh were reduced significantly less than those to vagal stimulation at frequencies up to and including 20 Hz.

Effect of Bay K 8644 on vagal responses

The amplitude of vagally-mediated increases in ILP was increased in the presence of Bay K 8644, 10^{-6} M (no flurbiprofen present) (Figures 3c and 6). The degree of augmentation varied considerably between tissues. At a stimulation frequency of 50 Hz, for example, increases ranged from 5–116%. Within a few minutes of commencing washout of Bay K 8644 an unexpected pattern of response was observed in 4 of 5 preparations with responses at low frequencies of stimulation (up to 5 Hz) transiently greatly increased in amplitude (Figure 3c). At higher frequencies the response amplitude was unchanged in the early stages of washout. Responses at all frequencies of stimulation declined in amplitude towards control levels over 30 min in drug-free Krebs solution. No augmentation of vagal responses was observed in 3 tissues exposed to lower concentrations of Bay K 8644 (10^{-7} M and 5×10^{-7} M).

Discussion

These results show that two Ca^{2+}-channel blockers, verapamil and nifedipine attenuate vagally-mediated constriction in the guinea-pig isolated trachea, indicating that a significant component of this response is due to Ca^{2+} influx through VOCs. Similar results have been obtained in bovine trachea with nifedipine (Cuss *et al.*, 1985) and in human airways with methoxyverapamil (Kannan & Davies, 1988). Farley & Miles (1978) observed in canine trachealis that responses to low concentrations of acetylcholine were more susceptible to inhibition by VOC blockers than responses to high concentrations. They concluded that there is significant involvement of Ca^{2+} influx through VOCs in airway constriction elicited by low concentrations of agonists but that at high concentrations constriction is due mainly to potential-independent mechanisms, involving influx of Ca^{2+} through receptor-operated Ca^{2+}-channels (ROCs) and release of intracellularly-stored Ca^{2+}. In the present work a significant VOC-dependent component was evident in responses at all frequencies of vagal stimulation.

In keeping with the result of Farley & Miles (1978) in canine trachea other groups have shown that low concentrations of cholinomimetics are more susceptible to Ca^{2+} antagonists than higher concentrations in species including guinea-pig, rabbit and dog (Weiss *et al.*, 1985; Baba *et al.*, 1986; Bengtsson *et al.*, 1987). In contrast neither verapamil nor nifedipine had significant effects on the constriction induced by ACh at any concentration in guinea-pig trachea in the work of Foster *et al.*, (1984) and Ahmed *et al.*, (1985). In the present work, both verapamil and nifedipine consistently reduced the response to a single dose of ACh, suggesting a significant contribution from VOC opening even at this relatively high concentration of ACh.

In the absence of flurbiprofen, attenuation of vagal responses increased with increasing concentrations of verapamil or nifedipine. At concentrations of 10^{-7} or 10^{-6} M of these drugs the responses to vagal stimulation and applied ACh were attenuated to a similar degree. This strongly suggests that their effects can be accounted for entirely by postjunctional blockade of the L type VOCs in the smooth muscle cell membrane (see Spedding, 1987). At the highest concentration of verapamil or nifedipine tested, 10^{-5} M, vagal responses were attenuated more than those to applied ACh, suggesting a prejunctional component to the inhibition. It has been demon-

strated, however, that Ca^{2+} antagonists selectively block L-type channels and do not affect the N-type channels which are thought to predominate in nerve-terminals and are involved in transmitter release (Miller, 1987; Spedding, 1987). Stimulation of the vagus nerve in this preparation is preganglionic and an effect on ganglionic transmission cannot be ruled out since L-type channels are thought to be present on neuronal cell bodies (Spedding, 1987). Alternatively, ACh released from nerve-terminals may have effects which differ from those of applied ACh. If, for example, the distribution of VOCs in the smooth muscle cell membrane were asymmetrical, with clustering at sites close to nerve terminals, a greater part of the action of neuronally-derived ACh might depend on VOC activation.

The development of spontaneous tone in guinea-pig trachea depends on cyclical influx of Ca^{2+} ions through VOCs, recorded as slow waves in single trachealis cells, since it is abolished in Ca^{2+}-free medium or reduced by VOC blockade (Small, 1982; McCaig, 1986). In the presence of cyclo-oxygenase inhibitors, such as indomethacin or flurbiprofen, slow wave activity continues unabated but is no longer coupled to tension development, indicating a requirement for cyclo-oxygenase products in the coupling process (Boyle *et al.*, 1988; McCaig & Rodger, 1988; McCaig, 1989). On the other hand endogenous cyclo-oxygenase products have been shown to suppress cholinergic neurotransmission in canine airways (Ito & Tajima, 1981) and cyclo-oxygenase inhibition augments vagal constriction in guinea-pig trachea (McCaig, 1989). In the present work it was found that flurbiprofen augmented vagal responses and prevented the deterioration in vagal response amplitude that occurred over several hours. Verapamil and nifedipine attenuated vagal responses both in the absence and presence of flurbiprofen. Nifedipine, however, was less effective in the presence of flurbiprofen, especially at lower frequencies of stimulation (<10 Hz). This suggests that a smaller component of the constriction is dependent on VOC opening in flurbiprofen-treated tissues where cholinergic neurotransmission is not being naturally dampened by endogenous cyclo-oxygenase products. This is not borne out by the results for verapamil, however, which were not significantly different in the presence of flurbiprofen. This differential effect of flurbiprofen on nifedipine could be related to the fact that nifedipine and verapamil each bind to specific but distinct sites within the Ca^{2+} channel (Towart & Schramm, 1984).

A reduction in resting tone of guinea-pig trachea in response to methoxyverapamil has been reported (Small, 1982) but neither nifedipine nor verapamil was found to affect resting tone significantly (Foster *et al.*, 1984; Ahmed *et al.*, 1985). In the present work both verapamil and nifedipine reduced baseline tone, which is consistent with the suppression of slow wave activity by these drugs. Bay K 8644 had variable effects on spontaneous tone. Similar results have been obtained in guinea-pig (Allen *et al.*, 1985) and human airways (Black *et al.*, 1989). In addition, Bay K 8644 had no effect on membrane potential or slow wave discharge in guinea-pig trachealis (Allen *et al.*, 1985). Bay K 8644 promotes Ca^{2+} influx through VOCs seemingly by binding to and activating the dihydropyridine binding site thereby prolonging the open state of the channel (Freedman & Miller, 1984; Reuter *et al.*, 1985). It seems, therefore, that Bay K 8644 has a limited capacity to activate VOCs in trachea at rest. In contrast Bay K 8644 augmented responses to vagal stimulation, sometimes by very large amounts, indicating that Bay K 8644 can activate VOCs involved in the cholinergic neurotransmission process. Whilst it is likely that these VOCs are associated with the smooth muscle cell membrane, a prejunctional effect on transmitter release cannot be excluded (Perney *et al.*, 1986).

In conclusion the Ca^{2+} antagonists verapamil and nifedipine reduce vagal constriction in guinea-pig isolated trachea, whilst the Ca^{2+}-channel activator Bay K 8644 augments this response. These results indicate significant involvement of VOCs in vagally-mediated constriction. The effects of verapamil and nifedipine occur whether or not the enzyme cyclo-oxygenase is inhibited but nifedipine is much less effective in reducing responses to low frequency vagal stimulation in the presence of flurbiprofen. At higher concentrations of verapamil or nifedipine attenuation may involve prejunctional effects on cholinergic neurotransmission.

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Renovascular hypertension impairs formation of endothelium-derived relaxing factors and sensitivity to endothelin-1 in resistance arteries

*Yasuaki Dohi, ††Leoluca Criscione & *,†,[1]Thomas F. Lüscher

Department of Research, *Laboratory of Vascular Research and Department of Medicine, †Divisions of Clinical Pharmacology and Cardiology, University Hospital Basel and ††Cardiovascular Research Department, Ciba-Geigy Ltd., Basel, Switzerland

1 Endothelium-dependent vascular regulation was investigated in mesenteric resistance arteries of Goldblatt two kidney-one clip (2K1C) renovascular hypertensive rats.

2 Third order branches of mesenteric arteries were dissected free and mounted on glass cannulae in an organ chamber. Changes in vascular diameter were measured in pressurized and perfused arteries with a video dimension analyzer.

3 Acetylcholine evoked endothelium-dependent relaxations that were much more pronounced with intraluminal than with extraluminal application. In 2K1C rats the relaxation induced by intraluminal, but not extraluminal acetylcholine was decreased compared to normotensive Wistar Kyoto rats (WKY). Increased duration of hypertension further decreased the response to intraluminal but not extraluminal acetylcholine.

4 Endothelin-1 and noradrenaline caused contractions which were augmented by removal of the endothelium. This augmentation was reduced in 2K1C rats compared to WKY; the difference was small with noradrenaline but more pronounced with endothelin-1.

5 In arteries without endothelium the sensitivity, but not the maximal contraction to endothelin-1 was lower in 2K1C rats, while the response to noradrenaline was not different in 2K1C rats and WKY. The sensitivity to the peptide was not further affected by increasing the duration of hypertension.

6 Thus, renovascular hypertension leads to an impaired intraluminal, but not extraluminal activation of the release of endothelium-derived relaxing factor and a decreased inhibitory effect of the endothelium against endothelin-1- and noradrenaline-induced contractions in mesenteric resistance arteries. Furthermore, the sensitivity, but not the maximal response of vascular smooth muscle to endothelin-1 was reduced.

Keywords: Acetylcholine; endothelin-1; endothelium-derived relaxing factor; noradrenaline; Wistar Kyoto rats; renovascular hypertension; resistance arteries

Introduction

Vascular smooth muscle tone is locally regulated by the endothelium (Lüscher, 1988; Lüscher & Vanhoutte, 1990). The endothelium releases endothelium-derived relaxing factor(s) [EDRF(s)] or endothelium-derived contracting factors in response to vasoactive substances and physical stimuli (Lüscher, 1988; Lüscher & Vanhoutte, 1990). The endothelium can reduce the effects of vasoconstrictor substances either through basal or stimulated release of relaxing factors (Lüscher, 1988; Lüscher & Vanhoutte, 1990; Dohi *et al.*, 1990). Alterations in this modulating function of the endothelium may play an important role in the development or maintenance of hypertension, particularly if occurring in small arteries which take part in the regulation of peripheral vascular resistance and organ blood flow (Lüscher, 1988; 1989; Lüscher & Vanhoutte, 1990). Indeed, endothelium-dependent relaxation to acetylcholine is decreased in isolated arteries of spontaneously hypertensive rats (Lüscher, 1988; 1989; Lüscher & Vanhoutte, 1990). However, in the resistance circulation it remains uncertain whether these changes observed in the spontaneously hypertensive rat are truely related to blood pressure. Thus, more information about endothelium-dependent responses in a secondary form of hypertension appears appropriate.

Besides the relaxing factors, the endothelium produces the potent vasoconstrictor endothelin (Yanagisawa *et al.*, 1988). The peptide evokes a strong and long-lasting contraction in isolated vascular tissue including that obtained from man (D'Orleans-Juste *et al.*, 1989; Lüscher *et al.*, 1990; Dohi & Lüscher, 1990a). However, the role of endothelin in hypertension is not clear since the responsiveness of arteries obtained from hypertensive animals to the peptide is still controversial. Increased (Tomobe *et al.*, 1988; Miyauchi *et al.*, 1989; Criscione *et al.*, 1990), decreased (Dohi & Lüscher, 1990b; Auch-Schwelk & Vanhoutte, 1989) and normal responses (Diederich *et al.*, 1989; Criscione *et al.*, 1990) to endothelin-1 have been reported in vessels from spontaneously hypertensive rats. The responsiveness of resistance arteries to endothelin-1 in renovascular hypertension would be of pathophysiological interest, since this type of hypertension is characterized by an increased activity of the renin-angiotensin system (Brunner *et al.*, 1962; Fourcade *et al.*, 1971; Ploth *et al.*, 1977; Huang *et al.*, 1981; Miyazaki *et al.*, 1986; Wilson *et al.*, 1989) and because angiotensin II stimulates the production of endothelin in cultured endothelial cells (Emori *et al.*, 1989).

The present study was designed to investigate (1) the modulatory function of the endothelium and (2) the response to endothelin-1 in mesenteric resistance arteries of renovascular hypertensive rats.

Methods

Animals

Male Wistar Kyoto rats (WKY) were obtained from IFFA CREDO (L'Arbresle, France). At the age of 4 weeks the left

[1] Author for correspondence at: Department of Medicine, University Hospital, CH-4031 Basel, Switzerland.

renal artery of the rat was clipped under anaesthesia with ether. The rats were maintained on a standard rat chow with free access to drinking water and used for experiments at the age of 12 or 16 weeks, respectively. Systolic blood pressure was measured in conscious rats by the tail-cuff method. Systolic blood pressures averaged 233 ± 7 mmHg and 266 ± 6 mmHg in 12 and 16 week old two kidney-one clip (2K1C) rats, respectively ($P < 0.01$) and 122 ± 5 mmHg in 12 week old WKY ($P < 0.0001$ versus age-matched 2K1C rats).

Experimental set-up

The mesentery was removed from rats anaesthetized with pentobarbitone (50 mg kg^{-1}, i.p.) and placed into cold buffer solution of the following composition (mM): NaCl 118.6, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.1, disodium calcium edetate 0.026, glucose 10.1. A small segment (3 mm in length) of the third order branch of the mesenteric artery was isolated under a dissection microscope. The artery was transferred to an arteriograph chamber (Halpern *et al.*, 1984; Dohi *et al.*, 1990; Dohi & Lüscher, 1990a) filled with buffer solution (95% O_2: 5% CO_2, 37°C).

The chamber contained two glass microcannulae. The proximal end of the artery was cannulated with the afferent cannula and secured with a surgical nylon suture. The distal end was attached to the inside of the efferent cannula. The artery was perfused with buffer solution containing 1% bovine serum albumin (supplied from a 500 ml reservoir) and equilibrated under an optimal transmural pressure (i.e. that pressure at which contractions to KCl were maximal) of 30 mmHg (Dohi *et al.*, 1990; Dohi & Lüscher, 1990a) for 1 h before experiments. A polyethylene catheter (internal diameter 50 μm) was placed in the afferent cannula and connected to a pressure transducer for measurement of transmural pressure or for intraluminal application of drugs. Transmural pressure was adjusted by changing the height of the reservoir supplying the perfusate.

The arteriograph was placed on a stage of a microscope which had a TV camera attached to the viewing tube. The signal derived from the video image of the vessel was processed by an electronic system (Living Systems Instrumentation, Burlington, VT, U.S.A.) for continuous measurement and recording of intraluminal diameter.

Endothelium removal

To remove the endothelium, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]1-propanesulphonate (CHAPS) was infused intraluminally for 30 s (Tesfamariam & Halpern, 1988; Dohi *et al.*, 1990; Dohi & Lüscher, 1990a). The resting intraluminal diameter and the contraction to KCl (100 mM) were not affected by endothelial removal. The presence or absence of the endothelium was confirmed by the presence or absence of the response to acetylcholine (1×10^{-6} M).

Vascular dimension in normotensive and hypertensive rats

In 12 week old WKY, the intraluminal diameter and wall thickness of the studied vessels were 199 ± 5 μm and 30 ± 1 μm, respectively. The wall thickness was greater in 12 week old 2K1C rats (38 ± 1 μm; $P < 0.005$) than in age-matched WKY, whereas the intraluminal diameter of 2K1C rats (196 ± 4 μm) was not significantly different. In 16 week old 2K1C rats, neither the wall thickness (30 ± 1 μm) nor the intraluminal diameter (197 ± 5 μm) differed from those of 12 week old 2K1C rats.

Protocols

Concentration-response curves to extraluminal noradrenaline and endothelin-1 were obtained in arteries with and without endothelium by cumulative application of the drug. To study endothelium-dependent relaxations to intraluminal and extraluminal acetylcholine, arteries were precontracted half-maximally with extraluminal noradrenaline. Flow through the vascular segment was repeatedly determined by counting the drops at the efferent cannula. In this experimental set-up, 20 drops corresponded to 1 ml; accordingly the volume of 1 drop equalled 0.05 ml. Depending on the contractile state of the blood vessel, the intraluminal flow rates ranged from 0.03–0.6 ml min^{-1}. The concentrations of intraluminally applied drugs required to construct a concentration-response curve were calculated for the actual level of flow under each experimental condition and the infusion rate (through the polyethylene catheter) of the solution containing the drugs was adapted accordingly (Syringe infusion pump 22, Harvard Apparatus, Boston, Mass., U.S.A.; infusion rates: 1–30 μl min^{-1}). Contractions or relaxations were expressed as percent decrease or increase in intraluminal vascular diameter, respectively. The diameter at the resting level was taken as 100%.

Drugs

The following drugs were used (unless otherwise stated, from Sigma Chemicals, St. Louis, Mo., U.S.A.): acetylcholine hydrochloride, 3-[(3-cholamidopropyl) dimethylammonio]1-propanesulphonate (CHAPS), endothelin-1 (Novabiochem AG, Switzerland), (−)-noradrenaline. Endothelin-1 was dissolved in distilled water containing 0.05% albumin and all other drugs in distilled water. The concentrations are expressed as final molar concentration.

Calculations and statistics

Data are given as mean ± s.e.mean. The concentration of an agonist causing half-maximal contraction (ED_{50} value) or half-maximal inhibition of a preceding contraction (IC_{50} value) was calculated for each experiment and expressed as negative log molar (pD_2 value). The shift of concentration-response curves was expressed as concentration shift at pD_2 values. In each set of experiments, n equals the number of animals studied. Statistical evaluation was done by unpaired Student's t test. Means were considered to be significantly different when P values were less than 0.05.

Results

Endothelium-dependent relaxation to acetylcholine

Intraluminal activation: Intraluminal acetylcholine (10^{-9}–10^{-6} M) evoked concentration-dependent relaxations in arteries with endothelium (Figure 1a). In 12 week old 2K1C rats both the pD_2 value and the maximal response were smaller than in age-matched WKY ($P < 0.05$; Table 1).

In 16 week old 2K1C rats the relaxation to 3×10^{-8} M (54 ± 7%) and 10^{-7} M of acetylcholine (80 ± 3%) were further reduced as compared to 12 week old 2K1C rats (78 ± 8% and 92 ± 3%, respectively; $P < 0.05$), while the pD_2 value and the maximal response were identical in the two groups (Table 1).

Extraluminal activation: In 12 week old rats, extraluminal acetylcholine was less effective in causing relaxation than intraluminal acetylcholine (Figure 1b). In WKY, the pD_2 value, but not the maximal response was smaller with extraluminal application of acetylcholine than with intraluminal application (concentration shift: 6.6 fold; $P < 0.005$; Table 1). In 2K1C rats the concentration shift comparing intra- and extraluminal acetylcholine was smaller (3.7 fold; $P < 0.05$); the maximal response also was similar to that seen with intraluminal activation (Table 1). With extraluminal application of acetylcholine, neither the pD_2 value nor the maximal response differed in WKY and 2K1C rats.

In 16 week old 2K1C rats, the relaxation to extraluminal acetylcholine was not different from that in 12 week old 2K1C

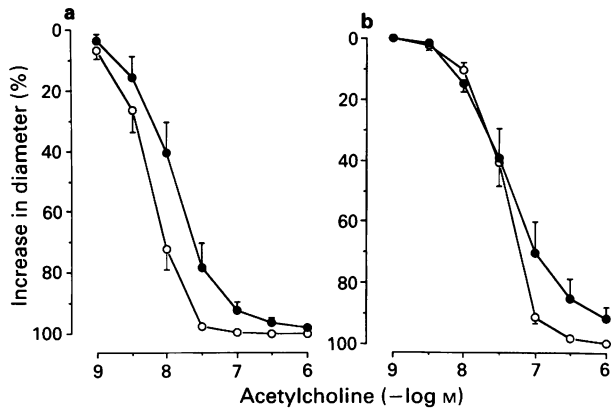

Figure 1 Concentration-response curves to intraluminal (a) and extraluminal acetylcholine (b) in mesenteric resistance arteries obtained from 12 week old Wistar Kyoto rats (control; ○) and age-matched two kidney-one clip hypertensive rats (2K1C; ●) which were clipped at the age of 4 weeks. Note that the relaxation induced by intraluminal, but not extraluminal acetylcholine was impaired in 2K1C. Each point represents the mean value from five experiments for control; 6 experiments for 2K1C; s.e.mean shown by vertical bars.

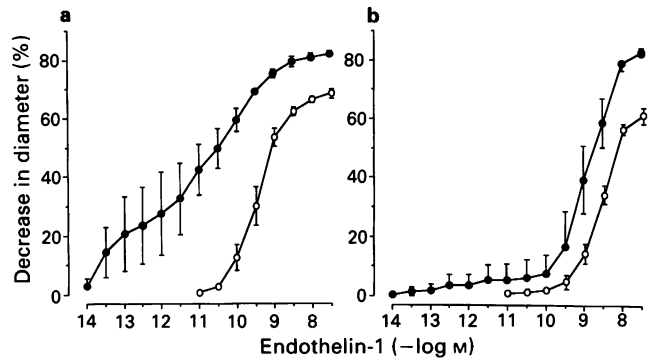

Figure 2 Concentration-response curves to extraluminal endothelin-1 in mesenteric resistance arteries with (○) and without endothelium (●) obtained from 12 week old Wistar Kyoto rats (control; a) and age-matched two kidney-one clip hypertensive rats (2K1C; b). Note that the sensitivity but not the maximal response to endothelin-1 is reduced in 2K1C. Each point represents the mean value from five experiments; s.e.mean shown by vertical bars.

rats and similar to the relaxation induced by intraluminal acetylcholine (Table 1).

Endothelin-1

In arteries with endothelium obtained from 12 week old rats, extraluminal endothelin-1 (10^{-11}–3×10^{-8} M) evoked contractions in a concentration-dependent manner (Figure 2a). The pD_2 value was smaller in 2K1C rats than in WKY ($P < 0.005$), while the maximal response did not differ in 2K1C and WKY (Table 2).

Removal of the endothelium enhanced the sensitivity to the peptide in both groups (Figure 2). The shift at the pD_2 value was strikingly smaller in 2K1C rats (2.7 fold; NS) than in WKY (150 fold; $P < 0.05$), but the enhancement of the maximal response was identical in 2K1C rats and WKY (Table 2). In preparations of 2K1C rats without endothelium, the pD_2 value ($P < 0.05$), but not the maximal response was markedly reduced as compared to WKY (Table 2).

In 16 week old 2K1C rats, the maximal response, but not the pD_2 value was different in the presence and absence of the endothelium (Table 2). Neither the pD_2 value nor the maximal response both in preparations with and without endothelium were different between 12 and 16 week old 2K1C rats.

Noradrenaline

Noradrenaline (3×10^{-8}–10^{-4} M) caused concentration-dependent contractions in WKY and 2K1C rats (Figure 3). In WKY, removal of the endothelium augmented both the pD_2 value ($P < 0.05$) and the maximal response to noradrenaline ($P < 0.005$; Figure 3a and Table 2), while in 2K1C rats only the maximal response ($P < 0.005$), but not the pD_2 value was increased by the removal of the endothelium (Figure 3b and Table 2). The contractions to the agonist did not differ between WKY and 2K1C rats in preparations with or without endothelium.

Similarly, in 16 week old 2K1C rats the maximal response, but not the pD_2 value was different in the presence and absence of the endothelium (Table 2). The contraction to noradrenaline was identical in 12 and 16 week old 2K1C rats both in arteries with and without endothelium.

Potassium chloride

The contraction induced by KCl (100 mM) in arteries with endothelium was identical in 12 week old WKY (75 ± 1%) and age-matched 2K1C rats (73 ± 1%) and 16 week old 2K1C rats (73 ± 1%).

Discussion

The major findings of the present study are reduced endothelium-dependent relaxation to intraluminal, but not extraluminal acetylcholine and an impaired inhibitory function of the endothelium against contractions induced by endothelin-1 and noradrenaline in mesenteric resistance arteries of renovascular hypertensive rats. In addition, renovascular hypertension is associated with a decreased response to endothelin-1 in the mesenteric resistance circulation.

Acetylcholine evoked potent endothelium-dependent relaxation in mesenteric resistance arteries of normotensive rats, confirming previous reports from other investigators

Table 1 The pD_2 values and maximal responses to intraluminal and extraluminal acetylcholine in mesenteric resistance arteries obtained from 12 week old Wistar Kyoto rats (WKY) and two kidney-one clip hypertensive rats (2K1C) at ages of 12 and 16 weeks

	12 week old				*16 week old*	
	WKY		*2K1C*[a]		*2K1C*[a]	
	pD_2[b]	Max[c]	pD_2	Max	pD_2	Max
Acetylcholine Intraluminal	8.2 ± 0.1	100 ± 0	7.9 ± 0.1*	98 ± 1*	7.6 ± 0.1	94 ± 2
Extraluminal	7.4 ± 0.1††	99 ± 1	7.3 ± 0.2†	92 ± 4	7.4 ± 0.1	91 ± 1

[a] The renal arterial clipping was performed at the age of 4 weeks.
[b] Negative logarithm IC_{50} value of acetylcholine.
[c] Maximal relaxation to acetylcholine (%).
* $P < 0.05$ as compared to WKY.
† $P < 0.05$ as compared to intraluminal application; †† $P < 0.005$ as compared to intraluminal application.

Table 2 The pD_2 values and maximal responses to endothelin-1 and noradrenaline in arteries with and without endothelium obtained from 12 week old Wistar Kyoto rats (WKY) and two kidney-one clip hypertensive rats (2K1C) at ages of 12 and 16 weeks

		12 week old				*16 week old*	
		WKY		*2K1C*[a]		*2K1C*[a]	
		pD_2[b]	*Max*[c]	pD_2	*Max*	pD_2	*Max*
	Endothelium						
Endothelin-1	with	9.4 ± 0.1	67 ± 2	8.6 ± 0.1**	60 ± 3	8.6 ± 0.1	59 ± 3
	without	11.6 ± 0.7†	82 ± 1††	9.0 ± 0.3*	82 ± 2††	8.9 ± 0.1	80 ± 1†††
Noradrenaline	with	5.8 ± 0.1	69 ± 1	5.9 ± 0.1	69 ± 2	5.9 ± 0.1	64 ± 1
	without	6.1 ± 0.1†	82 ± 1††	6.1 ± 0.1	84 ± 1††	6.1 ± 0.1	82 ± 1†††

[a] The renal arterial clipping was performed at the age of 4 weeks.
[b] Negative log ED_{50} value of agonists.
[c] Maximal contraction to agonists (%).
* $P < 0.05$ as compared to WKY; ** $P < 0.005$ as compared to WKY.
† $P < 0.05$ as compared to with endothelium; †† $P < 0.005$ as compared to with endothelium; ††† $P < 0.0001$ as compared to with endothelium.

(Tesfamariam & Halpern, 1988; Watt & Thurston, 1989) and our own group (Dohi *et al.*, 1990; Diederich *et al.*, 1990). Intraluminal acetylcholine caused a more powerful relaxation than did extraluminal acetylcholine. In renovascular hypertensive rats, as in spontaneously hypertensive rats (Dohi *et al.*, 1990), the relaxation induced by intraluminal, but not extraluminal acetylcholine was decreased as compared to control rats. This indicates that high blood pressure primarily alters the intraluminal activation of the endothelium with the muscarinic agonist regardless of the cause or type of hypertension. This is of particular interest as renovascular hypertension is a secondary form of hypertension where, in contrast to genetic hypertension of the rat, blood pressure is primarily maintained by an increased activity of the renin-angiotensin system (Brunner *et al.*, 1962; Fourcade *et al.*, 1971; Ploth *et al.*, 1977; Huang *et al.*, 1981; Miyazaki *et al.*, 1986; Wilson *et al.*, 1989). Although under these conditions the vascular renin-angiotensin system is also activated, this does not appear to be associated with a more pronounced inhibition of endothelium-dependent relaxations than in spontaneous hypertension.

Increased duration of hypertension (from 8 to 12 weeks) further decreased the relaxation induced by intraluminal acetylcholine (although this further deterioration of endothelium function was very small), while the relaxation induced by extraluminal acetylcholine remained unaltered. Although intraluminal activation of the endothelium with acetylcholine was more effective than extraluminal activation in normotensive and hypertensive rats, this difference was significantly smaller in the animals exposed to renovascular hypertension for 8 weeks as compared to age-matched normotensive rats, and after 12 weeks of hypertension intra- and extraluminal acetylcholine were similarly effective. This indicates that the functional alterations of the intraluminal surface of the endothelium become more pronounced with increasing duration of hypertension. On the other hand, the response of the extraluminal surface of the endothelium to acetylcholine is well preserved.

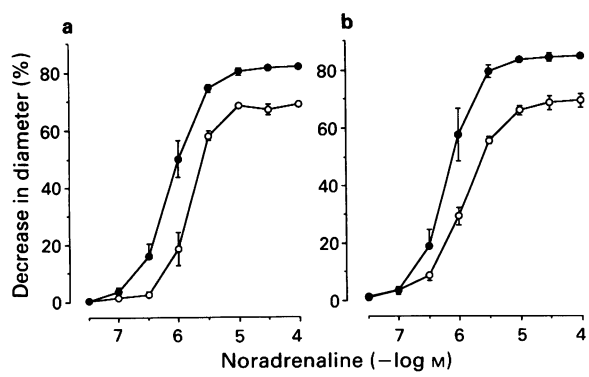

Figure 3 Concentration-response curves to extraluminal noradrenaline in mesenteric resistance arteries with (○) and without endothelium (●) obtained from 12 week old Wistar Kyoto rats (control; a) and age-matched two kidney-one clip hypertensive rats (2K1C; b). Note that the response to noradrenaline was not different between control and 2K1C. Each point represents the mean value from five experiments; s.e.mean shown by vertical bars.

Endothelin-1 is a very potent vasoconstrictor in mesenteric resistance arteries of normotensive rats (Dohi & Lüscher, 1990a, b; Criscione *et al.*, 1990). In preparations without endothelium the sensitivity, but not the maximal response to endothelin-1 was decreased in 2K1C rats as compared to WKY. In WKY, the biphasic shape of the concentration-response curve to endothelin-1 in arteries without endothelium suggests that two receptor subtypes might mediate the contractions, one with a high affinity and low efficacy and another one with a low affinity and higher efficacy. The decreased sensitivity in 2K1C rats was specific for endothelin-1, since the response of mesenteric resistance arteries without endothelium to noradrenaline did not differ in 2K1C rats and WKY. In the spontaneously hypertensive rat, the responsiveness of vascular smooth muscle to endothelin-1 relative to its normotensive controls is still controversial; increased (Tomobe *et al.*, 1988; Miyauchi *et al.*, 1989; Criscione *et al.*, 1990), decreased (Dohi & Lüscher, 1990b; Auch-Schwelk & Vanhoutte, unpublished observations) and normal responses (Diederich *et al.*, 1989; Criscione *et al.*, 1990) to endothelin-1 have been reported. Although some of the differences may be explained by the differences in the experimental conditions or the anatomical origin of the arteries used, the present study further supports the notion, at least in mesenteric resistance arteries, that hypertension is associated with a decreased sensitivity to the peptide. This decreased sensitivity may be due to downregulation of endothelin receptors on vascular smooth muscle particularly in the presence of increased vascular levels of the peptide. Indeed, downregulation of endothelin receptors occurs in cultured smooth muscle cells after exposure to the peptide (Hirata *et al.*, 1988; Roubert *et al.*, 1990). If so, it would appear that that receptor mediating the response to endothelin at lower concentrations is particularly affected. This could occur due to greater vascular endothelin production or pressure *per se*. Angiotensin II, which is elevated in renovascular hypertension (Brunner *et al.*, 1962; Fourcade *et al.*, 1971; Ploth *et al.*, 1977; Huang *et al.*, 1981; Miyazaki *et al.*, 1986), is known to stimulate the production of endothelin in cultured endothelial cells (Yanagisawa *et al.*, 1988; Emori *et al.*, 1989). However, plasma concentrations of endothelin are increased only in certain hypertensive patients (Cernàcek & Stewart, 1989; Saito *et al.*, 1990; Shichiri *et al.*, 1990; Kohno *et al.*, 1990) and appear normal or decreased in hypertension in the rat (Suzuki *et al.*, 1990). Alternatively, the circulating levels of endothelin may not reflect the local concentration of the peptide within the blood vessel wall (particularly since greater amounts of endothelin are released abluminally;

Yoshimoto *et al.*, 1990) or the catabolism of endothelin-1 by endogenous proteases may be increased in 2K1C rats.

Removal of the endothelium enhanced the sensitivity (pD_2 value) to the peptide in WKY. In 2K1C rats, the enhancement in the sensitivity to endothelin-1 was blunted, indicating a reduced inhibitory capacity of the endothelium in renovascular hypertension. This contrasts with the spontaneously hypertensive rat where the inhibitory function of the endothelium against contractions to extraluminal endothelin-1 is increased as compared to control rats (Dohi & Lüscher, 1990b). The contraction induced by extraluminal endothelin-1 both in arteries with and without endothelium was identical in 12 and 16 week old 2K1C rats. This indicates that, in contrast to the release of EDRF stimulated by acetylcholine, the inhibitory function of the endothelium against endothelin-1-induced contraction does not further deteriorate with prolonged renovascular hypertension.

The contraction induced by extraluminal noradrenaline in arteries without endothelium was identical in 2K1C rats and WKY, confirming previous data from other investigators (Duarte *et al.*, 1990). The presence of the endothelium slightly, but significantly reduced the sensitivity in WKY, but not in 2K1C rats though the maximal response was increased after endothelial removal in both groups. Thus, as in spontaneously hypertensive rats (Dohi *et al.*, 1990), the inhibitory function of the endothelium against the contraction induced by noradrenaline is decreased in renovascular hypertensive rats, although this difference between the two strains was rather small. Since noradrenaline does not stimulate the release of EDRF in this preparation (Dohi *et al.*, 1990), this indicates that renovascular hypertension reduces the basal release of ERDF in the resistance circulation. The contraction induced by extraluminal noradrenaline both in arteries with and without endothelium was identical in 12 and 16 week old 2K1C rats, indicating that basal release of EDRF does not further decrease during this period. Since this alteration in endothelial function was small, it is uncertain whether it contributes to the hypertensive process.

In conclusion, the intraluminal, but not extraluminal activation of the release of endothelium-derived relaxing factors in response to acetylcholine is impaired in 2K1C rats. The inhibitory function of the endothelium against extraluminal endothelin-1 and to a lesser degree against noradrenaline is decreased in 2K1C rats. These functional changes of the endothelium in hypertensive resistance arteries may play a role in the maintenance of increased peripheral vascular resistance that occurs in established hypertension.

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Pharmacological specificity of novel, synthetic, cyclic peptides as antagonists at tachykinin receptors

A.T. McKnight, J.J. Maguire, N.J. Elliott, A.E. Fletcher, A.C. Foster, R. Tridgett, B.J. Williams, J. Longmore & [1]L.L. Iversen

Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR.

1 The interaction at tachykinin receptors of a series of novel cyclic hexapeptides has been examined by use of radioligand binding assays (NK_1 and NK_3 sites in rat cortex, NK_2 sites in hamster urinary bladder) and functional pharmacological assays (guinea-pig ileum, rat vas deferens and rat portal vein for NK_1, NK_2 and NK_3 receptors, respectively).

2 The compounds cyclo(GlnTrpPhe(*R*)Gly[ANC-2]LeuMet) (L-659,837) and cyclo(GlnTrpPheGly-LeuMet) (L-659,877) were powerful and selective displacers of NK_2 binding (pIC_{50} 6.9 and 8.0, respectively), and were competitive antagonists of responses to stimulation of NK_2 receptors in rat vas deferens (pK_B for antagonism of responses to eledoisin 6.7 and 8.1, respectively). Responses in the NK_1 and NK_3 pharmacological assays were blocked only weakly, if at all.

3 In the longitudinal muscle of the small intestine of the rat, responses to stimulation of the putative NK_2 receptor by eledoisin, neurokinin A or neurokinin B were antagonized by both cyclo(GlnTrpPhe(*R*)-Gly[ANC-2]LeuMet) and cyclo(GlnTrpPheGlyLeuMet) in a manner consistent with the presence in this tissue of a uniform population of receptors, indistinguishable from the NK_2 receptor of the rat vas deferens.

4 The compounds cyclo(GlnTrpPheGlyLeuMet) and the lactam-containing analogue are among the most selective antagonists for the NK_2 receptor that have been described; their availability should be of value in the characterization of the receptors mediating responses to tachykinins, and in elucidating the physiological functions of the tachykinin receptors.

Keywords: Tachykinin receptors; tachykinin receptor antagonists; antagonists for NK_2-receptors

Introduction

It is now generally accepted that the diverse actions of the tachykinins (Table 1) are mediated by different types of receptor and, currently, the evidence is strongest for three types, termed NK_1, NK_2 and NK_3, using a nomenclature (Henry, 1987; Iversen *et al.*, 1990) that has superseded the original SP-P, SP-E and SP-N designations (Lee *et al.*, 1982; Laufer *et al.*, 1985). Among the endogenous mammalian tachykinins, substance P is the preferred ligand at the NK_1 receptor, while neurokinin A and neurokinin B are the preferred ligands for NK_2 and NK_3 receptors, respectively (see Watson, 1984; Iversen *et al.*, 1988; 1990; Quirion & Dam, 1988).

The characterizations of these receptor types was initially based solely on observed differences in the order of potencies of agonists in pharmacological studies and similar differences in agonist affinities observed in radioligand binding assays. Thus the pattern of activity that is displayed by the natural and synthetic tachykinin agonists for producing a contraction of the longitudinal muscle of guinea-pig ileum in the presence of atropine, the archetypal NK_1 assay, correlates quite well with the pattern in radioligand binding assays in preparations of peripheral tissues such as rat salivary gland, or of brain (Viger *et al.*, 1983; Cascieri *et al.*, 1981; 1986; Lee *et al.*, 1986; Iversen *et al.*, 1988; 1990) for displacement of the binding of the Bolton-Hunter conjugate of substance P labelled with ^{125}Iodine ([^{125}I-BH]-SP).

Table 1 Structures of naturally occurring mammalian and non-mammalian tachykinins

Tachykinin	*Amino acid sequence*
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met.NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met.NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met.NH_2
Eledoisin	Glp-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met.NH_2

Conserved residues are underlined. Glp = pyroglutamic acid.

[1] Author for correspondence.

The NK_2 pattern of activity seen with agonists in the rat vas deferens correlates well with the pattern for the inhibition of the binding of [^{125}I-BH]-eledoisin ([^{125}I-BH]-Ele) in membranes from hamster urinary bladder (Lee *et al.*, 1986), and we have found that the correlation between potency for agonists to displace the binding of [^{125}I-BH]-Ele in rat brain cortex (NK_3 site) and to contract the portal vein of the rat is highly significant (McKnight & Maguire, 1987).

The existence of multiple tachykinin receptor subtypes has been confirmed more recently by Nakanishi and co-workers who cloned and expressed in *Xenopus* oocytes the three tachykinin receptors: the rat brain NK_1 receptor, rat brain NK_3 receptor and bovine stomach NK_2 receptor. All three receptors belong to the G-protein coupled family, with several hydrophobic membrane spanning domains, and there is considerable homology in amino acid sequence among them (Masu *et al.*, 1987; Shigemoto *et al.*, 1990).

Although the existence of multiple tachykinin receptors is no longer in doubt, there remains a need for more potent and selective receptor antagonists. The problems associated with classification of receptors only on the basis of differences in rank order of potency of agonists are well known (Kenakin, 1984; Watson, 1984). Important progress has been made recently in the discovery of both peptide (Ward *et al.*, 1990) and non-peptide antagonists (Snider *et al.*, 1991) with selectivity for NK_1 receptors. Rovero *et al.* (1990) and Maggi *et al.* (1990) have also described novel linear peptides which act as selective antagonists at NK_2 receptors in rabbit pulmonary artery. The compound MEN 10207 (=[Tyr^5,D-Trp^{6,8,9}Arg^{10}]-NKA(4-10) had a pA_2 of 7.65 in this tissue.

We describe here a series of cyclic peptides, some of which also display high affinity and selectivity as NK_2 receptor antagonists. The most active compound, L-659,877, is among the most selective NK_2 antagonists available.

Some of these findings have been the subject of a preliminary communication (McKnight et al., 1988).

Methods

Radioligand binding assays

NK_1 and NK_3 binding sites in rat cortex were labelled with [^{125}I-BH]-SP (0.2 nM) and [^{125}I-BH]-Ele (0.5 nM), respectively; NK_2 sites in homogenates of hamster urinary bladder were labelled with [^{125}I-BH]-Ele (0.3 nM). Preparations of cortical synaptic plasma membranes from 250–350 g male Sprague-Dawley rats (Bantin & Kingman) and of homogenates of urinary bladder from 80–160 g male Golden Syrian hamsters (Intersimian Ltd) were as described previously (Lee et al., 1986; Foster & Tridgett, 1988); these were stored in a concentration of approximately 10 mg protein ml^{-1} at −80°C prior to use.

Incubations were for 60 min at room temperature for NK_2 and NK_3 binding, or for 30 min for NK_1, in 50 mM Tris-acetate buffer (pH 7.4) containing 1 mM $MnCl_2$. Chymostatin (50 μg ml^{-1} for NK_1 and NK_3, 20 μg ml^{-1} for NK_2) and bovine serum albumin (200 μg ml^{-1}) were present throughout; bacitracin (40 μg ml^{-1}) and leupeptin (4 μg ml^{-1}) were also present in the NK_2 assay. Assays were performed in duplicate in a total volume of 150 μl with 50 μg protein per tube; protein concentration was measured by the method of Lowry et al. (1951). Non-specific binding was determined for NK_2 and NK_3 sites in the presence of 5 μM eledoisin, and with 5 μM substance P for the NK_1 site. Incubations were terminated by filtration under vacuum through Whatman GF/C filter strips in a Brandel Cell Harvester, with washing in 6 ml of ice-cold buffer; the filters had previously been soaked in a solution of 0.3%(v/v) polyethyleneimine and 0.5%(v/v) Triton X-100 in buffer.

The ability of putative antagonists to inhibit the binding at all three sites was determined by use of at least five concentrations of compound, routinely between 0.001 and 10 μM, and estimates of affinity (IC_{50}) and the pseudo Hill slope factor (N_H) were obtained by the ALLFIT iterative four-parameter logistic model (De Lean et al., 1978).

Pharmacological assays

Smooth muscle preparations were obtained from male Sprague Dawley rats (200–300 g) or from male Dunkin-Hartley guinea-pigs (300–400 g, Bantin & Kingman). Unless otherwise stated, strips of smooth muscle were mounted for isometric tension recording in 3 ml siliconised organ baths containing Krebs Hensleit solution at 37°C and gassed with 95% O_2 and 5% CO_2. For guinea-pig and rat ileum and rat portal vein preparations, an initial resting tension of 1 g was applied. Tissues were allowed to equilibrate for 60 min before exposure to sequentially increasing concentrations of the appropriate agonist. Dose cycles of 10 min or 12–15 min were used for guinea-pig and rat ileum or for rat portal vein and vas deferens preparations, respectively. In each case the contractile response to the agonist was allowed to reach a peak (between 2–3 min for ileum and 3–5 min for rat vas deferens and portal vein) before washout. The tissues were then exposed to the appropriate concentration of the antagonist for 10–30 min before repetition of the agonist dose-response curve in the continuing presence of the antagonist. In all cases eledoisin was used for the initial estimate of antagonist affinity.

For the NK_1 assay, strips of longitudinal muscle were obtained from the small intestine of the guinea-pig. Agonist-induced contractile responses were measured in the presence of atropine (5 μM), mepyramine (5 μM), methysergide (5 μM) and indomethacin (1 μM), so that the response could be used as a measure of NK_1 stimulation.

For the NK_3 receptor assay, 2–3 cm longitudinal strips of rat portal vein were used (McKnight & Maguire, 1985). Contractile responses were measured as changes in the underlying baseline tension. In some preparations, where the level of spontaneous activity was unusually high, the bath temperature was reduced to 30°C. Such a reduction in temperature had little apparent effect on agonist potency.

Rat colon and vas deferens were used as NK_2 receptor assays. Strips of longitudinal muscle were obtained from the rat small intestine and contractile responses to the agonists were measured in the presence of atropine (5 μM), mepyramine (5 μM), methysergide (5 μM) and indomethacin (1 μM). The vas deferens was electrically stimulated with rectangular bipolar pulses of supramaximal voltage, delivered through platinum electrodes. Trains of electrical pulses were delivered for 1 s every 20 s (7 Hz, 70–80 V) with individual pulse duration of 0.5 ms. The increase in twitch height induced by the agonist was expressed as a percentage of the pre-dose level (Corbett et al., 1981; McKnight et al., 1985).

The affinity of the antagonists was derived in the conventional way, from the degree of rightward shift of the log dose-response curves to eledoisin, or other agonist, observed in the presence of antagonist, with respect to the control curve (dose-ratio, DR). Where possible, the measure of affinity, pK_B, was obtained by the line of best fit (least squares) to the Schild regression (Arunlakshana & Schild, 1959), using 3 or more observations at each of 3 or more concentrations of antagonist. Where the solubility or affinity of the test compound were limiting factors, estimates for pK_B were made using at least 3 observations, and more than one concentration of antagonist, from the standard equation for the Schild regression, assuming unit slope.

Drugs and solutions

The tachykinins, substance P, neurokinin A, neurokinin B, eledoisin, substance P-methyl ester, suc-[Asp^6,Me-Phe^8]SP(6-11) (senktide) and [Glp^6]SP(6-11) were obtained from Cambridge Research Biochemicals, or from Peninsula Laboratories. Assurances on the identity, purity and amount of material used, for these and other peptides, were based on amino acid analysis, high performance liquid chromatography (h.p.l.c.) analysis and FAB mass spectrometric analysis.

[Glp^6,L-Pro^9]SP(6-11) and the corresponding D-Pro^9-analogue were synthesized by solid-phase methods involving the use of Fmoc amino acids, and their purity confirmed in the Department of Chemistry. The hexapeptide tachykinin analogue containing a cyclic γ-lactam ([ANC-2]) unit, Glp-Phe-Phe-(*R*)Gly[ANC-2]Leu-Met-NH_2 (L-363,851) (Cascieri et al., 1986) was synthesized at our laboratories at West Point, Pennsylvania, U.S.A. The antagonist peptides used in this study were all designed, synthesized and purified in the Department of Chemistry at the Neuroscience Research Centre by solid phase methods described elsewhere (Williams et al., 1991).

[^{125}I-BH]-SP and [^{125}I-BH]-Ele (specific activities approximately 2000 Ci mmol^{-1}) were obtained from DuPont-New England Nuclear and the radiochemical purity of these ligands was confirmed by reverse-phase h.p.l.c.

Carbamyl choline chloride, histamine dihydrochloride, atropine sulphate, mepyramine maleate and indomethacin were obtained from Sigma; methysergide maleate was a gift from Sandoz.

Inhibitors of peptidases were obtained as follows: bestatin ([2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine), Cambridge Research Biochemicals; phosphoramidon (N-(α-rhamnopyranosyloxyhydroxyposphinyl)-L-Leu-L-Trp), Protein Research Foundation; leupeptin (acetyl-L-leucinyl-L-leucine) and chymostatin, Peninsula Laboratories Inc; bacitracin, Sigma Chemical Co.

Eledoisin, neurokinin A and substance P and its methyl ester were dissolved in a solution of 0.1 M acetic acid in water : methanol (1 : 1) containing 0.1%(w/v) bovine serum albumin (crystalline, Sigma) and stored in polypropylene tubes at −20°C. Stock solutions were 10 mM and dilutions were made initially in the same solvent and subsequently in assay

medium. The other agonists, and the antagonists, were dissolved in dimethylsulphoxide (DMSO).

The addition of agonists or antagonists to the bath was by Hamilton microsyringes that had been silanised (Sigmacote, Sigma) before use, in volumes usually not exceeding 9 μl. With neither solvent was there any marked effect on the responsiveness in the pharmacological or the biochemical assays, at a concentration of up to 1%(v/v) in the assay buffer.

The composition of the Krebs-Henseleit solution was (mM): NaCl 118, KCl 4.74, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.

Results

Rationale for the design of antagonists

It is well known that the potency of substance P at tachykinin receptors in many pharmacological models is largely retained by synthetic C-terminal analogues, such as [Glp^6]SP(6-11) (Table 2), and accordingly C-terminal sequences have commonly been chosen as the basis of the design of analogues, as agonists or antagonists at tachykinin receptors.

The comparatively minor change in the hexapeptide sequence that is produced in L-363,851 by the introduction of a two-carbon bridge between the α-carbon of glycine and the nitrogen of leucine to produce a cyclic lactam unit ([ANC-2]) results in a dramatic loss of potency at the NK_1 receptor (Cascieri *et al.*, 1986); potency and affinity at NK_2 and NK_3 sites, however, are significantly increased (Tables 2 and 3). The incorporation of the lactam ring in the [Glp^6]SP(6-11) sequence constrains the conformation of the peptide in a near cyclic configuration that appears to favour interaction with the NK_2 and NK_3 receptors, but is not favourable for optimal interaction with the NK_1 receptor. We have used this observation as the rationale of a programme for the design and synthesis of cyclic peptides in which the C-terminal methionine of the linear peptide is linked to the N-terminal glutamine by a peptide bond, as potentially selective antagonists for tachykinin receptors (Williams *et al.*, 1988; 1991).

Affinity of cyclic peptides for NK_1, NK_2 and NK_3 receptors

None of the cyclic peptides exhibited any efficacy at NK_1, NK_2 or NK_3 receptors in the pharmacological models at concentrations of up to 10 μM or greater: the upper limit for testing in the assays was determined by the solubility of the peptides in Krebs-Henseleit solution. For most of the novel peptides the solubility limit was 10 μM for pharmacological assays and 100 μM for binding assays.

When the original lactam-containing linear peptide L-363,851 was cyclised, the resulting compound cyclo(GlnPhePhe(*R*)Gly[ANC-2]LeuMet) had no detectable activity in any assay (Compound II, Table 3). By contrast, in the analogue in which L-tryptophan substituted for the 'first' phenylalanine (Compound III, L-659,837), although affinity at the NK_1 and NK_3 sites was not greatly increased, affinity at the NK_2 site was high (Table 3). The pharmacological assays confirmed the comparative lack of affinity at NK_1 and NK_3 receptors ($pK_B < 5$, not shown). Using eledoisin as the agonist we found that the Schild regression for the antagonism by this cyclic peptide had unit slope, and produced a value for pK_B of 6.71 (95% confidence limits 6.1, 7.2) (Figure 1); the value for pK_B at the NK_2 receptor agrees well with the corresponding value for pIC_{50} from the binding assay in hamster urinary bladder (Table 3).

In synthetic linear peptides, the most useful antagonists are those that contain substitutions by D-amino acids, most commonly tryptophan. In the cyclic peptide, however, this latter substitution reduced affinity at the NK_2 site (Compound IV, Table 3). Affinity at the NK_2 receptor in the pharmacological assay with this peptide, cyclo(GlntrpPhe(*R*)Gly[ANC-2]LeuMet) was correspondingly low (pK_B approximately 5, not shown). Some affinity at the NK_2 site was restored by substituting a methyl group on the nitrogen of the phenylalanine (Compound V, Table 3), and this was also observed in the pharmacological assay; the pK_B for cyclo(GlntrpPheMe(*R*)Gly[ANC-2]LeuMet) against eledoisin in the rat vas deferens was 6.4 $\pm$ 0.3 (n = 3, $\pm$s.e.mean).

The effect of the two-carbon [ANC-2] bridge between glycine and leucine on affinity was tested by synthesizing the peptide cyclo(GlnTrpPheGlyLeuMet) (Compound VI, L-659,877). The affinity of this analogue for the NK_2 site in hamster bladder was increased more than 10 fold compared to

Table 2 Relative potencies of natural and synthetic tachykinins at NK_1, NK_2 and NK_3 receptors in pharmacological models

	NK_2 (GPI)	NK_2 (RVD)	NK_3 (RPV)
Substance P	100	100	100
Neurokinin A	10	9100	940
Neurokinin B	60	2200	43100
Eledoisin	60	27200	42000
Substance P-methyl ester	65	<10	<1
[Glp^6]SP(6-11)	60	70	200
L-363,851	1	650	400
[Glp^6,L-Pro^9]SP(6-11)	35	<10	2
[Glp^6,D-Pro^9]SP(6-11)	7	2000	50

GPI = guinea-pig ileum, RVD = rat vas deferens, RPV = rat portal vein.
L-363,851 is GlpPhePhe(*R*)Gly[ANC-2]LeuMet.NH_2.
Values are for potencies of the peptides relative to that for substance P (= 100) in terms of values for EC_{50} (from more than three separate determinations); a high value denotes high potency (low EC_{50}). The geometric mean values for EC_{50} with substance P in the separate assays were: GPI 3.2 nM (range 1.1–6.6, n = 40), RVD 13.6 μM (8.5–1917, n = 6), RPV 0.6 μM (0.3–1.7, n = 8).

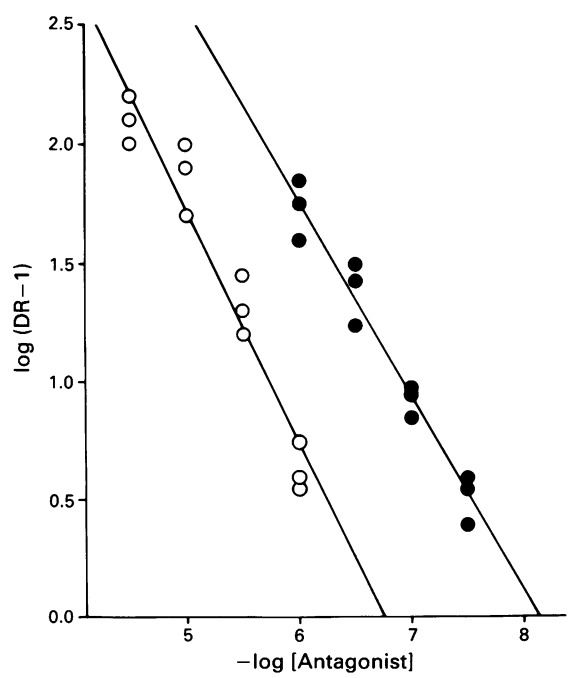

Figure 1 Arunlakshana–Schild plots for the interactions between the agonist eledoisin, and cyclo(GlnTrpPhe(*R*)Gly[ANC-2]LeuMet) (L-659,837, ○) or cyclo(GlnTrpPheGlyLeuMet) (L-659,877, ●) at NK_2 receptors in the rat isolated vas deferens. The lines are the best fit (least squares) to the data points; in neither case is the slope significantly different from unity.

Table 3 Affinity of synthetic peptides for NK_1, NK_2 and NK_3 binding sites

	NK_1 pIC_{50}	NK_2 pIC_{50}	NK_3 pIC_{50}
I GlpPhePhe(*R*)Gly[ANC-2]LeuMet.NH_2	5.4 ± 0.1 (0.5 ± 0.1)	7.0* (0.8)*	7.4 ± 0.1 (0.8 + 0.2)
II cyclo(GlnPhePhe(*R*)Gly[ANC-2]LeuMet)	<4	<4	<4
III cyclo(GlnTrpPhe(*R*)Gly[ANC-2]LeuMet)	<4	6.9 ± 0.1 (1.1 ± 0.4)	4.2 ± 0.1 (1.1 ± 0.2)
IV cyclo(GlntrpPhe(*R*)Gly[ANC-2]LeuMet)	<4	5.4 ± 0.04 (1.2 + 0.1)	5.2 ± 0.1 (1.1 + 0.2)
V cyclo(Glntrp(Me)Phe(*R*)Gly[ANC-2]LeuMet)	4.8* (0.8)	6.2 ± 0.2 (0.9 ± 0.1)	4.6* (1.1
VI cyclo(GlnTrpPheGlyLeuMet)	<4	8.0 ± 0.1 (0.8 ± 0.1)	<4
VII cyclo(GlnTrpPheProLeuMet)	4.3 ± 0.1 (0.9 ± 0.1)	<4	<4
VIII cyclo(GlnTrpPheproLeuMet)	<4	5.0 ± 0.1 (0.9 ± 0.1)	4.9 ± 0.2 (0.6 ± 0.1)

* Mean of two observations, otherwise values are means ± one s.e.mean of at least three determinations for $-\log_{10}IC_{50}$. Values in parentheses are mean ± one s.e.mean for the slope factor ('Hill coefficient') of the displacement curve.
NK_1: specific binding of [^{125}I-BH]-SP in membranes from rat brain cortex.
NK_2: specific binding of [^{125}I-BH]-Ele in membranes from hamster urinary bladder.
NK_3: specific binding of [^{125}I-BH]-Ele in membranes from rat brain cortex.
Compound I is the agonist L-363,851, compound III is L-659,837, compound VI is L-659,877.

the corresponding peptide containing the lactam, and since affinity was not detectable at the NK_1 and NK_3 sites in cortex ($IC_{50} > 100\,\mu M$), the selectivity of this cyclic peptide for the NK_2 site, over the latter sites, was more than 10,000 fold (Table 3). Pharmacological assays confirmed the selectivity of cyclo(GlnTrpPheGlyLeuMet). From the Schild regression for the interaction with eledoisin the antagonism appeared to be competitive with a value for pK_B of 8.1 (95% confidence limits 7.6, 8.4) (Figure 1). In the guinea-pig ileum and rat portal vein, responses to eledoisin were only weakly antagonised (values for pK_B were, respectively, 5.7 ± 0.1, $n = 4$ and 5.1 ± 0.2, $n = 4$). In the ileum, with substance P-methyl ester used as a selective agonist for the NK_1 receptor or, in the absence of atropine, with senktide as the selective NK_3 agonist (Table 2), there was no evidence of antagonism by up to $10\,\mu M$ of cyclo(GlnTrpPheGlyLeuMet).

Affinity at all three sites was essentially lost if proline was substituted for glycine in the cyclic peptide (Compound VII, Table 3); the same substitution in the agonist [Glp6]SP(6-11] also results in loss of affinity and efficacy at the NK_2 receptor (Table 2). While agonist affinity is restored in the linear peptide when the substitution is by D-proline (Table 2), the corresponding cyclic peptide cyclo(GlnTrpPhe-D-ProLeuMet) (Compound VIII, Table 3) in contrast had only weak activity.

Table 4 Potencies of natural and synthetic tachykinins to contract the longitudinal muscle of rat small intestine

Tachykinin	$EC_{50}(\mu M)$	*Range*
Substance P	4.1	2.0–5.4
Neurokinin A	0.02	0.01–0.05
Neurokinin B	0.06	0.02–0.10
Eledoisin	0.1	0.04–0.30
Substance P-methyl ester	>100	—
L-363,851	0.5	1.1–4.4
[Glp^6,D-Pro^9]SP(6-11)	3.5	1.7–4.9
[Glp^6,L-Pro^9]SP(6-11)	>100	—
suc-[Asp^6,Me-Phe^8]SP(6-11)	>100	—

Values for EC_{50} are geometric means of at least four determinations. Individual values for EC_{50} were obtained in preparations from which a value for EC_{50} for eledoisin was also obtained; the value for EC_{50} for the test peptide was normalised to the mean EC_{50} for eledoisin (pEC_{50} ± s.e.mean for eledoisin was 6.99 ± 0.05, $n = 40$).

Table 5 Antagonism of responses to tachykinins in rat small intestine by cyclic peptide antagonists

	Neurokinin A	*Neurokinin B*	*Eledoisin*
Antagonist peptide	pK_B	pK_B	pK_B
L-659,837	7.4 (7.0, 7.8)	7.5 (7.0, 8.0)	7.8 (7.1, 8.5)
L-659,877	8.0 (7.6, 8.4)	8.7 (8.0, 9.4)	8.5 (8.1, 8.9)

L-659,837 is cyclo(GlnTrpPhe(*R*)Gly[ANC-2]LeuMet)
L-659,877 is cyclo(GlnTrpPheGlyLeuMet)
Values are for pK_B derived from the Schild regression for the line of best fit (least squares) from the plots of $\log_{10}(DR - 1)$ against $-\log_{10}$[antagonist] using 3 or 4 observations for dose-ratio (DR) at each of 4 concentrations of the antagonist. Values in parentheses are 95% confidence limits. In no case did the slope of the regression line differ significantly from unity.

Specificity of action of L-659,837 and L-659,877

The selective NK_2 antagonists cyclo(GlnTrpPheGlyLeuMet) (L-659,877) and the lactam-containing analogue (L-659,837) were tested for specificity of action in the untreated guinea-pig ileum, where neither compound, at a concentration of $\leqslant 10\,\mu M$, had any effect on the responses to carbachol or histamine (not shown).

Characterization of the receptors mediating contraction by tachykinins in the longitudinal muscle from rat small intestine

The order of potencies of agonist peptides on the receptor for tachykinins that mediates contraction of the longitudinal muscle of the small intestine of the rat was similar to that present in the rat vas deferens: the NK_2 receptor. Most notably, the analogue [Glp^6,D-Pro^9]SP(6-11) was a full agonist of reasonable potency, while the L-Pro^9-analogue was inactive (Table 4). We used the two cyclic peptide antagonists that have high affinity and were highly selective for the NK_2 receptor in rat vas deferens (and hamster urinary bladder), to characterize the receptor in the rat intestine more rigorously. Both cyclo(GlnTrpPheGlyLeuMet) (Compound VI, L-659,877) and the lactam-containing analogue (Compound III, L-659,837)

were antagonists of responses to eledoisin in the rat intestine. From the Schild regressions for these interactions, the antagonism appeared to be competitive with both antagonists; the values for pK_B with cyclo(GlnTrpPheGlyLeuMet) and cyclo(GlnTrpPhe(*R*)Gly[ANC-2]LeuMet) in this tissue were higher, but not significantly different from those derived from the vas deferens (Table 5). When the nature of the interactions between these antagonists and the agonist eledoisin at the tachykinin receptors in rat small intestine was re-examined, in a few experiments with inhibitors of peptidases present (bestatin and phosphoramidon, both 10 μM), there appeared to be no effect of the addition of the peptidase inhibitors (data not shown). Compounds III and VI, L-659,837 and L-659,877, were competitive antagonists of responses to neurokinin A and neurokinin B in the rat intestine, and the antagonists blocked responses to either agonist with apparently equal affinity (Table 5).

Discussion

The rationale for the design of the present series of cyclic peptide analogues has been described in this paper and elsewhere (Williams *et al.*, 1988; 1991). The testing for activity among the series relied on established methods with radioligand binding assays for affinity at NK_1 and NK_3 sites in rat cortex, and in hamster urinary bladder for NK_2 sites. Functional models for these receptors in isolated tissue preparations were used to provide definitive observations on the nature of the interactions by the peptides under study at tachykinin receptors. Some of the novel cyclic peptides tested proved to be NK_2 selective antagonists of high affinity. NK_2 receptors were first defined in terms of the potencies of agonists in the vas deferens of the rat and, although it is possible to use this tissue in a binding assay for the NK_2 receptor (Lee *et al.*, 1986; Foster & Tridgett, unpublished), the urinary bladder of the hamster provides a more convenient source of material and appears to contain an indentical type of receptor (Lee *et al.*, 1986).

The use of the small intestine of the guinea-pig as an assay for NK_1 receptors is complicated by the presence, not only of an NK_3 receptor on myenteric cholinergic neurones (Laufer *et al.*, 1985), but apparently of another type of receptor for tachykinins as yet undefined (Lee *et al.*, 1982). The effect of stimulation of the NK_3 receptor in ileum can be blocked by atropine, but the remaining 'non-NK_1' receptor appears to be present on the longitudinal muscle, along with the NK_1 receptor. Our use of this preparation with the non-selective agonist eledoisin was intentional, however, to allow a more conservative approach to the discovery of antagonists; any effect of a novel compound to block the NK_1 receptor would be detected, but any affinity for the unknown type might also be observed. This 'non-NK_1' receptor is unlikely to be similar to the NK_2 receptor located on rat vas deferens, since the pK_B for cyclo(GlnTrpPheGlyLeuMet) was lower in the guinea-pig ileum than rat vas deferens (5.7 and 8.1, respectively). However, recently the existence of NK_2 receptor subtypes has been suggested (Maggi *et al.*, 1990) and it is possible that the 'non-NK_1' in guinea-pig ileum may reflect an NK_2 receptor subtype less sensitive to the effects of this peptide antagonist. It appears that the NK_{2A} receptor subtype, postulated to exist in rabbit pulmonary artery by Maggi *et al.* (1990) is sensitive to MEN 10207 but not to the cyclic peptide L-659,877 (Maggi *et al.*, unpublished). Conversely, the NK_{2B} receptor subtype in hamster trachea is sensitive to L-659,877 but much less susceptible to blockade by MEN 10207 (Maggi *et al.*, 1990 and unpublished). Whether these observations reflect the existence of additional tachykinin receptor subtypes or merely species differences in the NK_2 receptor remains unclear.

The present results show that, in terms both of the order of potencies of agonists and the affinities of two agonists, the receptor for tachykinins in the longitudinal muscle of the small intestine of the rat is not distinguishable from that in the prototypical NK_2 model, the vas deferens of the rat as previously suggested by others (Falconier Erspamer *et al.*, 1980; Watson *et al.*, 1983; Regoli *et al.*, 1987). In two respects the rat intestine is a superior model for measuring affinity and efficacy at NK_2 receptors in our hands: the absolute potencies of agonists are greater, and apparent potency remains stable throughout the day. With the vas deferens the effect of the agonist increases steadily with time, because of the decline through the day of the baseline response to stimulation. Thus we believe that the measures of affinity for antagonists that are obtained with the rat intestine will be more reliable than those from the rat vas deferens, and it is with the former model that we carried out the more extensive studies on the antagonism of agonists other than eledoisin (Table 5).

The urinary bladder of the hamster exists as a *de facto* assay for NK_2 binding sites, and we have used this assay to measure affinity of our cyclic peptides at the NK_2 site. It is clear from previous work that the criterion is fulfilled for the receptor mediating a functional response in this tissue with respect to the order of potency of agonists (e.g. Falconieri Erspamer *et al.*, 1980; Watson *et al.*, 1983; Lee *et al.*, 1986; Bristow *et al.*, 1987) but now, in view of the correspondence between observed affinity of the cyclic peptides at the binding site in the hamster urinary bladder and the receptor in the vas deferens, the binding site for tachykinins is, *ipso facto*, an NK_2 site. In further support of this view, we have also observed that the selective cyclic peptides are antagonists of responses to tachykinins in this tissue (Suman-Chauhan *et al.*, 1988; Maguire & McKnight, unpublished).

In conclusion, we have described the pharmacological profile of some novel, synthetic, cyclic peptides, two of which are high affinity selective antagonists for the NK_2 type of receptor for tachykinins in rat and hamster tissues. The use of these compounds has allowed the first definitive characterisations of receptors mediating the effects of tachykinin antagonists in commonly used models for NK_2 receptor function.

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Modulation of agonist-induced calcium mobilisation in bovine aortic endothelial cells by phorbol myristate acetate and cyclic AMP but not cyclic GMP

Kevin W. Buchan & [1]William Martin

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

1 In bovine aortic endothelial cells (BAEC), thrombin (1 u ml^{-1}), bradykinin (1–10 nM) and adenosine triphosphate (ATP) (0.3 μM–100 μM) each induced a biphasic elevation of cytosolic calcium ($[Ca^{2+}]_i$), consisting of an initial transient followed by a sustained plateau phase.

2 Pretreatment of BAEC with 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM) reduced the magnitude of the initial transient elevation of $[Ca^{2+}]_i$, induced by thrombin (1 u ml^{-1}), low concentrations of bradykinin (1 nM) or ATP (0.3 μM, 3 μM), but not by higher concentrations of the latter two agonists. Addition of PMA (100 nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (30 μM) resulted in a fall in $[Ca^{2+}]_i$.

3 The inhibitory effects of PMA (100 nM) were inhibited by staurosporine (100 nM) but not mimicked by the inactive phorbol ester, 4α-phorbol 12, 13-didecanoate (4α-PDD; 100 nM). Furthermore, staurosporine (100 nM) increased $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin or bradykinin. In contrast, staurosporine (100 nM) reduced $[Ca^{2+}]_i$when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (30 μM).

4 Pretreatment with forskolin (10 μM) had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM and 10 nM) or ATP (30 μM). In contrast, forskolin (10 μM) and isoprenaline (10 μM) each induced biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by the three agonists. Furthermore, in the presence of the inhibitor of calcium influx, nickel chloride (4 mM), these biphasic elevations were reduced to monophasic transient elevations.

5 8 Bromo cyclic GMP (30 μM), a membrane-permeant analogue of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (3 μM). Furthermore, 8 bromo cyclic GMP (30 μM) and sodium nitroprusside (1 μM), had no effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by the three agonists.

6 N^G-nitro-L-arginine (50 μM), an inhibitor of nitric oxide synthase, had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM) or ATP (3 μM), and had no effect on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by these agents.

7 These findings suggest that while activation of protein kinase C inhibits and elevation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) augments calcium mobilisation in bovine aortic endothelial cells, elevation of cyclic GMP appears to have no effect.

Keywords: Endothelial cell; bradykinin; ATP; thrombin; calcium; fura-2; cyclic AMP; phorbol ester; cyclic GMP

Introduction

Endothelium-derived relaxing factor (EDRF) has recently been identified as nitric oxide (Palmer *et al.*, 1987). The primary stimulus for production of EDRF is calcium (Singer & Peach, 1982), reflecting the calcium-dependence of the enzyme nitric oxide synthase (Mayer *et al.*, 1989). Recently, attempts have been made to determine if other intracellular second messenger systems such as protein kinase C and the cyclic nucleotides modulate calcium mobilisation in the vascular endothelium. For example, tumour-promoting phorbol esters, which activate protein kinase C (Castagna *et al.*, 1982) inhibit endothelium-dependent relaxation induced by histamine in guinea-pig pulmonary artery (Weinheimer *et al.*, 1986), acetylcholine and substance P in rabbit aorta (Lewis & Henderson, 1987) and acetylcholine in rabbit pulmonary artery (Cherry & Gillis, 1988). The inability of phorbol esters to inhibit relaxations induced by sodium nitroprusside (Lewis & Henderson, 1987) suggests that these compounds inhibit the production, but not the action, of EDRF. Not all studies on isolated endothelial cells are in agreement, however, since phorbol esters have been reported to inhibit bradykinin-stimulated production of EDRF by bovine (de Nucci *et al.*, 1988) but not by porcine aortic endothelial cells (Smith & Lang, 1990). Nevertheless, the finding that endothelium-dependent relaxation induced by the calcium ionophore, A23187, is unaffected (Weinheimer *et al.*, 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988) strongly suggests that phorbol esters inhibit EDRF production by blocking receptor-mediated calcium mobilisation, a concept supported by direct measurement of cytosolic calcium using fluorescent probes (Brock & Capasso, 1988; Carter *et al.*, 1989).

Whether the cyclic nucleotides, guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and adenosine 3′:5′-cyclic monophosphate (cyclic AMP), modulate calcium mobilisation in the endothelial cell is less clear. Endothelial cells respond to the EDRF they produce (Martin *et al.*, 1988) as well as to atrial naturiuretic factors (Leitman & Murad, 1986; Martin *et al.*, 1988), and the resulting elevations of cyclic GMP content are thought to inhibit EDRF production (Evans *et al.*, 1988; Hogan *et al.*, 1989). Elevation of cyclic GMP content inhibits thrombin-induced production of inositol 1,4,5-trisphosphate by porcine aortic endothelial cells (Lang & Lewis, 1991), but it is not known if calcium mobilisation is inhibited. Furthermore, elevation of cyclic AMP content has variously been reported in bovine aortic endothelial cells to inhibit

[1] Author for correspondence.

bradykinin-and ATP-stimulated calcium mobilisation (Lückhoff *et al.*, 1990) or to augment ATP-induced mobilisation (Brock *et al.*, 1988), whereas in human umbilical vein endothelial cells it has been reported to have no effect on histamine-stimulated calcium mobilisation (Carson *et al.*, 1989).

In view of the differing reports on the actions of phorbol esters and cyclic nucleotides we wished to examine more fully the effects of these agents on a single endothelial cell type, namely, bovine aortic endothelial cells (BAEC). Specifically, we wished to examine their effects upon calcium mobilisation induced by a range of agonists over a wide range of concentrations. Furthermore, we wished to examine separately the effects of these regulators on both the initial transient elevation of calcium resulting from intracellular release and the plateau phase resulting from calcium influx.

Methods

Culture of endothelial cells

Bovine aortae were obtained from a local abattoir. Immediately after removal from the animal, the aorta was flushed with sterile saline containing benzyl penicillin (100 u ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). The vessel was ligated at one end and cannulated at the other with a 60 ml syringe containing the same sterile saline. This saline was infused into the lumen and the aorta transported to the laboratory. The endothelial cells were then isolated as described previously (Buchan & Martin, 1991). Briefly, after ligating the intercostal arteries, collagenase (0.1%, type II, Sigma, in Dulbecco's modification of Eagle's medium (DMEM)) was infused into the aortic lumen and the blood vessel incubated for 25 min at 37°C. The endothelial cells from each aorta were then harvested, washed twice and finally resuspended in bicarbonate-buffered DMEM containing foetal bovine serum (10%), newborn bovine serum (10%), glutamine (4 mM), benzyl penicillin (200 u ml^{-1}) and streptomycin (200 $\mu g\,ml^{-1}$) and seeded into 80 cm^2 tissue culture flasks. The culture medium was replaced the day after isolation and every 2 days subsequently. All tissue culture reagents were obtained from Gibco (Paisley, UK). Upon reaching confluence (3–5 days), the cells were detached by exposure to trypsin (0.05%) and EDTA (0.02%) (Flow Laboratories, Irvine, UK), washed twice in DMEM, seeded onto sterile glass coverslips (11 × 42 mm) and grown to confluence, reached after 2–3 days.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as previously described (Buchan & Martin, 1991). Briefly, confluent bovine aortic endothelial cell monolayers were incubated for 45 min with the penta-acetoxymethyl ester form of fura-2 (2 μM, Novabiochem) at 37°C in HEPES (20 mM)-buffered DMEM (Northumbria Biologicals, Cramlington, UK) containing 1% bovine serum albumin (Fraction V, Sigma). Fura-2 loaded cells were then transferred to HEPES (10 mM)-buffered Krebs solution (pH 7.4) containing (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1, $NaHCO_3$ 2.4, glucose 11, HEPES 10 and $CaCl_2$ 1.8, and left at room temperature for 20 min in order to maximize conversion to the Ca^{2+}-sensitive acid form. For experiments involving nickel, $MgSO_4$ was substituted with $MgCl_2$ (1 mM) and $NaHCO_3$ was omitted. The coverslips were placed in a quartz cuvette containing HEPES (10 mM)-buffered Krebs solution in a Perkin Elmer LS3B fluorimeter, at an angle of 30° to the incident light. The beam irradiated the monolayer without passing through the coverslip. The cuvette was maintained at 37°C and stirred continuously. The excitation monochromator was computer-driven between 340 nm and 380 nm every 3.8 s and fluorescence emission collected at 509 nm. Background auto-fluorescence was determined at the end of each experiment by permeabilizing the cells to divalent cations with ionomycin (1 μM) and adding Mn^{2+} (2 mM) to quench intracellular fura-2 fluorescence. Following subtraction of auto-fluorescence, the corrected fluorescence values obtained following excitation at 340 nm were divided by those obtained at 380 nm, giving a corrected ratio (R). $[Ca^{2+}]_i$ was then calculated by the computer from the equation of Grynkiewicz *et al.* (1985):

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

the maximal (R_{max}) and minimal (R_{min}) fluorescence ratios of fura-2 obtained in medium containing saturating concentrations of calcium and in calcium-free medium (with 40 mM EGTA) were determined to be 16.3 and 0.8 respectively. S_{f2} and S_{b2} are the fluorescence values obtained at 380 nm in the absence of calcium and the presence of saturating levels of calcium, respectively, and the S_{f2}/S_{b2} ratio was determined to be 7.3. The K_d for the fura-2-Ca^{2+} complex was assumed to be 225 nM at 37°C.

Drugs

Adenosine triphosphate (ATP; sodium salt); bradykinin triacetate, 8 bromo cyclic GMP, ethylene glycol *bis* (β-aminoethyl ethene N,N,N′,N′-tetraacetic acid (EGTA), (±)-isoprenaline hemisulphate, N^G-nitro-L-arginine (L-NNA), 4α-phorbol 12, 13-didecanoate (4α-PDD), 4β-phorbol 12-myristate 13-acetate (PMA), sodium nitroprusside and thrombin (bovine) were obtained from Sigma. Fura-2 penta-acetoxymethyl ester (fura-2/AM), forskolin, ionomycin and staurosporine were obtained from Novabiochem. All drugs were dissolved in distilled water except for fura-2/AM, ionomycin, forskolin and staurosporine which were dissolved in dimethyl sulphoxide (DMSO) and PMA and 4α-PDD which were dissolved in 100% ethanol.

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by Student's *t* test or by the Mann-Whitney test when there was unequal variance in samples. A probability of 0.05 or less was considered significant. The term '*n*' refers to the number of experiments conducted, usually on different batches of cells.

Results

Phorbol esters

In the presence of 1.8 mM extracellular calcium, the resting level of $[Ca^{2+}]_i$ in bovine aortic endothelial cells was 88 ± 5 nM ($n = 37$). The addition of thrombin (Figure 1), bradykinin or adenosine triphosphate (ATP) resulted in a biphasic elevation of $[Ca^{2+}]_i$ consisting of a rapid, transient component which peaked within 30 s, followed by a lower sustained component. Pretreatment with 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min), an activator of protein kinase C, had no effect on resting $[Ca^{2+}]_i$ (Table 1). It did, however, significantly inhibit the transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), low concentrations of bradykinin (1 nM) or ATP (0.3 μM and 3 μM), but had no effect on that induced by higher concentrations of bradykinin or ATP (Figure 1b, Figure 2, Table 1). 4α-Phorbol 12, 13-didecanoate (4α-PDD; 100 nM, 5 min), which does not activate protein kinase C, had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Figure 1c, Table 1).

Pretreatment with staurosporine (100 nM, 5 min) an inhibitor of protein kinase C, had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the thrombin-induced initial transient elevation of $[Ca^{2+}]_i$ (Table 1), but did inhibit the ability of PMA

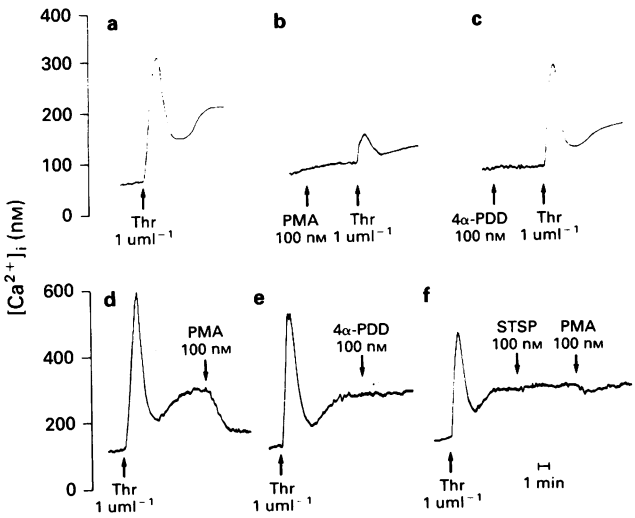

Figure 1 Individual traces showing (a) the biphasic elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) in bovine aortic endothelial cells. Also shown are the effects of pretreatment with (b) 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min) and (c) 4α-phorbol 12, 13 didecanoate (4α-PDD; 100 nM, 5 min) on the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin as well as the effects of (d) PMA (100 nM), (e) 4α-PDD (100 nM) and (f) PMA (100 nM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin in the presence of staurosporine (STSP, 100 nM).

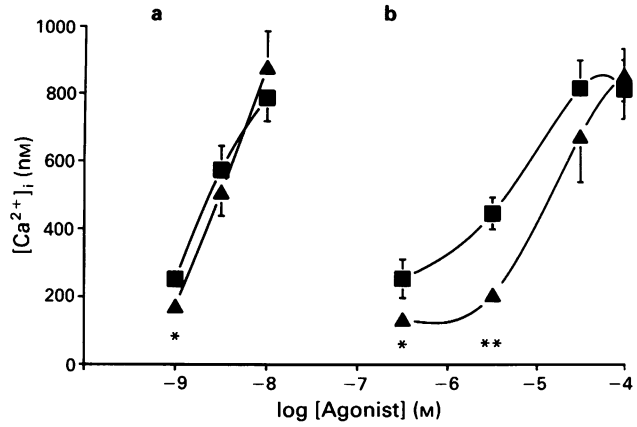

Figure 2 Concentration-effect curves showing the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by (a) bradykinin (BK) and (b) adenosine triphosphate (ATP) in bovine aortic endothelial cells in the absence (squares) and presence (triangles) of 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min). Individual points represent the mean of 4–10 observations and vertical bars represent the s.e.mean. Where error bars are not seen, they are encompassed within the symbols. Asterisks indicate a significant difference from the response obtained in the absence of PMA; $*P < 0.05$; $**P < 0.01$.

(100 nM) to attenuate this initial transient elevation of $[Ca^{2+}]_i$ (Table 1).

When PMA (100 nM) was added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}; Figure 1d), bradykinin (10 nM) and ATP (30 μM), falls in $[Ca^{2+}]_i$ of 89 ± 26 nM ($n = 4$), 35 ± 14 nM ($n = 4$) and 161 ± 29 nM ($n = 6$), respectively, were obtained. Addition of 4α-PDD (100 nM) during the thrombin-induced plateau phase of the increase in $[Ca^{2+}]_i$ had no effect on $[Ca^{2+}]_i$ ($n = 4$; Figure 1e).

Staurosporine (100 nM) added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) and bradykinin (10 nM) resulted in increases in $[Ca^{2+}]_i$ of 10 ± 3 nM ($n = 13$) and 43 ± 12 nM ($n = 4$), respectively. In contrast, staurosporine (100 nM) induced a fall in $[Ca^{2+}]_i$ of 76 ± 14 nM ($n = 4$) when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (30 μM). Furthermore, staurosporine (100 nM), when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), reduced the PMA (100 nM)-induced fall in $[Ca^{2+}]_i$ to 19 ± 3 nM ($n = 4$; Figure 1f).

Table 1 Effects of phorbol esters and staurosporine on the increase in $[Ca^{2+}]_i$ stimulated by thrombin

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)		n
None (control)	None	88 ± 5		37
None	PMA (100 nM)	97 ± 9		10
None	STSP (100 nM)	102 ± 7		13
None	PMA (100 nM) +STSP (100 nM)	122 ± 4*		7
Thr (1 u ml^{-1}) (control)	None	333 ± 29	***	14
Thr (1 u ml^{-1})	PMA (100 nM)	191 ± 20		10
Thr (1 u ml^{-1})	STSP (100 nM)	274 ± 34	*	6
Thr (1 u ml^{-1})	PMA (100 nM) +STSP (100 nM)	274 ± 24		9
None (control)	None	76 ± 6		12
None	4α-PDD (100 nM)	87 ± 6		6
Thr (1 u ml^{-1})	None	237 ± 18		6
Thr (1 u ml^{-1})	4α-PDD (100 nM)	232 ± 26		6

Bovine aortic endothelial cells were pretreated with either 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min), 4α-phorbol 12, 13 didecanoate (4α-PDD; 100 nM, 5 min), staurosporine (STSP; 100 nM, 5 min) or with a combination of STSP (100 nM, 10 min) and PMA (100 nM; 5 min). The effects of these pretreatments on both resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) are shown. Values are the mean ± s.e.mean. Asterisks denote a significant difference from the control or a difference between groups joined by a bracket; ($*P < 0.05$; $***P < 0.001$).

Forskolin and isoprenaline

Pretreatment with forskolin (10 μM, 5 min), an activator of adenylate cyclase, had no effect on resting $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM and 10 nM) or ATP (30 μM; Table 2).

Addition of a maximal concentration of forskolin (10 μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) resulted in a biphasic elevation of $[Ca^{2+}]_i$ (Figure 3b): the maximum increase was 66 ± 8 nM ($n = 9$) and this decayed to 41 ± 6 nM after 5 min. Forskolin (10 μM) induced similar biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phases induced by both bradykinin

Table 2 Effects of forskolin on the increases in $[Ca^{2+}]_i$ stimulated by thrombin, bradykinin and ATP

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)	n
None (control)	None	97 ± 5	51
None	For (10 μM)	110 ± 8	22
Thr (1 u ml^{-1})	None	347 ± 34*	11
Thr (1 u ml^{-1})	For (10 μM)	362 ± 52*	9
BK (1 nM)	None	298 ± 43*	6
BK (1 nM)	For (10 μM)	264 ± 26*	5
BK (10 nM)	None	809 ± 104*	8
BK (10 nM)	For (10 μM)	708 ± 122*	4
ATP (30 μM)	None	1038 ± 77*	4
ATP (30 μM)	For (10 μM)	1153 ± 112*	4

The effects of pretreatment of bovine aortic endothelial cells with forskolin (For; 10 μM, 5 min) on resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}), bradykinin (BK; 1 nM and 10 nM) and adenosine triphosphate (ATP; 30 μM) are shown. Values are the mean ± s.e.mean. Asterisks denote a significant difference from control; $*P < 0.001$.

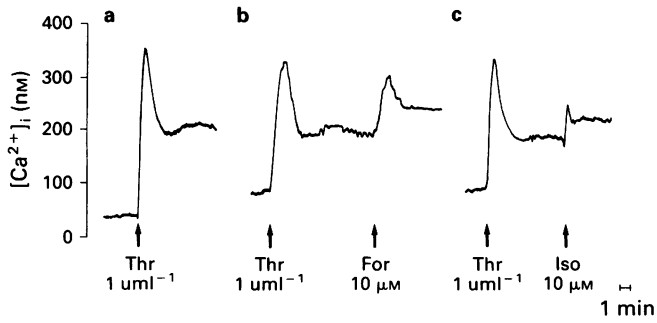

Figure 3 Individual traces showing (a) the biphasic elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) in bovine aortic endothelial cells and the effects of (b) forskolin (For; 10 μM) and (c) isoprenaline (Iso; 10 μM) when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin.

(10 nM) and ATP (3 μM): with bradykinin (10 nM) the maximum increase was 101 ± 8 nM ($n = 4$) which decayed to 35 ± 8 nM after 5 min, while with ATP (3 μM) the maximum increase was 74 ± 13 nM ($n = 7$) and this decayed to 26 ± 7 nM within 5 min. A maximal concentration of isoprenaline (10 μM) induced a biphasic elevation of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}; Figure 3c): the maximum increase was 61 ± 6 nM ($n = 6$) and this decayed to 25 ± 6 nM after 5 min. In the presence of nickel (4 mM), which blocks agonist-induced influx of calcium (Hallam *et al.*, 1988), the first component of the biphasic response induced by forskolin or isoprenaline was unaffected, but the second prolonged phase was abolished (data not shown).

In the presence of staurosporine (100 nM), the ability of forskolin (10 μM) to enhance the thrombin (1 u ml^{-1})-induced plateau phase of the increase in $[Ca^{2+}]_i$ was not significantly attenuated: the maximum increase was 53 ± 7 nM ($n = 8$) and this decayed to 33 ± 3 nM after 5 min.

Pretreatment with forskolin (10 μM, 5 min) did not affect the ability of PMA (100 nM) to induce a fall in the plateau phase of the thrombin (1 u ml^{-1})-induced increase in $[Ca^{2+}]_i$: the fall induced by PMA in untreated and forskolin-treated cells was 88 ± 8 nM ($n = 5$) and 114 ± 18 nM ($n = 5$), respectively.

8 Bromo cyclic GMP, sodium nitroprusside and N^G-nitro L-arginine

Pretreatment with 8 bromo cyclic GMP (30 μM, 5 min) had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (3 μM) (Table 3). Furthermore, neither 8 bromo cyclic GMP (30 μM) nor sodium nitroprusside (1 μM) had any effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (30 μM) (data not shown, $n > 4$ for each).

Table 3 Effects of 8 bromo cyclic GMP on the increases in $[Ca^{2+}]_i$ stimulated by thrombin, bradykinin and ATP

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)	n
None (control)	None	99 ± 6	36
None	8BrcGMP (30 μM)	114 ± 8	15
Thr (1 u ml^{-1})	None	228 ± 16*	9
Thr (1 u ml^{-1})	8 BrcGMP (30 μM)	238 ± 28*	6
BK (10 nM)	None	909 ± 104*	8
BK (10 nM)	8 BrcGMP (30 μM)	952 ± 245*	4
ATP (3 μM)	None	745 ± 101*	4
ATP (3 μM)	8 BrcGMP (30 μM)	892 ± 99*	5

The effects of pretreatment of bovine aortic endothelial cells with 8 bromo cyclic GMP (8 BrcGMP; 30 μM, 5 min) on resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}), bradykinin (BK; 10 nM) and adenosine triphosphate (ATP; 3 μM) are shown. Values are mean ± s.e.mean. Asterisks denote a significant difference from control; *$P < 0.001$.

Table 4 Effects of N^G-nitro-L-arginine on the increases in $[Ca^{2+}]_i$ stimulated by thrombin, bradykinin and ATP

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)	n
None (control)	None	118 ± 5	36
None	L-NNA (50 μM)	124 ± 5	18
Thr (1 u ml^{-1})	None	299 ± 35*	6
Thr (1 u ml^{-1})	L-NNA (50 μM)	285 ± 39*	6
BK (1 nM)	None	515 ± 78*	6
BK (1 nM)	L-NNA (50 μM)	395 ± 16*	6
ATP (3 μM)	None	528 ± 61*	6
ATP (3 μM)	L-NNA (50 μM)	549 ± 113*	6

The effects of pretreatment of bovine aortic endothelial cells with N^G-nitro-L-arginine (L-NNA; 50 μM, 5 min) on resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}), bradykinin (BK; 1 nM) and adenosine triphosphate (ATP; 3 μM) are shown. Values are the mean ± s.e.mean. Asterisks denote a significant difference from control; *$P < 0.001$.

Pretreatment with the inhibitor of nitric oxide synthase, N^G-nitro L-arginine (L-NNA; 50 μM, 5 min) had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM) or ATP (3 μM) (Table 4). Furthermore, L-NNA (50 μM) had no effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}; $n = 3$), bradykinin (10 nM; $n = 4$) or ATP (30 μM; $n = 1$) (data not shown).

Discussion

Agonist-induced elevations of intracellular calcium ($[Ca^{2+}]_i$) in the vascular endothelium are biphasic, consisting of a large transient followed by a lower, more sustained, plateau phase (Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987; Brock & Capasso, 1988; Buchan & Martin, 1991). The initial transient results from the release of calcium from intracellular stores and our novel observations show that the ability of the phorbol ester, PMA, to inhibit this component in bovine aortic endothelial cells depends upon the agonist studied. Specifically, PMA inhibited the ability of a maximally active concentration of thrombin or low concentrations of bradykinin and ATP to induce release of calcium from intracellular stores but, in contrast, had not effect on release induced by high concentrations of bradykinin or ATP. Therefore, in bovine aortic endothelial cells, the ability of PMA to inhibit agonist-induced release of calcium from intracellular stores depends not only on the agonist studied, but on the agonist concentration. Our findings in bovine aortic endothelial cells contrast with the ability of phorbol esters to inhibit intracellular release of calcium induced by maximal concentrations of thrombin, bradykinin and ATP in human umbilical vein endothelial cells (Brock & Capasso, 1988, Carter *et al.*, 1989) and highlights a species or site difference.

The actions of PMA probably result from activation of protein kinase C as they were attenuated by staurosporine, an inhibitor of this enzyme, and were not mimicked by the inactive phorbol ester, 4α-phorbol 12, 13-didecanoate. The transient elevation of $[Ca^{2+}]_i$ is likely to result from inositol 1,4,5-trisphosphate (IP_3)-induced release of calcium from intracellular stores (Berridge, 1984). The ability of phorbol esters to inhibit thrombin- and histamine-induced IP_3 production in

human umbilical vein endothelial cells (Brock & Capasso, 1988) is therefore likely to account for their ability to inhibit mobilisation of intracellular calcium. Our finding that PMA inhibits differentially release of intracellular calcium stimulated by a range of agonists may reflect variations in the extent to which receptor-mediated IP_3 production can be inhibited by protein kinase C. Alternatively, at low agonist concentrations, where IP_3 generation may be limited, other mechanisms may contribute to the inhibitory actions of PMA: for example, stimulation of protein kinase C may promote IP_3 breakdown (Connolly *et al.*, 1986) or raise the threshold for activation of the IP_3 receptor (Willems *et al.*, 1989).

The sustained plateau phase of the increase in $[Ca^{2+}]_i$ induced by agonists results from calcium influx (Hallam *et al.*, 1988) through receptor- but not voltage-operated channels (Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987; Johns *et al.*, 1987). Our study shows that addition of PMA during the sustained plateau phase of the increase in $[Ca^{2+}]_i$ induced by maximal concentrations of thrombin, bradykinin and ATP in bovine aortic endothelial cells results in a fall in $[Ca^{2+}]_i$ with each of the agonists examined. Therefore, this component of the biphasic elevation of $[Ca^{2+}]_i$ is much more sensitive to the inhibitory actions of PMA than is the intracellular release component. As observed with the intracellular release of calcium, the sustained plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin was less sensitive to the inhibitory actions of PMA than those induced by thrombin or ATP. As EDRF production is calcium-dependent (Singer & Peach, 1982), the ability of phorbol esters to inhibit EDRF release (Weinheimer *et al.*, 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988; de Nucci *et al.*, 1988; Smith & Lang, 1990) is likely to result from their ability to inhibit endothelial calcium mobilisation. However, differential agonist sensitivity to the inhibitory actions of the phorbol esters on EDRF release appears to occur: for example, Smith & Lang (1990) have shown that bradykinin-induced production of EDRF by pig aortic endothelial cells is unaffected, whereas that induced by substance P or ATP is inhibited. This differential sensitivity may be explained by our finding that bradykinin-induced calcium mobilisation in bovine aortic endothelial cells is less sensitive to the inhibitory actions of phorbol esters than that induced by other agonists.

Our finding that staurosporine increases the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin and thrombin may reflect the ability of these agonists to activate protein kinase C themselves and so inhibit their own actions by a negative feedback loop. In contrast, staurosporine induced a fall in the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP. This may result from the ability of staurosporine to interact with the ATP binding sites of other proteins (Davis *et al.*, 1989).

Our study also provides novel evidence that cyclic AMP enhances agonist-induced mobilisation of calcium in bovine aortic endothelial cells. Forskolin, a direct activator of adenylate cyclase, and isoprenaline, a β-adrenoceptor agonist, each induced biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phase of agonist-induced increases in $[Ca^{2+}]_i$; this consisted of a large transient elevation followed by a lower more sustained plateau phase. Neither forskolin nor isoprenaline had any effect on basal $[Ca^{2+}]_i$. The initial transient and sustained phase of the increases in $[Ca^{2+}]_i$ are likely to result from intracellular calcium release and calcium influx, respectively, since only the latter was abolished in the presence of nickel, a calcium entry blocker (Hallam *et al.*, 1988). Therefore, in bovine aortic endothelial cells, cyclic AMP appears to enhance agonist-induced mobilisation of calcium from both intracellular and extracellular stores. The mechanisms underlying this augmentation are unclear but from our finding that pretreatment with forskolin did not attenuate the ability of PMA to inhibit the plateau phase of agonist-induced increases in $[Ca^{2+}]_i$, it is unlikely that cyclic AMP mediates its actions via inhibition of protein kinase C activity.

Although elevating intracellular cyclic AMP during the plateau phase of the agonist-induced increase in $[Ca^{2+}]_i$ clearly augments intracellular release of calcium, our finding in bovine aortic endothelial cells and those of Carson *et al.*, (1989) in human umbilical vein endothelial cells show that agents which elevate endothelial cyclic AMP content have no effect on the magnitude of the initial agonist-induced transient elevation of $[Ca^{2+}]_i$. This inability to observe an augmentation of the initial transient elevation of calcium is likely to result from the small magnitude of this augmentation relative to the magnitude of the discharge that normally occurs. In one study, elevating cyclic AMP content has been reported to augment slightly the ATP-induced initial transient elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells (Brock *et al.*, 1988). All of these findings clearly conflict with those of Lückhoff *et al.* (1990) who reported that elevating cyclic AMP content inhibits endothelial calcium mobilisation. We have no explanation for this anomaly.

Elevation of endothelial cyclic GMP, by 8 bromo cyclic GMP (Evans *et al.*, 1988) or atrial natriuretic factor (Hogan *et al.*, 1989) is reported to inhibit release of EDRF. Agonists which induce EDRF release also elevate endothelial cyclic GMP content (Martin *et al.*, 1988) and, as a consequence, EDRF release may be regulated via a negative feedback mechanism involving cyclic GMP. Cyclic GMP has been demonstrated to inhibit IP_3 production in pig aortic endothelial cells (Lang & Lewis, 1991), but we found in bovine aortic endothelial cells that elevation of cyclic GMP content does not modulate either basal $[Ca^{2+}]_i$ or elevations of $[Ca^{2+}]_i$ induced by thrombin, bradykinin or ATP. As these agonists are known to increase endothelial cyclic GMP content via the generation of EDRF, we considered whether endogenously generated cyclic GMP was capable of inhibiting endothelial calcium mobilisation. N^G-nitro-L-arginine, an inhibitor of EDRF production (Moore *et al.*, 1990) did not, however, modulate either the magnitude of the agonist-induced initial transient elevation of $[Ca^{2+}]_i$ or the plateau phase of the increase in $[Ca^{2+}]_i$. These findings suggest cyclic GMP does not modulate calcium mobilisation, at least in bovine aortic endothelial cells.

In conclusion, our study suggests that stimulation of protein kinase C inhibits, elevation of cyclic AMP enhances and elevation of cyclic GMP has no effect on calcium mobilisation in bovine aortic endothelial cells.

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Investigation of the effects of IVth ventricular administration of the 5-HT_2 agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), on autonomic outflow in the anaesthetized cat

Sara L. Shepheard, *David Jordan & [1]Andrew G. Ramage

Academic Departments of Pharmacology and *Physiology Royal Free Hospital School of Medicine, Rowland Hill St, Hampstead, London NW3 2PF

1 The effects of IVth ventricular injections of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) on renal, splanchnic and cardiac sympathetic nerve activities, phrenic nerve activity, arterial blood pressure, heart rate, femoral arterial conductance, tracheal and intragastric pressures were investigated in α-chloralose anaesthetized, neuromuscular blocked and artificially ventilated cats.

2 Cumulative doses of DOI (80, 160 and 320 nmol kg^{-1}) injected into the IVth ventricle caused an increase in mean arterial blood pressure, a fall in femoral arterial conductance, an increase in tracheal pressure and a decrease in the rate of phrenic nerve bursts but did not affect any of the other variables recorded.

3 Even after i.v. administration of the peripheral 5-HT_2 antagonist BW501C67 (2 mg kg^{-1}) following the highest dose of DOI there was still a significant pressor response, a fall in femoral arterial conductance and small increase in tracheal pressure.

4 In control experiments, intravenous infusion of noradrenaline to raise blood pressure to the levels obtained during the cumulative doses of DOI caused large falls in renal, splanchnic and cardiac nerve activities which were all significantly lower than those recorded during the cumulative doses of DOI.

5 The results of this study provide evidence for a brainstem site of action of DOI in producing hypertension and further support the hypothesis that central 5-HT_2 receptors are involved in the control of skeletal muscle and skin vascular beds.

Keywords: DOI; 5-HT_2 receptors; sympathetic nerve activity, phrenic nerve activity; blood pressure; tracheal pressure; anaesthetized cats; IVth ventricle

Introduction

Central administration of 5-hydroxytryptamine (5-HT) and its precursors have complex effects on autonomic nerve activity, blood pressure and heart rate (see Mir & Fozard, 1990; Coote, 1990) and this may reflect different roles of 5-HT in different brain regions. Investigations into the role of central 5-HT_{1A} and 5-HT_2 receptors in cardiovascular regulation have indicated that these receptors have opposing actions on efferent autonomic outflow. Activation of central 5-HT_{1A} receptors in the cat by the injection of 5-HT_{1A} agonists such as 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) into the IVth ventricle (Shepheard *et al.*, 1989) produces a similar profile of effects to that seen when these compounds are administered intravenously, i.e. hypotension and differential sympathoinhibition (Ramage & Wilkinson, 1989). In addition, evidence is accumulating that the location of the central 5-HT_{1A} receptors involved in mediating these effects is within the brainstem, and in particular within access of the rostral ventrolateral medulla (Gillis *et al.*, 1989; Laubie *et al.*, 1989; Mandal *et al.*, 1989). In contrast, activation of central 5-HT_2 receptors by intravenous administration of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) has been shown to produce sympathoexcitation (McCall *et al.*, 1987; McCall & Harris, 1988).

The aim of the present study was to investigate whether the activation of central 5-HT_2 receptors by administration of DOI into the IVth ventricle would cause similar effects to those observed when DOI is given i.v. A preliminary account of some of these observations has been given (Shepheard *et al.*, 1991).

[1]Author for correspondence.

Methods

Experiments were performed on male adult cats anaesthetized with a mixture of α-chloralose (70 mg kg^{-1}) and pentobarbitone sodium (6 mg kg^{-1}) i.v.; supplementary doses of α-chloralose (10–15 mg kg^{-1}) were given as required. Following a tracheotomy low in the neck, the animals were intubated and artificially ventilated (rate 30 per min, tidal volume 17–20 ml) using positive pressure after neuromuscular blockade with vecuronium bromide (200 μg kg^{-1}). End-tidal CO_2 was maintained between 6–7% by varying the rate and tidal volume of the ventilator. Arterial blood pressure, heart rate, body temperature and arterial blood gases and pH were monitored as previously described (Ramage, 1984). A constant infusion of a solution comprising 500 ml plasma substitute (Gelofusine, Consolidated Chemicals Ltd), 500 ml H_2O, 8.4 g $NaHCO_3$ and 2 g glucose was given at a rate of 6 ml kg^{-1} h^{-1} into a brachial vein to maintain blood volume and to counteract the development of non-respiratory acidosis. During the experiments, arterial blood values were in the range pH 7.24–7.35, P_{CO_2} 41–48 mmHg and P_{O_2} 112–130 mmHg.

Simultaneous recordings were made of right inferior cardiac, splanchnic and renal nerve activities plus left phrenic nerve activity. The right inferior cardiac nerve was exposed by deflecting the scapula and removing the second rib retropleurally. The splanchnic and renal nerves were exposed by a retroperitoneal approach through the right flank. The left phrenic nerve was exposed low down in the neck at the level of the 4th and 5th spinal nerves. Whole nerve activity was recorded from the central cut or crushed ends of these nerves with bipolar silver hook electrodes as previously described (Ramage & Wilkinson, 1989). In all experiments sympathetic nerve activity was tested to see if it was under baroreceptor

modulation by checking that activity in these nerves was increased by a fall in blood pressure caused by sodium nitroprusside (2 $\mu g\,kg^{-1}$, i.v.) or decreased by a rise in blood pressure caused by noradrenaline (0.5 μg per animal, i.v.). In addition, femoral arterial flow, from which arterial conductance was calculated, was measured with an electromagnetic flow probe placed on the right femoral artery. Tracheal pressure was monitored by a pressure transducer connected to a side arm of the tracheal cannula. Gastric motility was measured by inserting a rubber balloon into the stomach via the oesophagus. This balloon was then filled with 30 ml of saline and connected to a pressure transducer.

To give microinjections into the IVth ventricle the animal's head was placed in a stereotaxic frame, the dorsal surface of the skull exposed, and a small hole made in the bone on the midline, 7 mm anterior to the interaural line. The cannula, angled at 28°, was lowered in a caudal direction through the hole for 22–27 mm from the surface of the dura so that its tip lay in the IVth ventricle. In addition, the caudal brainstem was exposed by a dorsal approach. After removal of the muscles on the back of the neck the occipital bone was removed and the dura mater overlying the brainstem cut. The position of the cannula tip within the IVth ventricle was verified by observing the free flow of artificial CSF through the cannula and out through the cisterna magna. The position of the cannula was confirmed at the end of the experiment by injecting pontamine sky blue dye (20 μl over 1 min). When the cannula was correctly placed it was found that the dye was distributed over the floor of the IVth ventricle from its anterior end to the level of the lateral recesses and from there through the lateral recesses, down on to the ventral surface of the medulla.

All variables were recorded for a 20 min stabilization period. A microinjection of vehicle ('initial' vehicle injection) was then given into the IVth ventricle (i.c.v.) followed 10 min later by either DOI in the DOI experiments or vehicle in the control experiments (Figure 1). All i.c.v. injections were given in a volume of 20 μl over a period of 1 min.

In the DOI experiments cumulative doses of DOI of 80, 160, 320 $nmol\,kg^{-1}$ were given i.c.v. at 10 min intervals. The 5-HT_2 antagonist BW501C67 (1 $mg\,kg^{-1}$) was then given i.v. 10 min after the last dose of DOI, followed 3 min later by a second dose of BW501C67 (1 $mg\,kg^{-1}$). A bolus dose of atropine methonitrate (0.1 $mg\,kg^{-1}$, i.v.) was given 3 min after the second dose of BW501C67 to determine the amount of vagal tone that was present. A second bolus injection of noradrenaline (0.5 μg per animal) was given between the last dose of DOI and before the first dose of BW501C67 to determine if a rise in blood pressure would still cause a baroreceptor mediated decrease in sympathetic nerve activity (Figure 1). The time between injections represents the time taken for the changes in blood pressure and nerve activities to stabilize.

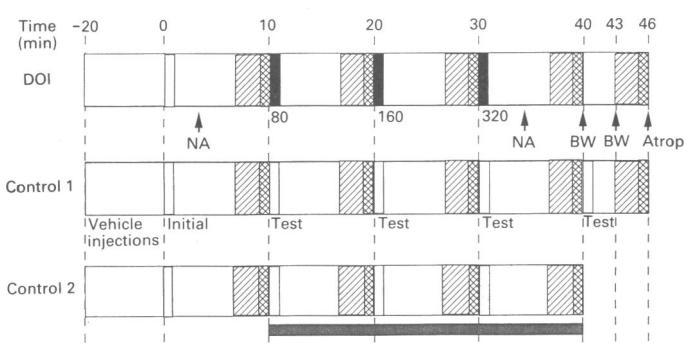

Figure 1 Diagram representing the infusion, injection and measurement protocols in the 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and two control sets of experiments. Infusions (20 μl in 1 min) of DOI (80, 160 and 320 $nmol\,kg^{-1}$) (solid blocks) or vehicle (stippled blocks) were given into the IVth ventricle at 10 min intervals, followed by two injections of BW501C67 (1 $mg\,kg^{-1}$, i.v.) (BW) and atropine (0.1 $mg\,kg^{-1}$ i.v.) (Atrop) in the DOI experiments. Bolus injections of noradrenaline (0.5 μg) (NA) were used to assess baroreceptor function. In the second control set of experiments a slow i.v. infusion of noradrenaline (18 $\mu g\,ml^{-1}$) was given during the experimental period (dark stippled strip).

Measurements of gut motility were made in the 3 min periods preceding injections shown by the hatched blocks. All other variables were measured in the last 1 min periods shown by the cross hatched blocks: N.B. these overlap the ends of the 3 min gut measurement periods.

Two control sets of experiments were performed. In the first set (control 1) four test i.c.v. injections of vehicle were given at 10 min intervals to correspond in time to the three i.c.v. injections of DOI and the first i.v. injection of BW501C67. In the second set (control 2) three test i.c.v. injections of vehicle were given at 10 min intervals to correspond in time to the three i.c.v. injections of DOI and in addition, a slow i.v. infusion of noradrenaline (18 $\mu g\,ml^{-1}$) was given during the period of these three vehicle injections, to raise blood pressure to levels equivalent to those seen during the injections of the cumulative doses of DOI. The rise in blood pressure produced by noradrenaline could be accurately controlled by adjusting the flow rate of the infusion pump.

Analysis of data

Cardiac, splanchnic and renal nerve activities were quantified by rectifying and integrating the signals above background noise over 5 s periods with solid state electronic integrators. The outputs were then displayed on a Grass polygraph recorder in arbitrary units. Phrenic nerve activity was quantified by integrating the amplitude and frequency of the action potentials in each inspiratory burst (see Holtman *et al.*, 1986) or, if activity was continuous, by integrating the amount of activity in 5 s periods, again by use of a solid state electronic integrator the output of which was displayed on the Grass polygraph in arbitrary units. The first method of quantifying phrenic nerve activity gives an indication of both the amount of activity in each inspiratory burst and the frequency of inspiratory bursts. The validity of the threshold settings used to quantify the nerve activities was verified at the end of each experiment after intravenous administration of pentobarbitone sodium (60 mg per animal) or by crushing the nerves to block all activity. All nerve activity is reported as the mean level over 1 min in arbitrary units. Intragastric pressure was quantified by displaying the output from the pressure transducer on the Grass polygraph recorder. The total line length of the ink trace over a 3 min period was measured with an IBAS IPS image analyser (Kontron U.K.) and converted to give mean line length in $mm\,min^{-1}$. Total line length gives an indication of changes in both frequency and amplitude of gastric contractions (see Andrews & Wood, 1986).

In the DOI experiments, measurements of all variables except gastric motility were made in the minute before each injection of DOI, the first injection of BW501C67 and then 6 min after that (Figure 1). Measurements of gastric motility were made in the 3 min period before each injection of DOI, the first injection of BW501C67 and then 3 min after that. For both sets of control experiments, measurements of all variables were made at times corresponding to those at which measurements were made in the DOI experiments except in the noradrenaline infusion experiment where no further measurements were made 10 min after the third 'test vehicle' injection (Figure 1). In all three sets of experiments baseline values were those values measured just before the first injection of DOI or test vehicle. All results are expressed as changes from these baseline values. In order to normalise the data, changes in integrated nerve activity are given as percentage changes from baseline. The changes in all other variables are presented as actual changes.

For all parameters, statistical analysis by Student's unpaired t test was performed to compare the changes from baseline values induced by administration of DOI with the changes from baseline values induced during the control 1 or

control 2 experiments by test vehicle. The changes caused by BW501C67 were compared with those caused by the final vehicle injection in the control 1 experiments also by use of an unpaired t test. All values given are the mean ± s.e.mean. Differences are considered significant when $P < 0.05$.

Drugs

Drugs used were DOI HCl ((±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride) (Semat Tech., U.K.) and BW501C67 (2-anilino-N-[2-(3-chlorophenoxy) propyl] acetamidine hydrochloride) (a gift from Wellcome Laboratories, U.K.). All drugs were dissolved in saline. DOI was found to have a pH of 4.2–6.1, so vehicle solutions were saline, matched to the same pH as the DOI by the addition of 0.1 N HCl. The composition of the artificial CSF used to check the position of the cannula tip in the IVth ventricle was (mM): KH_2PO_4 2.2, $MgSO_4.7H_2O$ 1.2, KCl 2.0, glucose 10, $NaHCO_3$ 25, NaCl 115 and $CaCl_2.2H_2O$ 2.5. All doses except noradrenaline refer to the salts of the drug.

Results

Baseline values for all experimental groups are shown in Table 1.

Effects of cumulative doses of DOI (80–320 nmol kg^{-1})

Cumulative doses of DOI (80–320 nmol kg^{-1}) into the IVth ventricle caused a significant ($n = 5$; $P < 0.05$) increase in mean arterial blood pressure reaching a maximum of 14 ± 2 mmHg (mean ± s.e.mean), from a baseline of 119 ± 11 mmHg, by the highest dose. This was accompanied by a significant decrease in femoral arterial conductance of $-22 \pm 7 \times 10^{-3}$ ml $mmHg^{-1}$ min^{-1} from a baseline of $83 \pm 18 \times 10^{-3}$ ml $mmHg^{-1}$ min^{-1} and an increase in tracheal pressure of 2.4 ± 0.2 cmH_2O from a baseline of 6.3 ± 0.5 cm H_2O, both by the highest dose (Figure 3). Changes in both blood pressure and tracheal pressure could be observed by the end of the injection period. In those experiments in which the phrenic nerve activity was rhythmic ($n = 3$) there was a small significant reduction in mean inspiratory rate of −1.8 ± 0.8 bursts min^{-1} from a baseline rate of 9.7 ± 1.7 bursts min^{-1} by the highest dose, although there were no significant effects on the mean amount of activity in each inspiratory burst since the mean change in activity by the highest dose was 6 ± 22%. There were no changes in total phrenic nerve activity when it was continuous ($n = 2$). There were no significant changes in any of the other variables recorded. A baroreceptor-mediated decrease in sympathetic nerve activity in response to a bolus intravenous injection of noradrenaline, could still be elicited after the highest dose of DOI (see Figure 2).

Effects of BW501C67 after cumulative doses of DOI

Administration of BW501C67 (2 mg kg^{-1} in total, i.v.) following the 320 nmol kg^{-1} dose of DOI reduced the DOI-evoked increases in mean blood pressure and tracheal pressure and the decrease in femoral arterial conductance by 5 ± 2 mmHg, 1.4 ± 0.2 cmH_2O and $5 \pm 1 \times 10^{-3}$ ml $mmHg^{-1}$ min^{-1} respectively (Figure 3). More importantly, however, these variables were still significantly different from their appropriate controls following such blockade of peripheral 5-HT_2 receptors. Following BW501C67 there was a further reduction in inspiratory rate to a total significant decrease of −2.3 ± 1.2 bursts min^{-1} from baseline. BW501C67 had no effect on any of the other variables. Injection of atropine methonitrate after BW501C67 had no effect on heart rate.

Effects of control injections of saline into the IVth ventricle

Neither the 'initial' vehicle injections into the IVth ventricle nor the 'test' vehicle injections in the control 1 experiments had any effect on any of the variables recorded.

In the control 2 experiments, the slow intravenous infusion of noradrenaline caused a gradual increase in mean blood pressure reaching a maximum of 14 ± 2 mmHg after the final vehicle injection. This increase in mean arterial blood pressure was similar to the increase caused by the DOI (see Figure 3). Accompanying the increase in blood pressure were decreases in cardiac, splanchnic and renal nerve activities which reached maxima of −42 ± 15%, −67 ± 11% and −53 ± 15% respectively after the final injection. These decreases became significantly different from the nerve activity in the DOI experiments by the second dose of DOI (see Figure 3; only data for cardiac nerve are illustrated). There was also a decrease in femoral arterial conductance which reached $-19 \pm 5 \times 10^{-3}$ ml $mmHg^{-1}$ min^{-1} by the time of the final injection. This was similar to that caused by DOI (Figure 3). There was no significant change in heart rate or in the magnitude or rate of phrenic nerve activity.

Discussion

Application of DOI to the IVth ventricle produced a rise in blood pressure and a decrease in femoral arterial conductance which, surprisingly, were associated with no significant changes in heart rate or sympathetic nerve activity. In addition, DOI evoked an increase in tracheal pressure and decreased central respiratory frequency. The effects on blood pressure, femoral arterial conductance and tracheal pressure could have been caused by activation of peripheral 5-HT_2 receptors by DOI that had leaked out of the brainstem, across the blood-brain barrier, into the circulation. To test this possibility, the 5-HT_2 antagonist BW501C67, which is a compound that has been reported not to cross the blood-brain barrier (Mawson & Whittington, 1970; Fuller *et al.*, 1986), was administered intravenously. A dose of 2 mg kg^{-1} of BW501C67 is known not to have any effects *per se* on either bronchial smooth muscle or the cardiovascular variables recorded, and has also been shown to block the bronchoconstrictor action of 1 mg kg^{-1} of intravenously administered DOI (Ramage *et al.*, 1991). Since the highest dose of DOI given into the IVth ventricle in this study (320 nmol kg^{-1}) is approximately equivalent to only 0.1 mg kg^{-1}, it was presumed that any peripheral action of DOI would be reversed. It was found that intravenously administered BW501C67 did cause an immediate reduction of the increase in tracheal pressure, indicating that DOI had leaked out of the brainstem and

Table 1 Baseline values of heart rate (HR), mean arterial blood pressure (BP), femoral arterial conductance (FAC), gastric motility (GM), trachael pressure (TP) and inspiratory rate (Insp. rate)

	n	*HR* (beats min^{-1})	*BP* (mmHg)	*FAC* (ml $mmHg^{-1}$ min^{-1} × 10^{-3})	*GM* (linelength mm min^{-1})	*TP* (cmH_2O)	*Insp. rate* (bursts min^{-1})	
Control 1	5	208 ± 10	124 ± 11	63 ± 8	60 ± 4	6.9 ± 0.3	10.0 ± 0.5	
DOI	5	191 ± 10	119 ± 11	83 ± 18	58 ± 6	6.3 ± 0.5	9.7 ± 1.7	($n = 3$)
Control 2	5	204 ± 14	113 ± 9	70 ± 11	55 ± 5	6.3 ± 0.3	8.5 ± 0.9	($n = 4$)

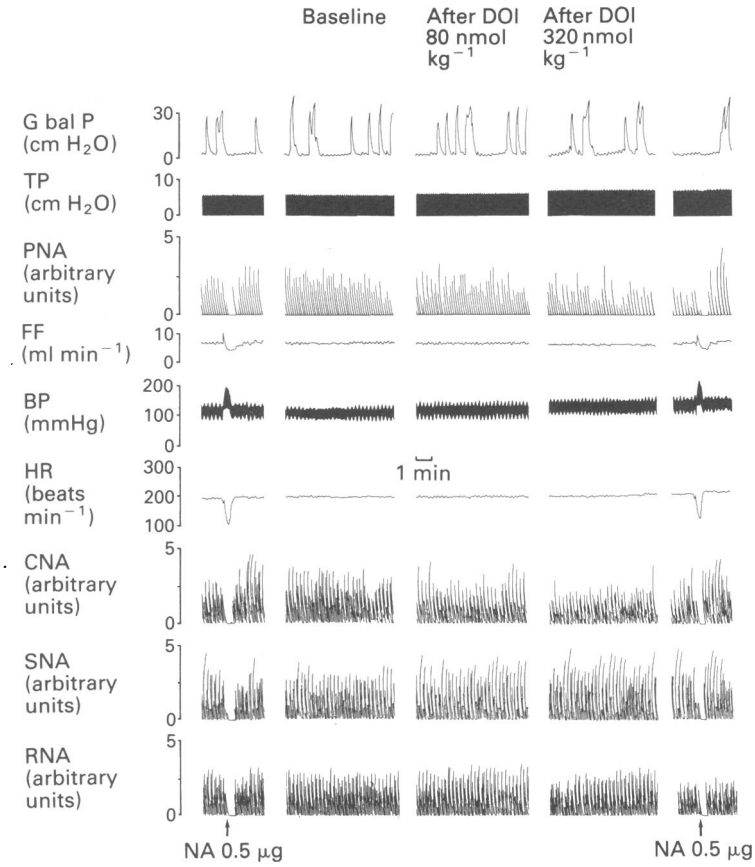

Figure 2 Traces showing the recordings of gastric balloon pressure (G bal P), tracheal pressure (TP), integrated phrenic nerve activity (PNA), femoral arterial flow (FF), blood pressure (BP), heart rate (HR), integrated cardiac (CNA), splanchnic (SNA) and renal (RNA) nerve activities in an anaesthetized cat. Centre three panels show the effects of the 'initial' vehicle injection and then 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 80 and 320 nmol kg^{-1} cumulative) into the IVth ventricle. The outer panels show the effect of a bolus i.v. dose of noradrenaline (NA, 0.5 μg per animal).

activated peripheral 5-HT_2 receptors on bronchial smooth muscle. However, the increases in tracheal and arterial pressure and the decrease in femoral arterial conductance could only be partially reduced by BW501C67 suggesting that these changes were due not only to the activation of peripheral 5-HT_2 receptors on the vasculature, but also to a central action of DOI, presumably by changing sympathetic vasoconstrictor drive to the hindlimb and airways.

The present results differ from previous reports of intravenous administration of DOI (McCall *et al.*, 1987; McCall & Harris, 1988). In those studies, application of doses of DOI similar to those used in the present experiments caused a rise in blood pressure and approximately a 159% increase in cardiac nerve activity. In the present study, DOI given into the IVth ventricle evoked a pressor response of similar size but no sympathoexcitation in the cardiac nerve. However, when an equivalent rise in blood pressure was induced by the infusion of noradrenaline, sympathetic activity decreased by approximately 50%, presumably as a result of activation of baroreceptors. As there was no overall change in the sympathetic outflows recorded in the present experiments during administration of DOI, the expected baroreceptor-mediated sympathoinhibition caused by the rise in blood pressure must be masking a sympathoexcitatory action of DOI on these sympathetic outflows. Whilst it is possible that DOI may have induced a resetting of the baroreceptor reflex, this is unlikely as there were no gross changes in the sympathoinhibition elicited by a rise in blood pressure following a bolus (i.v.) of noradrenaline (see Figure 2). These results therefore suggest that at the level of the IVth ventricle, activation of 5-HT_2 receptors causes sympathoexcitation. This sympathoexcitation would seem to be greater in the outflow to the skeletal muscle and/or skin vasculature than that of the other sympathetic outflows. This may be explained if the sympathetic outflow to the hindlimb is more susceptible to the DOI-evoked sympathoexcitation than the other sympathetic outflows. On the other hand, it may be less sensitive to the baroreceptor evoked sympathoinhibition. What evidence there is would not support this latter hypothesis (see Oberg, 1976). Finally in this respect, intravenous administration of 5-HT_2 antagonists characteristically increase femoral arterial conductance in anaesthetized cats (Ramage, 1985; 1988) and IVth ventricular application of the 5-HT_2 antagonist cinanserin in the present experimental system (Shepheard *et al.*, 1990) caused an increase in femoral arterial conductance.

The present results suggest that activation of 5-HT_2 receptors at the level of the brainstem can cause sympathoexcitation. However, the level of sympathoexcitation is considerably less than that observed with equivalent doses given intravenously (McCall & Harris, 1988) suggesting that other sites are also important for the sympathoexcitatory action of DOI. However, this may simply reflect the difference in the accessibility of DOI to different nuclei of the brainstem when administered by these different routes. Recent evidence has suggested that areas accessible from the rostral ventral surface of the medulla are sites at which DOI (Mandal *et al.*, 1990) and also another 5-HT_2 agonist, quipazine, (Courchay *et al.*, 1991) can cause central sympathoexcitation in anaesthetized cats. Interestingly, iontophoretic application of DOI failed to alter the activity of presumed sympathoexcitatory neurones in this area though they were excited by DOI given intravenously (Clement & McCall, 1990). Thus, the precise role of the rostral ventrolateral medulla in the sympathoexcitatory action of DOI and other 5-HT_2 agonists remains to be determined.

In the present experiments, DOI caused a decrease in the

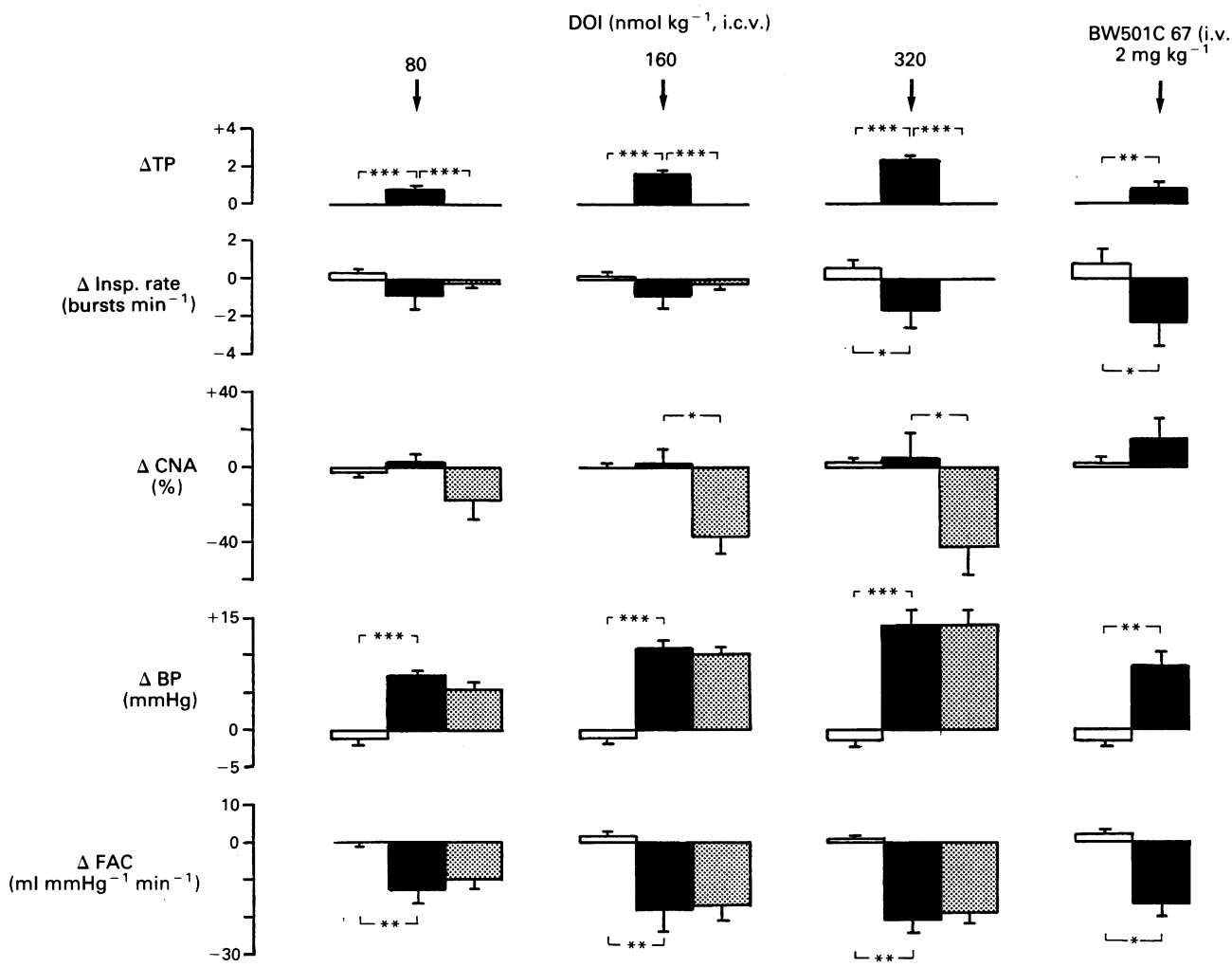

Figure 3 Anaesthetized cats: histograms comparing changes from the appropriate baseline values caused by injections into the IVth ventricle of cumulative doses 80, 160 and 320 nmol kg^{-1}, of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, closed columns) with those of control 1 (injection of vehicle alone; open columns) and control 2 (vehicle injection during noradrenaline infusion; stippled columns) on tracheal pressure (TP), inspiratory rate (Insp. rate), cardiac nerve activity (CNA), blood pressure (BP) and femoral arterial conductance (FAC), $n = 5$ except for DOI Insp. rate where $n = 3$ and control 2 Insp. rate where $n = 4$. The effects of DOI have been compared with both sets of controls by Student's unpaired t tests. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The last column shows the change from baseline caused by i.v. injection of BW501C67 (2 mg kg^{-1}) after the last dose of DOI compared with the equivalent change in control 1. As DOI had little effect on the other variables that were recorded the data on these variables have been omitted for the sake of clarity.

frequency of phrenic bursts but since arterial blood gases could not be monitored continuously, it is always possible that this small change was an indirect effect of alterations in these respiratory stimuli. However, the present results do confirm a recent report of King & Holtman (1990) who applied DOI directly onto the ventral surface of the brainstem. The physiological role of the 5-hydroxytryptaminergic innervation of this region remains to be determined since activation of 5-HT-containing pathways originating in raphe nuclei have been shown both to suppress (Lalley, 1986) and excite (Holtman *et al.*, 1987) inspiratory outflow.

The present experiments support the view that 5-HT_2 receptors in the brainstem are sympathoexcitatory and also have inhibitory actions on central inspiratory drive. Further, 5-HT_2 receptors would seem to have a greater influence on the control of sympathetic drive to skeletal muscle and/or the skin vasculature. However, whether the brainstem is the major site of action for the sympathoexcitatory effect of intravenously administered DOI is doubtful.

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The relationship of KCl- and prostaglandin $F_{2\alpha}$-mediated increases in tension of the porcine coronary artery with changes in intracellular Ca^{2+} measured with fura-2

Joseph L. Balwierczak

Research Dept., Pharmaceuticals Div., Ciba-Geigy Corp., Summit, N.J. 07901, U.S.A.

1 Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured simultaneously with changes in muscle tension by use of the fluorescent Ca^{2+} indicator, fura-2, in coronary arterial rings of the pig.

2 Changes in $[Ca^{2+}]_i$ were measured by monitoring the ratio of fluorescence due to excitation at 340 nm (F_{340}) to that at 380 nm (F_{380}).

3 Increases in tension of the porcine coronary artery induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (2–30 μM) and KCl (25–70 mM) were accompanied by increases in the F_{340}/F_{380} fluorescence ratio of fura-2.

4 KCl-induced increases in muscle tension, equivalent to those produced by $PGF_{2\alpha}$, were observed to occur in the presence of a higher $[Ca^{2+}]_i$.

5 Nearly complete relaxation of KCl-induced contractions by sodium nitroprusside (SNP) was accompanied by only a partial reversal of the increase in $[Ca^{2+}]_i$ that occurred during contraction.

6 Complete relaxation of the $PGF_{2\alpha}$-contracted coronary artery by cromakalim (BRL 34915) was accompanied by a nearly complete reversal of the increase in $[Ca^{2+}]_i$ caused by the contractile agent.

7 The contractile state of smooth muscle is not an indicator of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$-tension relationship is dependent upon the type of pharmacological agent that is used to change muscle tension.

Keywords: Intracellular Ca^{2+}; smooth muscle contraction; porcine coronary artery; fura-2; prostaglandin $F_{2\alpha}$; vasodilators; sodium nitroprusside; cromakalim

Introduction

An increase in cytosolic calcium is a prerequisite for smooth muscle contraction, and for many years contraction has been used as a convenient indicator of changes in intracellular calcium concentration ($[Ca^{2+}]_i$). However, there have been reports where smooth muscle contraction was not associated with the mobilization of Ca^{2+}. Rooke *et al.* (1984) showed that the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-mediated contraction of the canine coronary artery was dependent on extracellular Ca^{2+}. However, when $PGF_{2\alpha}$ was added to a maximally contracted (60 mM KCl) canine coronary artery, an additional contraction occurred which was not sensitive to calcium channel blockers and was not associated with any release of intracellular Ca^{2+}. The authors concluded that either changes in cytoplasmic Ca^{2+} caused by release from intracellular stores were too small to be detected or that the increased contraction by $PGF_{2\alpha}$ occurred independently of changes in the cytoplasmic concentration of Ca^{2+}.

In the last few years, natural (aequorin) and synthetic (quin-2, fura-2) Ca^{2+} indicators have been developed along with techniques needed for their incorporation into the intracellular environment of muscle cells (Tsien, 1980; Morgan & Morgan, 1982) in order to provide a quantitative relationship between contraction and $[Ca^{2+}]_i$ in intact muscle. The tetracarboxylate fluorescent dye, fura-2, is one of the more recently developed examples of these Ca^{2+} indicators (Grynkiewicz *et al.*, 1985).

In work which used the aequorin-loaded porcine coronary artery (Bradley & Morgan, 1987), KCl-induced contractions were associated with increases in aequorin luminescence whereas $PGF_{2\alpha}$-mediated contractions were not. This meant that the contraction induced by $PGF_{2\alpha}$ occurred with no increase in intracellular Ca^{2+} concentration. These results lend support to the findings of Rooke *et al.* (1984).

Other studies which have shown inexplicable correlations between muscle tension and $[Ca^{2+}]_i$ have been the reports by Karaki *et al.* (1988) and Gerthoffer *et al.* (1989). These authors have shown that noradrenaline and carbachol caused contractions of the rat aorta and canine trachea, respectively, that were dependent on increases in $[Ca^{2+}]_i$ but in a manner different from that found for KCl. Contractions mediated by KCl that were equivalent in magnitude to agonist induced contractions occurred in the presence of higher $[Ca^{2+}]_i$ as measured by either fura-2-fluorescence or aequorin luminescence.

In order to provide more data to determine if $PGF_{2\alpha}$-mediated contractions of coronary smooth muscle are correlated with increases in $[Ca^{2+}]_i$, studies were undertaken to measure the $[Ca^{2+}]_i$-tension relationship for KCl and $PGF_{2\alpha}$ in the coronary artery of the pig. Furthermore, this relationship was examined in the presence of different vasorelaxants. The studies were accomplished by the simultaneous measurement of tension and $[Ca^{2+}]_i$ by use of the fluorescent Ca^{2+} indicator, fura-2.

Methods

Pig hearts were purchased from a local abattoir and delivered to the laboratory in isotonic saline within 1 h after removal from the pigs. The left circumflex and anterior descending coronary arteries were dissected free of the heart, cut into 5 mm segments and placed into a Krebs-HCO_3 physiological salt solution (PSS) which contained (in mM): NaCl 127, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 18 and dextrose 11. The solution was bubbled with 95% O_2; 5% CO_2, maintaining the pH at 7.4. The adventitia of the coronary artery were removed by careful dissection and the resulting medial ring was placed in a 5 ml bath thermostatically controlled at 37°C and held in a horizontal configuration under 2.0 g of tension by stretching between two stainless steel triangles. One end of the tissue was connected to a force transducer for the measurement of isometric tension (model

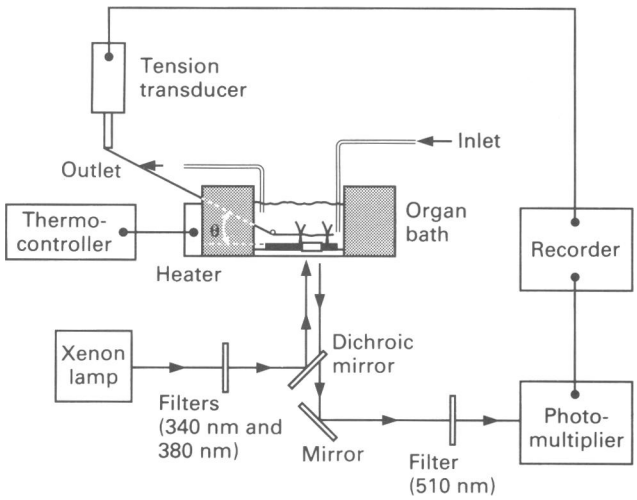

Figure 1 Diagram of the optical system of the JASCO CAF-100 fluorometer and the tissue setup used for the simultaneous recording of fura-2 fluorescence and muscle tension. The light source is a xenon lamp and alternating excitation of the tissue sample at 340 nm and 380 nm was attained by use of filters of appropriate band width placed on a rotating wheel (48 Hz) situated in the light path. The excitation light is reflected by a dichroic mirror and then focused onto the tissue sample by means of a lens which is positioned directly beneath the tissue. The reflected sample fluorescence is collected by a mirror beneath the sample and focused with a lens onto a filter (510 nm). Another lens then focuses the filtered emission fluorescence onto a photomultiplier. The emission resulting from excitation at each of the wavelengths (F_{340} and F_{380}) as well as their ratio were recorded continuously on a polygraph recorder (Model 79, Grass Instruments Co., Quincy, MA, U.S.A). Muscle tension was measured also with the same polygraph recorder with the use of a force transducer (Model 797159-2, Gould Inc.) and recorded simultaneously with fura-2 fluorescence. Oxygenated PSS (37°C) was perfused into the organ bath (5 ml) which was thermostatically controlled at 37°C. The thread connecting the coronary ring to the transducer made an angle of 37° (θ) relative to the horizontal position of the tissue by passing the thread around a small pulley positioned in the bath.

797159-2, Gould Inc., Cleveland, OH, U.S.A.) and the other end connected to a tissue bath (Figure 1). The thread connecting the transducer to the tissue was at an angle of 37° with respect to the horizontal plane of the tissue. Recorded tension was divided by cos 37° (0.8) in order to derive the tension that developed in the direction of tissue contraction.

$[Ca^{2+}]_i$ was measured with the fluorescent dye fura-2 (Grynkiewicz *et al.*, 1985). The tissue bath was part of a fluorometer (CAF-100, JASCO Calcium Analyzer, Tokyo, Japan) which measures fura-2/Ca^{2+} fluorescence by alternately exciting the tissue (frequency of 48 Hz) at 340 nm (F_{340} fluorescence) and 380 nm (F_{380} fluorescence). After allowing the coronary ring to equilibrate for 1 h, it was contracted twice with PSS containing 72 mM KCl (iso-osmotic substitution for NaCl). The porcine coronary tissue was loaded then with fura-2AM by incubating in 10 μM fura-2AM for 2 h while under tension. The fura-2AM solution was made by mixing 40 μl of 2.5 mM fura-2AM (Molecular Probes, Eugene, OR, U.S.A.) with 40 μl Cremophor EL (Sigma Chemical Co., St. Louis, MO, U.S.A.), combining this with 10 ml of oxygenated HEPES-PSS (10 mM HEPES substituted for $NaHCO_3$) containing 5% bovine serum albumin and finally sonicating for 5 × 5 s. Half of the loading solution was added to the tissue bath and the other half was kept oxygenated. These solutions were interchanged every 30 min in order to keep the tissue oxygenated during loading. After loading, the tissue was washed free of extracellular dye by perfusing the tissue bath with oxygenated PSS until the F_{340} and F_{380} fluorescence values were stable. Tension and fluorescence emission (510 nm) from excitation at 340 nm (F_{340}) and 380 nm (F_{380}), as well as the F_{340}/F_{380} ratio, were recorded simultaneously with a model 79E polygraph (Grass Instruments Co., Quincy, MA, U.S.A.).

Relative changes of $[Ca^{2+}]_i$ were determined by measuring the F_{340}/F_{380} fluorescence ratio of the fura-2-Ca^{2+} complex (Sato *et al.*, 1988; Hisayama *et al.*, 1990). Fura-2 fluorescence was quenched by adding 1 mM $MnCl_2$ to the tissue in the presence of 10 μM ionomycin for the measurement of autofluorescence. Autofluorescence was subtracted from all fluorescence values before determination of the F_{340}/F_{380} ratio.

Since absolute values of $[Ca^{2+}]_i$ were not calculated, a comparison of the changes in the fura-2 F_{340}/F_{380} ratio between tissues necessitates the use of a standard agent. Fluorescence changes are expressed as a percentage of that seen with the standard agent. In the comparison of the fluorescence changes seen in the porcine coronary artery wtih $PGF_{2\alpha}$ and KCl, the changes in the F_{340}/F_{380} ratio and tension seen with these two contractile agonists were expressed as a percentage of that obtained with 70 mM KCl. Experimental values are given as mean ± s.e.mean.

The following chemicals and drugs were used: sodium nitroprusside (SNP) (Fisher Scientific Co., Fairlawn, NJ, U.S.A.), prostaglandin $F_{2\alpha}$ (Cayman Chemical Co., Ann Arbor, MI, U.S.A.), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (Boehringer Mannheim, Indianapolis, IN.), Cremophor EL (Sigma Chemical Co., St. Louis, MO, U.S.A.) and cromakalim (BRL 34915), supplied by Dr Paul Wenk, Ciba-Geigy Ltd., Basle, Switzerland. All other reagents used were of analytical grade.

Results

Changes in tension and $[Ca^{2+}]_i$ of the pig coronary artery induced by KCl depolarization and prostaglandin $F_{2\alpha}$

The fluorescence changes measured at 510 nm due to excitation at 340 nm (F_{340}) and 380 nm (F_{380}) during contraction of the pig coronary artery that was not loaded with fura-2 were negligible. Occasionally, increases in both F_{340} and F_{380} were seen during contraction of the unloaded muscle; however, the percentage increases at both excitation wavelengths were similar resulting in no change of the F_{340}/F_{380} ratio (data not shown). The F_{340} and F_{380} fluorescence increased ~7–10 fold after loading.

Similar contractions of the porcine coronary artery were obtained in the presence of 25 mM KCl or 10 μM $PGF_{2\alpha}$; however, these two contractile agents caused different changes in the fura-2 F_{340}/F_{380} ratio that occurred simultaneously with the increases in tension (Figure 2). The contraction induced by 25 mM KCl occurred simultaneously with an increase in F_{340}, a decrease in F_{380} and consequently an increase in the F_{340}/F_{380} ratio from 0.44 to 0.59 (Figure 2). A similar contraction of the same muscle induced by 10 μM $PGF_{2\alpha}$ coincided with a smaller increase in F_{340}, a smaller decrease in F_{380}, and less of an increase in the F_{340}/F_{380} ratio when compared to the changes caused by 25 mM KCl. The $PGF_{2\alpha}$-mediated increase of the F_{340}/F_{380} ratio was from 0.45 to 0.51. These results suggested a different $[Ca^{2+}]_i$-tension relationship existing for contractions mediated by KCl vs those mediated by $PGF_{2\alpha}$.

In order to determine whether this was the case, the $[Ca^{2+}]_i$-tension relationship was determined for KCl and $PGF_{2\alpha}$ by measuring simultaneous increases of the fura-2 F_{340}/F_{380} ratio and tension during cumulative increases of each of these agonists. Cumulative additions of $PGF_{2\alpha}$ (2–10 μM) caused corresponding increases in the F_{340}/F_{380} ratio and tension. The increases in the ratio were due to an increase in F_{340} and a decrease in F_{380} (Figure 3). The ratio increased from a value of 0.44 at the start of the addition of $PGF_{2\alpha}$ to 0.60 when the $PGF_{2\alpha}$ concentration was 10 μM.

Figure 4 shows the simultaneous changes in F_{340}, F_{380}, F_{340}/F_{380} ratio and tension of the fura-2 loaded coronary artery during stimulation with cumulative increasing doses of

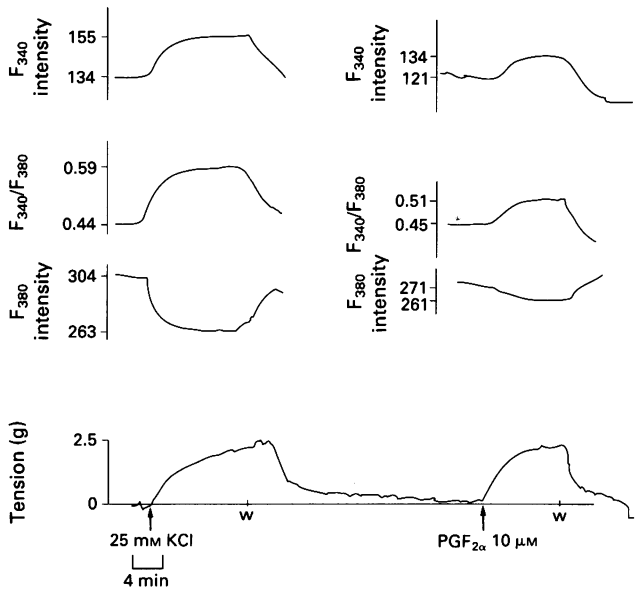

Figure 2 Representative simultaneous recordings of fluorescence (510 nm) and tension development induced by 25 mM KCl and 10 μM prostaglandin $F_{2\alpha}$ in the fura-2 loaded pig coronary arterial ring. The first and third traces from the top show changes in fluorescence intensities obtained at 340 nm (F_{340}) and 380 nm (F_{380}) excitation. The second trace shows changes in the fluorescence ratio, F_{340}/F_{380}, and the bottom trace shows force development. These results are representative of three experiments which were performed.

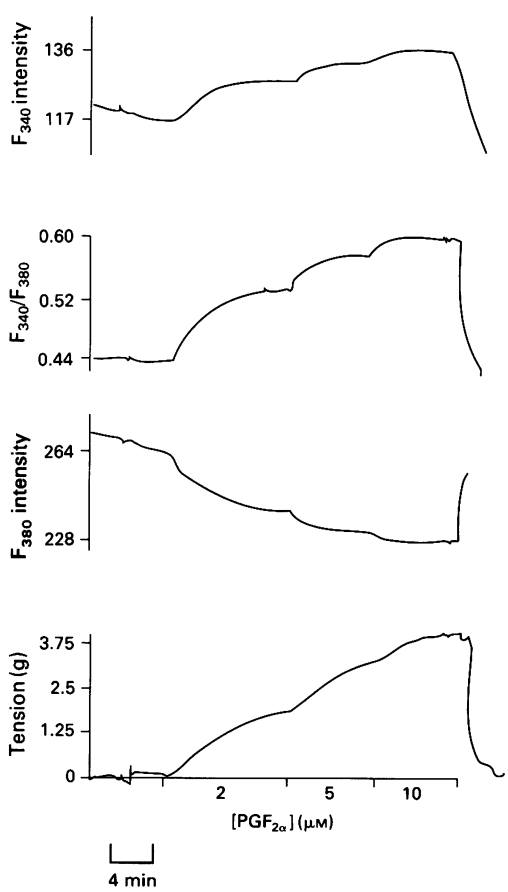

Figure 3 Representative simultaneous recordings of fluorescence (510 nm) and tension development induced by cumulative additions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the fura-2 loaded porcine coronary arterial ring. The traces show changes in fluorescence intensities obtained at 340 nm (F_{340}) and 380 nm (F_{380}) excitation and the F_{340}/F_{380} ratio. These results are representative of five experiments which were performed.

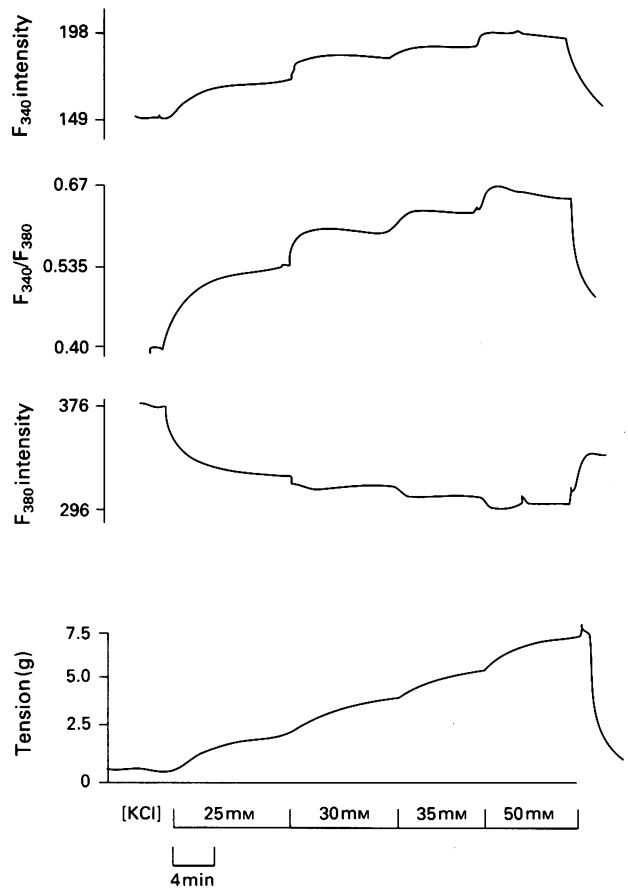

Figure 4 Representative simultaneous recordings of fluorescence (510 nm) and tension development induced by cumulative additions of KCl in the fura-2 loaded porcine coronary arterial ring. The traces show changes in fluorescence intensities obtained at 340 nm (F_{340}) and 380 nm (F_{380}) excitation and the F_{340}/F_{380} ratio. These results are representative of five experiments which were performed.

KCl. With the highest concentration of KCl (50 mM) the F_{340}/F_{380} ratio was 0.67, an increase of 67.5% from the value when the tissue was at resting tension (0.40).

The $PGF_{2\alpha}$ and KCl dose-dependent changes in muscle tension and the fura-2 F_{340}/F_{380} ratio that was seen in five experiments are summarized in Table 1. The changes in the F_{340}/F_{380} ratio and tension obtained with $PGF_{2\alpha}$ and KCl

Table 1 Changes in tension and $[Ca^{2+}]_i$ of the porcine coronary artery induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and KCl

[$PGF_{2\alpha}$] (μM)	*Increase* F_{340}/F_{380}	*Contraction* (%)
2	0.061 ± 0.008	27 ± 2.5
5	0.097 ± 0.011	57 ± 2.0
10	0.123 ± 0.012	80 ± 2.0
30	0.138 ± 0.022	98 ± 1.5
[*KCl*] (mM)	***Increase*** F_{340}/F_{380}	***Contraction*** (%)
25	0.144 ± 0.012	19 ± 4.2
30	0.195 ± 0.015	36 ± 3.3
35	0.245 ± 0.015	59 ± 2.0
50	0.293 ± 0.017	89 ± 2.5
70	0.298 ± 0.017	100

Contractions are expressed as a percentage of the maximum tension obtained with each contractile agent. Values are the mean ± s.e.mean from five experiments.

were expressed as a percentage of the changes seen in these tissues with 70 mM KCl. Expressed in this manner, the changes in the F_{340}/F_{380} ratio were plotted against the corresponding changes in tension. The plot for the $PGF_{2\alpha}$-mediated responses was found to lie to the left of that for KCl (Figure 5).

Effect of sodium nitroprusside on KCl and $PGF_{2\alpha}$-induced changes in mechanical activity and $[Ca^{2+}]_i$ of the porcine coronary artery

Figure 6a shows the simultaneous changes in tension and the F_{340}/F_{380} ratio of the fura-2 loaded porcine coronary artery contracted with 35 mM KCl and subsequently relaxed with sodium nitroprusside (SNP). Contraction occurred simultaneously with increases in $[Ca^{2+}]_i$. In three experiments, the average (±s.e.mean) increase in the fura-2 F_{340}/F_{380} ratio and tension caused by 35 mM KCl was 0.24 ± 0.01 (from 0.40 to 0.64) and 7.5 ± 0.7 g, respectively. In Figure 6a, the cumulative addition of SNP after the artery had reached peak tension with KCl caused up to 90% relaxation, but only a 24% decrease in $[Ca^{2+}]_i$, the F_{340}/F_{380} ratio decreasing from 0.62 to 0.56. The changes in tension and the F_{340}/F_{380} ratio caused by SNP on the KCl-precontracted porcine coronary artery from three experiments are summarized in Table 2.

Figure 7 shows the correlation between the average decreases in the fura-2 F_{340}/F_{380} ratio and the decreases in tension of the KCl-contracted porcine coronary artery caused by the addition of increasing concentrations of SNP. The effects of each of the concentrations of vasodilator used are expressed as a percentage change of the increase in tension and F_{340}/F_{380} ratio caused by 35 mM KCl. The rather steep linear relationship between these two parameters indicates that the percentage relaxation correlated with a much smaller percentage change in $[Ca^{2+}]_i$.

When 5 μM $PGF_{2\alpha}$ was the contractile agent, the average (±s.e.mean) increase in the fura-2 F_{340}/F_{380} ratio and tension from three experiments was 0.13 ± 0.01 (from 0.43 to 0.56) and 7.8 ± 2.0 g, respectively. The cumulative addition of SNP caused decreases in the fura-2 F_{340}/F_{380} ratio which correlated much more closely with the extent of relaxation than

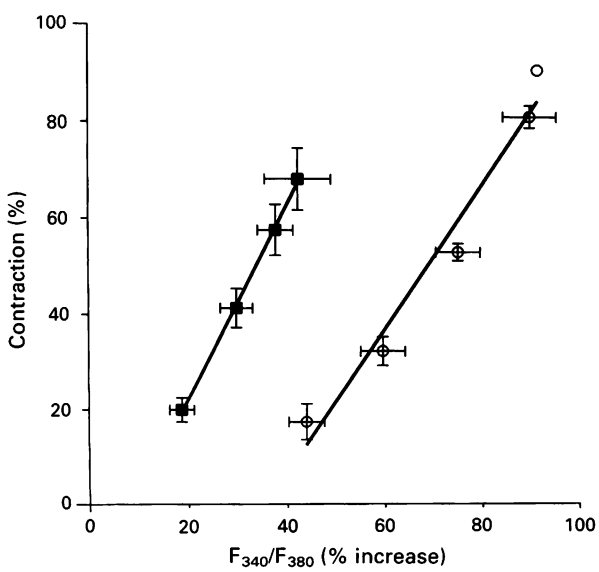

Figure 5 Relationship of KCl (○)- and prostaglandin $F_{2\alpha}$ (■)-stimulated increases in F_{340}/F_{380} ratio and tension in fura-2 loaded porcine coronary arterial rings. The changes in tension and F_{340}/F_{380} ratio that were seen with each contractile agent were expressed as a percentage of the changes that were seen with 70 mM KCl in the individual tissues. Data are the average of five experiments (±s.e.mean) which were similar to those described in Figures 3 and 4.

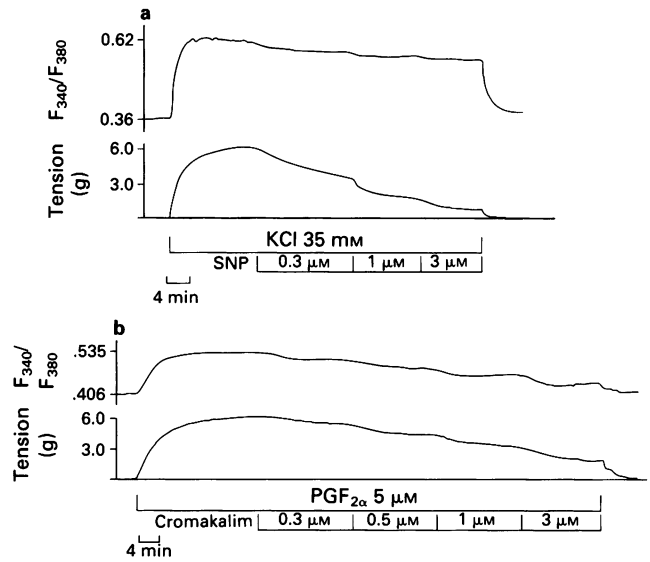

Figure 6 Simultaneous recording of F_{340}/F_{380} ratio and force development from the fura-2 loaded porcine coronary artery: (a) during contraction by 35 mM KCl and subsequent relaxation with increasing concentrations of sodium nitroprusside (SNP); (b) during contraction with 5 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and subsequent relaxation with increasing concentrations of cromakalim. These results are representative of three experiments which were performed.

was seen when KCl was the contracting agent. The changes in tension and the F_{340}/F_{380} ratio caused by SNP on the $PGF_{2\alpha}$-precontracted coronary artery from three experiments are summarized in Table 2.

Figure 7 shows an almost 1:1 correlation between the changes in the F_{340}/F_{380} ratio and tension caused by the cumulative addition of SNP. The effects of each concentration

Table 2 Effect of sodium nitroprusside (SNP) and cromakalim on tension and $[Ca^{2+}]_i$ of $PGF_{2\alpha}$- or KCl-precontracted porcine coronary arterial rings

SNP relaxation of $PGF_{2\alpha}$-induced contraction

[*SNP*] (μM)	*Decrease* F_{340}/F_{380}	*Relaxation* (%)
0.3	0.023	19
1	0.047 ± 0.016	51 ± 5
3	0.067 ± 0.028	76 ± 4
10	0.089 ± 0.038	91 ± 4

Cromakalim relaxation of $PGF_{2\alpha}$-induced contraction

[*Cromakalim*] (μM)	*Decrease* F_{340}/F_{380}	*Relaxation* (%)
0.3	0.024 ± 0.004	8.8 ± 1.2
1.0	0.063 ± 0.009	45 ± 0.5
3.0	0.102 ± 0.006	73 ± 0.4

SNP relaxation of KCl-induced contraction

[*SNP*] (μM)	*Decrease* F_{340}/F_{380}	*Relaxation* (%)
0.3	0.026 ± 0.004	36 ± 4.6
1	0.041 ± 0.003	65 ± 4.5
3	0.052 ± 0.006	86 ± 2.5
10	0.060	94

The amount of relaxation seen with each concentration of SNP or cromakalim is expressed as a percentage of the agonist-induced increase in tension. Values are the mean ± s.e.mean from three experiments.

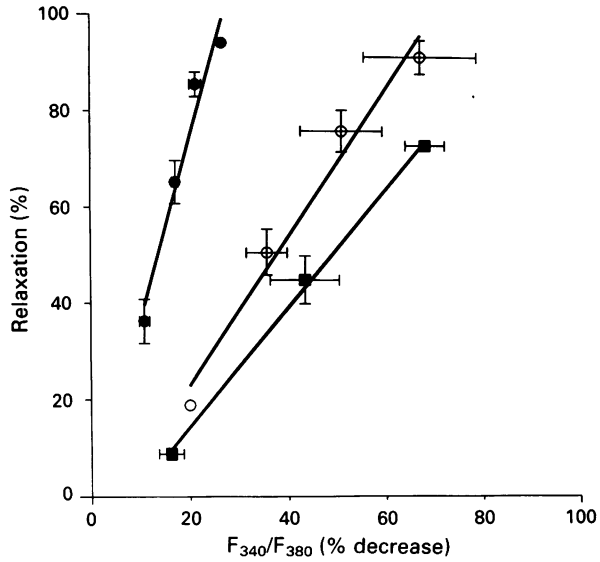

Figure 7 The relationship of the changes in the F_{340}/F_{380} ratio and tension of the pre-contracted fura-2 loaded porcine coronary artery caused by sodium nitroprusside (SNP) or cromakalim. The coronary artery was precontracted with either prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or 35 mM KCl. (●) Relaxation of the 35 mM KCl- or (○) 5 μM $PGF_{2\alpha}$-precontracted artery by cumulative additions of SNP. (■) Relaxation of the 5 μM $PGF_{2\alpha}$-precontracted artery by cumulative additions of cromakalim. The decrease in tension and F_{340}/F_{380} ratio is expressed as the percentage of the agonist-induced increases in tension and F_{340}/F_{380}. The data represent the average of three experiments.

of SNP are expressed as a percentage change of the increase in tension and F_{340}/F_{380} ratio caused by 5 μM $PGF_{2\alpha}$.

Effect of cromakalim on prostaglandin $F_{2\alpha}$-induced changes in mechanical activity and $[Ca^{2+}]_i$ in the porcine coronary artery

Figure 6b shows the simultaneous changes in the F_{340}/F_{380} ratio and tension of the fura-2 loaded porcine coronary artery during contraction by 5 μM $PGF_{2\alpha}$ and relaxation with the potassium channel opener, cromakalim. Contraction occurred with a concomitant increase in the F_{340}/F_{380} ratio from 0.406 to 0.534. The average (±s.e.mean) increase in the F_{340}/F_{380} ratio and tension in three experiments was 0.16 ± 0.01 (from 0.39 to 0.55) and 7.0 ± 0.7 g, respectively. The cumulative addition of cromakalim almost completely reversed both the increase in tension and the increase in the F_{340}/F_{380} ratio caused by $PGF_{2\alpha}$. The F_{340}/F_{380} ratio decreased to 0.44 with the highest concentration of cromakalim, 3 μM. This meant that 77% of the increase in $[Ca^{2+}]_i$ caused by $PGF_{2\alpha}$ was reversed by cromakalim. The changes in tension and the F_{340}/F_{380} ratio caused by cromakalim on the $PGF_{2\alpha}$-precontracted coronary artery from three experiments are summarized in Table 2.

Figure 7 shows an almost 1:1 correlation between the changes in the F_{340}/F_{380} ratio and tension caused by cromakalim. The effect of cromakalim is expressed as a percentage change of the increase in tension and F_{340}/F_{380} ratio caused by 5 μM $PGF_{2\alpha}$.

Discussion

This paper shows that coincident with contraction of the porcine coronary artery induced by KCl and $PGF_{2\alpha}$ there is an increase in $[Ca^{2+}]_i$ as measured by fura-2 fluorescence. Contractions obtained immediately after dye loading were similar to those obtained before loading, indicating no evidence of an alteration of the contractility of the preparation due to presence of the dye. Others who have used fura-2 fluorescence to show that contraction of smooth muscle correlates with increases in intracellular Ca^{2+} include Sato *et al.* (1988) and Bruschi *et al.* (1988). This correlation is a predictable confirmation of the obligatory role that Ca^{2+} plays in muscle contraction. However, there have been reports of an absence of this correlation in coronary artery preparations. Rooke *et al.* (1984) and Bradley & Morgan (1987) did not find intracellular Ca^{2+} to increase during a $PGF_{2\alpha}$-mediated contraction of the canine and porcine coronary, respectively. The reasons for the discrepancy between these reports and the present findings can be many. Rooke *et al.* (1984) did not actually use any techniques to measure $[Ca^{2+}]_i$ but rather measured the dependence of a contraction by $PGF_{2\alpha}$ on intra- and extracellular Ca^{2+} while using a depolarized preparation. Bradley & Morgan (1987) used the luminescent protein, aequorin, to estimate changes in $[Ca^{2+}]_i$ caused by $PGF_{2\alpha}$. The methods used in each of these studies may not have been sensitive enough to detect the small $PGF_{2\alpha}$-mediated changes in $[Ca^{2+}]_i$ seen in the present study.

It was also shown that similarly-sized $PGF_{2\alpha}$- and KCl-mediated contractions corresponded to different levels of intracellular Ca^{2+}. Contractions to $PGF_{2\alpha}$, equivalent to those produced by KCl occurred in the presence of a lower $[Ca^{2+}]_i$. A similar observation has been made by a number of investigators using other agonists in comparison with KCl. In the rat aorta, Karaki *et al.* (1988) and Bruschi *et al.* (1988) noted a disparity in the $[Ca^{2+}]_i$-tension relationship for noradrenaline and KCl, where noradrenaline was shown to cause contractions of the aorta, but at a lower $[Ca^{2+}]_i$ than seen with KCl. Gerthoffer *et al.* (1989) and Rembold & Murphy (1988) measured the ability of contractile agonists to increase $[Ca^{2+}]_i$ and myosin phosphorylation in smooth muscle and found that contraction and phosphorylation caused by agonists occurred at a lower $[Ca^{2+}]_i$ than with KCl. Gerthoffer *et al.* (1989) explained this result by suggesting an agonist-induced change in Ca^{2+} sensitivity of the contractile proteins. Rembold & Murphy (1988) speculated that ionic changes occurring during depolarization or an agonist-specific modulation of myosin phosphorylation, possibly through the action of protein kinase C, could explain the variant actions of KCl and contractile agonists.

This varying relationship between $[Ca^{2+}]_i$ and force in smooth muscle was further exemplified by the effects seen with SNP. The increases in contractile force of the porcine coronary artery caused by KCl could be largely reversed by SNP; however, this relaxant agent had smaller effects on the $[Ca^{2+}]_i$. The nearly complete relaxation by SNP occurred with only a small decrease in $[Ca^{2+}]_i$. The changes in tension and $[Ca^{2+}]_i$ were more closely correlated when $PGF_{2\alpha}$ was the contractile agonist. Yanagisawa *et al.* (1989) found that nitroglycerin, an agent which has a similar mechanism of action to that of SNP, caused relaxation of the KCl contracted canine coronary without any change in $[Ca^{2+}]_i$, while Karaki *et al.* (1988) reported that nitroprusside caused relaxation of a KCl-induced contraction of the rat aorta that was accompanied by a small decrease in $[Ca^{2+}]_i$ and in $^{45}Ca^{2+}$ uptake. Rashatwar *et al.* (1987) found that 8-bromo-cyclic GMP, a membrane permeable form of cyclic GMP which is believed to mediate the effects of SNP and nitroglycerin, lowers $[Ca^{2+}]_i$ in vascular smooth muscle cells. However, our data and that of others suggest that a significant part of the relaxation by SNP could also be via a mechanism which is independent of a change in $[Ca^{2+}]_i$. Itoh *et al.* (1985) and Pfitzer *et al.* (1984) showed that cyclic GMP can inhibit contraction at an intermediate $[Ca^{2+}]_i$ and that this may be due to a reduction in myosin light chain phosphorylation (Rapoport *et al.*, 1982).

In contrast to this, relaxation with cromakalim correlated quite well with decreases in $[Ca^{2+}]_i$. Cromakalim is a K^+ channel opener which is known to cause membrane hyperpolarization (Cook, 1988). Some of the contractile mechanisms

that may have been affected by the membrane hyperpolarizing activity of K^+ channel openers include dihydropyridine-insensitive voltage-sensitive Ca^{2+} channels, electrogenic ion transport systems (Na^+-Ca^{2+} exchange) and membrane bound Ca^{2+} release (Cook *et al.*, 1988). Our data do not help to clarify which of these mechanisms is responsible for mediating their effects. Further work must be done to elucidate the relationship between the enhanced permeability of K^+ channels by cromakalim and its effects on decreasing $[Ca^{2+}]_i$. In summary, the relationship of $[Ca^{2+}]_i$ and tension varies, depending on the contractile agent which is used. Vasodilator agents can cause relaxation by decreasing $[Ca^{2+}]_i$ (cromakalim) or by mechanisms which are independent of $[Ca^{2+}]_i$ (SNP).

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Inhibition of the tocolytic activity of atrial natriuretic factor by progesterone and potentiation by progesterone receptor antagonist RU486 in rats

W. Potvin, *S. Mulay & [1]D.R. Varma

Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada H3G 1Y6 and * Departments of Physiology and Medicine, McGill University and Endocrine Laboratory, Royal Victoria Hospital, Montreal, Canada

1 The influence of progesterone on the activity of atrial natriuretic factor (ANF) on rat myometrial motor activity was determined *in vitro*.

2 ANF inhibited the tension development by myometrium from cycling or oestrogen-treated rats in a dose-dependent manner; maximal inhibition was 100%.

3 Injections of progesterone into rats inhibited the tocolytic activity of ANF in a dose- and time-dependent manner. The tocolytic effects of ANF were completely abolished by 3 daily injections of $1\,mg\,kg^{-1}$ progesterone.

4 Pregnancy-related increase in plasma progesterone was accompanied by a corresponding decrease in the tocolytic effects of ANF; myometria from gestational day 10 to 21 were completely refractory and those from earlier gestational age and immediate postpartum were responsive to ANF to varying degrees.

5 Treatment of pregnant rats with the progesterone antagonist, RU486, caused abortions and vaginal bleeding, decreased plasma progesterone concentrations and restored the tocolytic activity of ANF. Tocolytic activity of ANF on virgin rat myometria was potentiated by RU486.

6 Progesterone also inhibited the effects of ANF on myometria from ovariectomzed rats.

7 Tocolytic activity of isoprenaline was not modified by progesterone, pregnancy, RU486 or ovariectomy.

8 It is concluded that progesterone antagonizes myometrial effects of ANF by an oestrogen-independent mechanism and the pregnancy-induced refractoriness to the tocolytic effects of ANF is caused by progesterone.

Keywords: Progesterone; atrial natriuretic factor; RU486; plasma progesterone in pregnancy; tocolytic activity of atrial natriuretic factor; progesterone-atrial natriuretic factor interactions; ovariectomy

Introduction

Since the demonstration of the natriuretic and hypotensive activities of atrial extracts (deBold *et al.*, 1981), atrial natriuretic factor (ANF) has been shown to produce various biological effects (Brenner *et al.*, 1990). Homeostasis of ANF during pregnancy and its effects on female organs have also been investigated. ANF inhibits myometrial motor activity (Bek *et al.*, 1988; Potvin & Varma, 1990) and oocyte maturation (Tornell *et al.*, 1990); it increases placental blood flow (Chemtob *et al.*, 1989) and does not cross the placenta (Mulay & Varma, 1989). Receptors for ANF are present in placenta blood vessels (McQueen *et al.*, 1990) and tissues (Sen, 1986; Hatjis & Grogan, 1988; 1989), rat mammary glands (Pelletier, 1988) and myometrium (Potvin & Varma, 1991). Plasma concentrations of ANF increase during pregnancy and parturition in man (Otsuki *et al.*, 1987; Rutherford *et al.*, 1987; Steegers *et al.*, 1987; Thomsen *et al.*, 1987; Elias *et al.*, 1988; Hatjis *et al.*, 1989; Miyamoto *et al.*, 1989) and animals (Cheung *et al.*, 1987; Castro *et al.*, 1989; Olsson *et al.*, 1989). Vascular (St-Louis *et al.*, 1988; Chemtob *et al.*, 1989) and renal (Kristensen *et al.*, 1986) effects of ANF and placental vascular ANF receptors (McQueen *et al.*, 1990) are modified by pregnancy and pregnancy hypertension.

We recently showed that ANF inhibited the motor activity and increased guanylate cyclase activity and guanosine 3′:5′-cyclic monophosphate levels of myometria from virgin rats; these effects of ANF were abolished or greatly attenuated by progesterone treatment and pregnancy (Potvin & Varma, 1990). On the basis of these results, we suggested that pregnancy-induced refractoriness to the tocolytic effects of ANF might be caused by an increase in endogenous progesterone levels. The purpose of this study was to examine in greater detail the possible causal relationship between plasma progesterone during pregnancy and the inhibition of the tocolytic effects of ANF.

[1] Author for correspondence.

Methods

Animals and treatments

Rats were used in this study following the approval of the protocol by the McGill University Animal Care Committee.

Adult virgin Sprague-Dawley female rats (220–225 g) were purchased from Charles River, St. Constant, Quebec, Canada and maintained on Purina rat diet and tap water *ad libitum*. Animals were housed at 22–25°C, 50–70% humidity and a 12 h light (07 h 00 min–19 h 00 min) and 12 h dark (19 h 00 min–07 h 00 min) schedule. Presence of sperms in vaginal washing after overnight cohabitation with males denoted day zero of pregnancy. Bilateral ovariectomy was performed two weeks before experiments under ether anaesthesia. The stage of oestrous cycle was determined by microscopic examination of vaginal smears. Oestrogen (17β-oestradiol) and progesterone were dissolved in peanut oil and injected intraperitoneally. The dose of oestrogen was $1\,mg\,kg^{-1}\,day^{-1}$ for 2 days and similar to that used previously by us (Potvin & Varma, 1990) and others (Bek *et al.*, 1988). Doses of progesterone were 0.25, 0.5, 1.0 and $2.0\,mg\,kg^{-1}\,day^{-1}$ for 3 days or $2\,mg\,kg^{-1}$ for 1, 2 or 3 days. Control rats were treated with peanut oil. A group of virgin and 17-day pregnant rats were treated orally once a day for two days with $10\,mg\,kg^{-1}$ RU486 suspended in 10% carboxymethyl cellulose (volume $1\,ml\,kg^{-1}$); controls for this

group of studies were treated with 10% carboxymethyl cellulose. Drugs were administered between 09 h 00 min–10 h 00 min and myometrial activity determined 24 h after the last injection.

Determination of tocolytic activity in vitro

Rats were decapitated and uterine horns removed and set up in 50 ml tissue baths for recording isometric myometrial contractions on a Grass polygraph exactly as previously described (Potvin & Varma, 1990). Cumulative tocolytic concentration-response curves to ANF and isoprenaline were determined on separate strips from the same uterus. Myometrial tension development was quantitated by integration as described by Granger *et al.* (1985) and previously used by us (Potvin & Varma, 1990). Complete suppression of tension development was treated as 100% inhibition. Tocolytic potencies of ANF and isoprenaline were expressed as the $-\log$ molar concentration of drugs causing 50% inhibition in tension development ($-\log$ M EC_{50}) and calculated from the regression line of the probit of percent effect versus $-\log$ M drug concentration for each experiment (Granger *et al.*, 1985). In cases where the maximal tocolytic effect was less than 100% but greater than 40%, $-\log$ M EC_{50} was calculated by extrapolation. EC_{50} was not calculated if the maximal tocolytic activity of ANF was less than 40%.

Plasma progesterone assays

In order to determine plasma progesterone profile during pregnancy, 0.3 ml blood was serially collected from the tail artery under brief periods (~2 min) of ether anaesthesia (Varma & Ramakrishnan, 1985) from 5 pregnant rats on days 0, 4, 7, 10, 14, 17, 19, 20 and 21 of gestation and within 12–24 h *postpartum*. Blood was collected from carboxymethyl cellulose-treated controls and RU486-treated animals just prior to killing them for the removal of uterine tissues. Plasma was separated by centrifugation (5000 r.p.m. for 2 min).

Plasma progesterone was measured by radioimmunoassay with highly specific antibodies as previously described (Mulay *et al.*, 1982). Approximately 800 c.p.m. of the tracer was added to the plasma and then extracted with methylene chloride; the extract was dissolved in phosphate-saline buffer. An aliquot was counted to estimate recovery and the remaining extract was incubated with approximately 6000–8000 c.p.m. of the tracer and the antibody for 2 h at 4°C. Separation of bound from free hormone was by adsorption onto dextran-coated charcoal. Intra- and interassay coefficients of variance were 8.5% and 8.1%, respectively. Each assay was done in duplicate and corrected for recovery.

Drugs and chemicals

Progesterone antagonist RU486(17β-hydroxy-11β-(4-dimethylaminophenyl-1)-17α(prop-1-ynyl)estra-4,9-dien-3-one) was a gift from Roussel UCLAF, France. The following agents were purchased: synthetic rat atrial natriuretic factor (Hukabel, Montreal, Quebec, Canada), progesterone, oestrogen (17β-oestradiol) and isoprenaline (Sigma Chemical, St. Louis, Missouri, U.S.A.), antiprogesterone antibodies (Endocrine Science, Tarzana, California, U.S.A.), [^{3}H]-progesterone (100 Ci mmol^{-1}, Amersham, Oakville, Ontario, Canada), all other chemicals (BDH, Montreal, Quebec, Canada).

Statistics

Two means were compared by Student's t test; F values (variance ratios) did not differ at 5% probability. Multiple means were compared by one-way analysis of variance followed by comparison of each pair in the group (Bonferroni). A probability of less than 0.05 was assumed to denote a significant difference. Throughout this paper, means ± s.e.mean are presented.

Results

Effects of atrial natriuretic factor on myometria from cycling rats

ANF inhibited the spontaneous motor activity of the uterus from cycling rats regardless of the stage of the oestrous cycle (Figure 1). However, the tocolytic potency of ANF was significantly lower on myometria from rats in metoestrus and dioestrus than on tissues from oestrogen-treated rats or rats in oestrus and pro-oestrus (Table 1).

Effect of progesterone on the tocolytic activities of atrial natriuretic factor

Treatment with progesterone inhibited the tocolytic activity of ANF in a dose-dependent (Figure 2a) and time-dependent (Figure 2b) manner. The tocolytic effect of ANF was completely abolished by 3 daily doses of 1 mg kg^{-1} progesterone. However, ANF exerted some tocolytic effect when rats were treated with even a higher dose of progesterone (2 mg kg^{-1}) only for 1 or 2 days (Figure 2b, Table 2).

Effect of RU486 on the tocolytic effects of atrial natriuretic factor

Treatment of virgin cycling rats with RU486 significantly ($P < 0.05$) increased the tocolytic potency of ANF (Figure 3a) relative to that on myometria from vehicle-treated as well as oestrogen-treated animals (Table 3).

Administration of RU486 to 4 pregnant rats on days 17 and 18 of gestation caused expulsion of all foetuses in 2 rats and 6 of the 13 foetuses in 1 rat by day 19. The fourth rat retained all its foetuses but vaginal bleeding was noted. The mean plasma concentration of progesterone was significantly reduced relative to that on the corresponding day in vehicle-treated pregnant rats (Table 3). ANF was completely ineffective in vehicle-treated rats (Figure 3b, 4a). On the other hand, ANF caused 100% inhibition of the spontaneous motor activ-

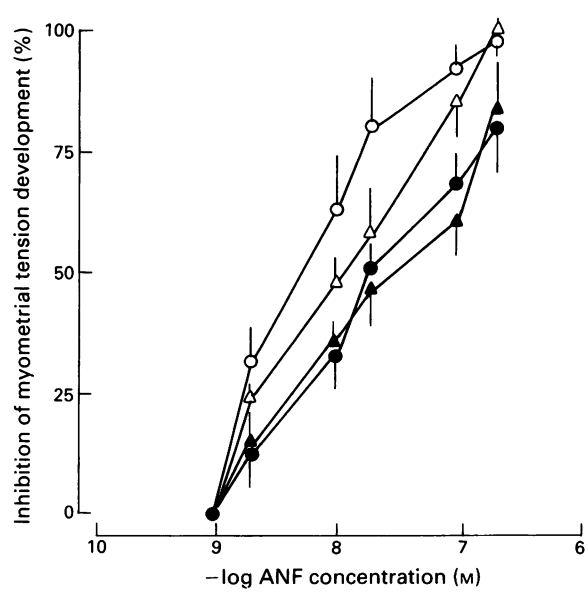

Figure 1 Tocolytic concentration-response curves to atrial natriuretic factor on myometrial strips from rats in oestrus (○), pro-oestrus (△), metoestrus (●) and dioestrus (▲). Data are means of 4 experiments; vertical lines show s.e.mean.

Table 1 Tocolytic potency and effectiveness of atrial natriuretic factor on myometrial strips from oestrogen-treated, ovariectomized and cycling rats

Animals	n	*Tocolytic* ($-\log$ M EC_{50})	*Tocolytic effectiveness* (%)
Oestrogen-treated	19	8.01 ± 0.11[a]	100[a]
Pro-oestrus	4	7.86 ± 0.24[a]	100[a]
Oestrus	4	7.98 ± 0.14[a]	100[a]
Metoestrus	4	7.46 ± 0.11	80 ± 11
Dioestrus	4	7.49 ± 0.17	83 ± 16
Ovariectomized	11	7.22 ± 0.12	50 ± 8
Ovariectomized plus progesterone-treated	4	>6	0

Oestrogen ($1\,mg\,kg^{-1}\,day^{-1} \times 2$) and progesterone ($2\,mg\,kg^{-1}\,day^{-1} \times 3$) were injected intraperitoneally and ovariectomy was performed 2 weeks before experiments. Data are means ± s.e.mean.

[a] Different ($P < 0.01$) from all values in the same column without the superscript but not from each other.

ity of myometrium from pregnant rats treated with RU486 (Figures 3b and 4b).

Effect of progesterone on the tocolytic activity of atrial natriuretic factor in ovariectomized rats

Relative to responses of myometria from cycling and oestrogen-treated virgin rats, ovariectomy caused a significant ($P < 0.05$) reduction in both the tocolytic effectiveness and potency of ANF. Treatment of ovariectomized rats with progesterone ($2\,mg\,kg^{-1} \times 3$) abolished the tocolytic activity of ANF (Table 1).

Plasma progesterone and tocolytic activity of atrial natriuretic factor

Injection of RU486 into virgin rats caused a small but insignificant increase in plasma progesterone. In contrast, administration of RU486 into pregnant rats caused a significant ($P < 0.001$) decrease in plasma progesterone (Table 3).

Plasma progesterone in pregnant rats progressively increased with gestational duration reaching a peak on day 14

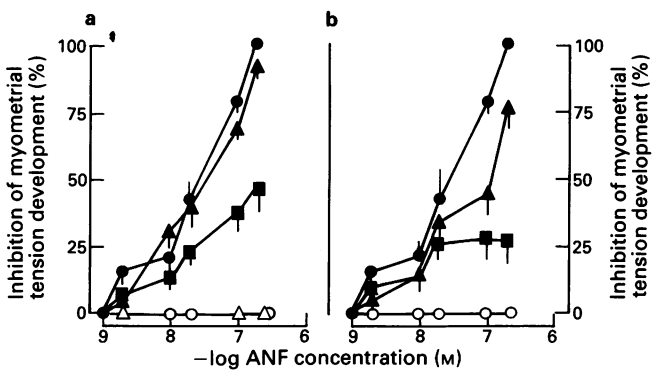

Figure 2 Tocolytic concentration-response curves to atrial natriuretic factor on myometrial strips from virgin rats treated intraperitoneally with progesterone at different dose levels (a) and time-periods (b). (a) Vehicle (peanut-oil)-treated controls (●); treated daily for 3 days with progesterone at dose levels of $0.25\,mg\,kg^{-1}$ (▲), $0.5\,mg\,kg^{-1}$ (■), $1\,mg\,kg^{-1}$ (○) and $2\,mg\,kg^{-1}$ (△). (b) Vehicle (peanut-oil)-treated controls (●); treated with $2\,mg\,kg^{-1}$ progesterone for 1 day (▲), 2 days (■) and 3 days (○). Injections were made intraperitoneally between 9 h 00 min–10 h 00 min and experiments done 24 h after the last injection. Data are means ± s.e.mean of 4–5 experiments.

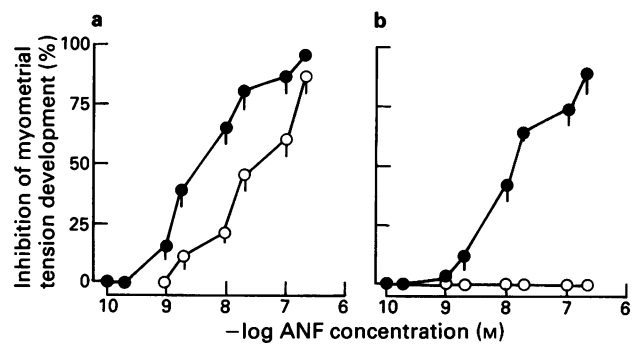

Figure 3 Effects of the antiprogesterone agent RU486 ($10\,mg\,kg^{-1}\,day^{-1} \times 2$) on the tocolytic concentration-response curves to atrial natriuretic factor on myometrial strips from (a) virgin and (b) pregnant rats. RU486 (●) and the vehicle, 10% carboxymethyl cellulose (○) were administered orally. Pregnant rats were treated on days 17 and 18 and experiments were done on day 19 of gestation. Data are means ± s.e.mean of 4 experiments; s.e.mean shown by vertical lines.

Table 2 Effects of progesterone injections into virgin rats on the tocolytic activity of atrial natriuretic factor *in vitro*

Progesterone *Dose* ($mg\,kg^{-1}$)	*Duration* (days)	*Tocolytic* ($-\log$ M EC_{50})	*Tocolytic effectiveness* (%)
0 (peanut oil)	3	7.69 ± 0.13[a]	100[a]
0.25	3	7.38 ± 0.16	91 ± 3
0.50	3	6.67 ± 0.11	47 ± 8
1.00	3	>6	0
2.00	3	>6	0
2.00	1	7.07 ± 0.18	76 ± 8
2.00	2	>6	22 ± 9
2.00	3	>6	0

Progesterone was dissolved in peanut oil and injected intraperitoneally between 9 h 00 min–10 h 00 min and experiments done 24 h after the last injection.

Data are means ± s.e.mean of 4 experiments.

[a] Different ($P < 0.01$) from all other values in the same column.

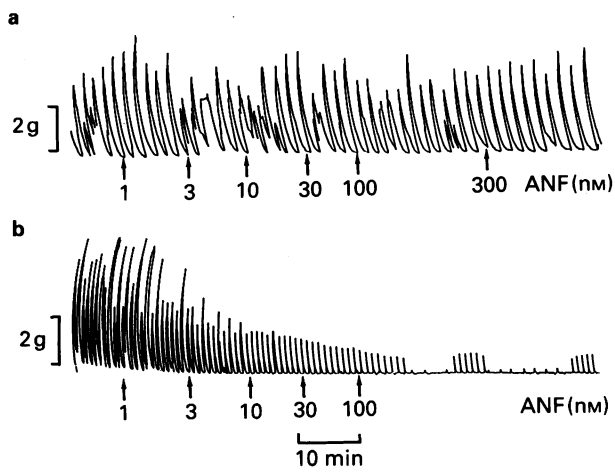

Figure 4 An example of the effect of atrial natriuretic factor (ANF) on the spontaneous motor activity of myometrial strips from (a) vehicle (10% carboxymethyl cellulose)-treated and (b) anti-progesterone agent RU486-treated pregnant rats. Vehicle or RU486 were administered orally on days 17 and 18 and the experiment done on day 19 of gestation. Concentrations of ANF (nM) are indicated at the arrows.

and decreased to below day 0 levels within 24 h *postpartum* (Figure 5). The increase in plasma progesterone was associated with a progressive decrease in the tocolytic activity of ANF (Figure 5). Myometria from day 10 to day 21 pregnant rats were totally refractory to ANF. A significant tocolytic activity of ANF was observed on *postpartum* day 1. By *postpartum* day 2, the tocolytic effect of ANF was comparable to that found in cycling virgin rats (Figure 5).

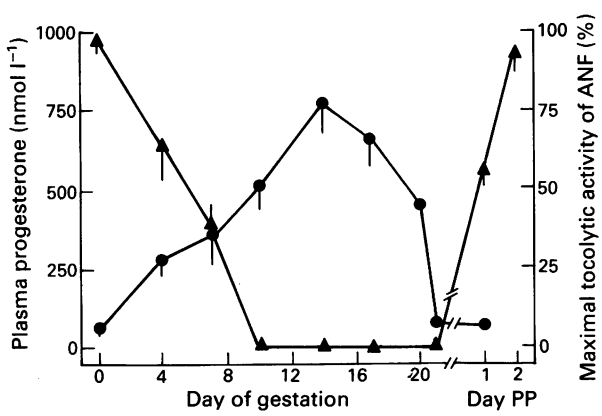

Figure 5 Relationship between plasma progesterone concentrations (●) and the tocolytic efficacy of atrial natriuretic factor, (ANF, ▲) in pregnant rats. Blood was collected serially from the same 5 rats on different days of gestation and 12–24 h postpartum (PP) for the plasma progesterone assays. Tocolytic concentration-response curves to ANF were determined on myometrial strips of 4–5 rats killed on different days of gestation and on days 1 and 2 postpartum (PP). Vertical lines show s.e.mean.

Tocolytic activity of isoprenaline

The tocolytic potency ($-\log$ M EC_{50}) and effectiveness (%) of isoprenaline on myometria from oestrogen-treated rats ($n = 13$) was 9.58 ± 0.11 and 100%, respectively, and was not significantly altered by the stage of oestrous cycle, pregnancy, ovariectomy, progesterone treatment and RU486 treatment (data not shown).

Discussion

In an earlier paper, we had suggested that the pregnancy-induced refractoriness to the tocolytic activity of atrial natriuretic factor (ANF) is mediated by progesterone (Potvin & Varma, 1990). The main objective of the present study was to test this hypothesis by examining the relationship between plasma progesterone and the tocolytic activity of ANF. We also argued that if our hypothesis is correct, progesterone antagonist RU 486 (Baulieu, 1989) should restore the tocolytic effects of ANF on myometrium from pregnant rats. The data of this study confirm all related observations described earlier (Potvin & Varma, 1990) and provide strong support for our hypothesis. On the other hand, the uterus is exposed to varying concentrations of oestrogen and progesterone under different physiological states in man (Danforth & Scott, 1986) and rats (Shaikh, 1971; Morishige *et al.*, 1973; Butcher *et al.*, 1974). Oestrogen and progesterone exert antagonistic effects on myometrial activity (Downing *et al.*, 1981) and on the tocolytic activity of ANF (Potvin & Varma, 1990). Thus endogenous oestrogen may mask by varying degree depending upon its plasma titre the influence of progesterone on the tocolytic activity of ANF. If so, progesterone dominance would be required for a full expression of its influence on the myometrial activity of ANF.

The sensitivity of the myometrium to ANF was greater during pro-oestrus and oestrus than during metoestrus and dioestrus (Table 1). Although oestrogen levels are highest during pro-oestrus (Shaikh, 1971; Butcher *et al.*, 1974), the highest progesterone levels are achieved just before oestrus (Butcher *et al.*, 1974). One would have expected a decreased response of the myometrium from rats in oestrus to ANF; the opposite, however, was the case. It is logical to assume that there is a lag period between the exposure of the uterus to these steroids and their influence on the tocolytic activity of ANF; indeed, this is suggested by our data that a single injection of a rather high dose of progesterone ($2\,mg\,kg^{-1}$) caused far less inhibition of the effects of ANF than did a smaller dose of $1\,mg\,kg^{-1}$ administered for three consecutive days (Table 2).

Notwithstanding the above, results of this study clearly show that an increase in plasma progesterone by exogenous administration (Figure 2) or increased endogenous production

Table 3 Effect of antiprogesterone agent RU486 on plasma progesterone levels and the tocolytic activity of atrial natriuretic factor on myometrial strips from virgin and day 19 pregnant rats

Animals	*Treatment*	*Plasma progesterone* ($nmol\,l^{-1}$)	*Tocolytic* ($-\log$ M EC_{50})	*Tocolytic effectiveness* (%)
Virgin	Oestrogen	174 ± 77	8.01 ± 0.09	100
Virgin	Vehicle	64 ± 13	7.61 ± 0.15	100
Virgin	RU486	204 ± 96	8.29 ± 0.10[a]	100
Pregnant	Vehicle	456 ± 66	>6	0
Pregnant	RU486	71 ± 30[a]	7.79 ± 0.15[a]	100

Vehicle ($1\,ml\,kg^{-1}$ of 10% carboxymethyl cellulose) and RU486 ($10\,mg\,kg^{-1}$) were administered orally once a day for 2 days, on days 17 and 18 of gestation in the case of pregnant rats and experiments were done 24 h after the second treatment. Oestrogen was administered at a dose of $1\,mg\,kg^{-1}\,day^{-1}$ for 2 days.

Data are means ± s.e.mean of 4–5 experiments except for the tocolytic activity in oestrogen-treated group, which is derived from 19 experiments.

[a] Different ($P < 0.01$) from the immediate top value.

such as during pregnancy (Figure 5), results in a marked decrease in the tocolytic activity of ANF. The profile of plasma progesterone as determined by serial blood collections from the same animals from day 0 to postpartum period are very similar to values described by other workers (Morishige *et al.*, 1973) and the increase in plasma progesterone is temporally related to a decrease in the tocolytic effects of ANF. Likewise a rapid decrease in plasma progesterone just preceding the parturition corresponds with a recovery of the tocolytic activity of ANF in the *postpartum* period.

A more direct evidence that pregnancy-induced refractoriness to the tocolytic activity of ANF is caused by progesterone comes from studies using the progesterone receptor antagonist RU486 (Baulieu, 1989). Thus the myometrium totally refractory to ANF on day 19 of gestation was fully responsive to it after the administration of RU486. Although the administration of RU486 caused a decrease in plasma progesterone due to obvious or impending abortion, the restoration of the tocolytic effects of ANF appears to be due to a blockade of progesterone receptors by RU486 rather than a consequence of abortion. This inference is supported by two observations. Firstly, the myometrium of one rat which had not aborted was responsive to ANF and second, RU486 potentiated the tocolytic effects of ANF in virgin rats without significantly altering plasma progesterone levels.

The exact mechanism by which progesterone inhibits the tocolytic effects of ANF is not clear from our studies. Oestrogen and progesterone appear to exert antagonistic effects on myometrial activity (Downing *et al.*, 1981). It may therefore be inferred that progesterone inhibits the activity of ANF by antagonizing oestrogen. However, our results suggest that this is not an absolute requirement for progesterone-induced refractoriness to the effects of ANF since this influence of progesterone was also observed in ovariectomized rats. This observation along with data that the effects of progesterone could be abolished by RU486 suggest that the inhibition of the tocolytic effects of ANF by progesterone involves an action of the steroid on its receptors leading to induction of certain factors, possibly proteins, which cause a decrease in myometrial ANF receptors (Potvin & Varma, 1991) and in turn a decrease in the tocolytic effects of ANF.

In summary, data of the present study strongly support the hypothesis that the pregnancy-induced inhibition of the tocolytic activity of ANF results from the exposure of the uterus to relatively high concentrations of progesterone. Plasma concentrations of ANF increase during pregnancy. Whereas this increase in plasma ANF during pregnancy might be a physiological response to fluid expansion, the decrease in the tocolytic activity of ANF might be desirable for foetal development and parturition.

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Clonidine inhibits ATP-sensitive K^+ channels in mouse pancreatic β-cells

[1]*T.D. Plant, †J-C. Jonas & *†J.C. Henquin

*I. Physiologisches Institut, Universität des Saarlandes, D6650 Homburg (Saar), Germany and †Unité de Diabétologie et Nutrition, University of Louvain, UCL 54.74, B 1200 Brussels, Belgium

1 The effects of clonidine and adrenaline on adenosine 5'-triphosphate (ATP)-sensitive K^+ channels were studied in pancreatic β-cells from normal mice.

2 When perifused with a medium containing 1 mM glucose, many of the ATP-sensitive K^+ channels in the β-cell membrane are open. Under these conditions, clonidine (5–100 μM) reversibly decreased ^{86}Rb efflux from the islets, whereas adrenaline was ineffective at concentrations up to 100 μM.

3 In 6 mM glucose, most of the ATP-sensitive K^+ channels in the β-cell membrane are closed. Opening these channels by diazoxide (100 μM) caused a marked acceleration of ^{86}Rb efflux from the islets, which was attenuated by 100 μM clonidine.

4 ATP-sensitive K^+ currents were measured in single β-cells by the whole cell mode of the patch-clamp technique. At concentrations above 4 μM, clonidine reversibly inhibited the ATP-sensitive K^+ current in a dose-dependent manner.

5 Voltage-sensitive K^+ currents were unaffected by 20 μM but decreased slightly by 100 μM clonidine.

6 Calcium currents, measured by the whole cell or perforated patch technique, were unaffected by clonidine at concentrations up to 100 μM.

7 It is concluded that high concentrations of the α_2-adrenoceptor agonist clonidine, but not of adrenaline, can inhibit ATP-sensitive K^+ channels in pancreatic β-cells. Other ionic channels are only slightly affected or unaffected.

Keywords: α-Adrenoceptors; clonidine; adrenaline; K^+ channels; pancreatic β-cells; insulin release

Introduction

A wide variety of chemically-unrelated substances stimulate insulin secretion by closing adenosine 5'-triphosphate (ATP)-sensitive K^+ channels in pancreatic β-cells whereas others inhibit secretion by opening these channels (Garrino *et al.*, 1989; Cook & Quast, 1990; Henquin, 1990).

We recently observed that the α-adrenoceptor blockers phentolamine (α_1, α_2) and yohimbine (α_2) can inhibit ATP-sensitive K^+ channels in β-cells (Plant & Henquin, 1990). Although this occurs at concentrations of the antagonists higher than those necessary to block the receptors, the effect is intriguing because agonists of the α_2-adrenoceptors appear to hyperpolarize the β-cell membrane by increasing its K^+ permeability (Drews *et al.*, 1990; Rorsman *et al.*, 1991). We therefore felt it of interest to evaluate the effects of two agonists of α_2-adrenoceptors, clonidine and adrenaline, on ATP-sensitive K^+ channels in mouse β-cells.

Methods

^{86}Rb efflux experiments

These experiments were carried out at 37°C with islets obtained by collagenase digestion of the pancreas of fed female NMRI mice. After isolation, the islets were loaded with ^{86}Rb (^{86}Rb used as tracer for K) for 90 min in a medium containing 15 mM glucose and supplemented with $^{86}RbCl$ (1.5 to 3 MBq ml^{-1}; sp.act. 7.4 to 18.5 TBq mol^{-1}). The Rb concentration never exceeded 0.4 mM (Garrino & Henquin, 1988). ^{86}Rb efflux was then monitored in a dynamic perifusion system (Henquin, 1978). The radioactivity lost by the islets was measured immediately by the Cerenkov radiation (Henquin, 1978) in the effluent fractions collected at 2 min intervals. From the sum of the radioactivity remaining in the islets at the end of the experiments and the accumulated effluent radioactivity, the fractional efflux rate was calculated for each period (radioactivity lost by tissue during the time interval/radioactivity present in the tissue during that time interval) and expressed as per cent per minute. The solutions used had the following ionic composition (in mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24, and were gassed with O_2/CO_2 (94:6) to maintain a pH of 7.4. They were supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany).

Electrical recordings

After isolation of the islets, islet cells were dispersed and cultured for 1–2 days as previously described (Plant, 1988). Patch-clamp experiments were performed at room temperature (20–24°C) on single β-cells. For measurements of ATP-sensitive and voltage-dependent K^+ currents the bath solution contained (in mM): NaCl 135, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10, and was titrated to pH 7.4 with NaOH. Pipettes were filled with a solution containing (in mM): KCl 135, $MgCl_2$ 4, $CaCl_2$ 2, EGTA 10, Na_2ATP 0.65, HEPES 20, and titrated to pH 7.15 with KOH. Details of the recording technique and the separation of ATP-sensitive and voltage-dependent currents from other membrane currents have been described previously (Garrino *et al.*, 1989; Plant & Henquin, 1990). In brief, ATP-sensitive K^+ currents were measured by recording the currents at 15 s intervals at the holding potential (−70 mV) and during 100 ms pulses to −60 and −80 mV which were separated by an interval of 100 ms. Under the conditions used, the current which develops with time during dialysis is almost entirely ATP-sensitive K^+ current. To measure voltage-dependent K^+ currents, cells were held at −70 mV and depolarized at 15 s intervals to 0 mV to activate K^+ currents in a bathing solution which was

[1] Author for correspondence.

supplemented with 100 μM tolbutamide to block ATP-sensitive K^+ currents. A short (50 ms) hyperpolarization to −100 mV, applied 100 ms before the test pulse, was used to estimate the leakage current. Currents were also measured (sampling rate: 2.5 kHz) during voltage ramps of 480 ms duration from −120 to 30 mV, applied at intervals of 15 s.

Calcium currents were recorded by the conventional whole-cell and perforated patch technique (Horn & Marty, 1988). The latter preserves second messenger and metabolic systems of the cell and reduces run-down of the Ca^{2+} current compared to the conventional whole cell technique. Cells were bathed in a solution containing (in mM): NaCl 115, tetraethylammonium chloride (TEACl) 20, $CaCl_2$ or $BaCl_2$ 10, $MgCl_2$ 1.2, tolbutamide 0.1, glucose 15, HEPES 10, which was titrated to pH 7.4 with NaOH. For whole-cell experiments, the pipette solution contained (in mM): CsCl 50, *N*-methyl-D-glucamine 70, HCl 58, $MgCl_2$ 4, Na_2ATP 3, EGTA 10, $CaCl_2$ 2, HEPES 10, titrated to pH 7.15 with CsOH. For perforated patch experiments, the pipette solution contained (in mM): Cs_2SO_4 70, NaCl 10, KCl 10, $MgCl_2$ 7, HEPES 10, titrated to pH 7.4 with NaOH (Smith *et al.*, 1989). The tip of the pipette was first filled by dipping the pipette briefly in this solution, then back-filled with the same solution supplemented with 100 or 150 $\mu g\,ml^{-1}$ nystatin in 0.2% dimethylsulphoxide (DMSO). Perforation of the patch was monitored by the change in the capacity transient during a 10 mV depolarizing pulse from a holding potential of −70 mV, and the value of the series resistance obtained from the capacity transient cancellation on the patch clamp amplifier. Measurements were started when the series resistance was less than 50 MΩ, which was normally within 10 to 20 min after seal formation.

Chemicals

Clonidine hydrochloride was obtained from Sigma (Deisenhofen, Germany) or Boehringer (Ingelheim, Germany), adrenaline D-hydrogentartrate from Serva (Heidelberg, Germany), tolbutamide from Hoechst A.G. (Frankfurt, Germany) and diazoxide was provided by Schering Corp. (Bloomfield, NJ, U.S.A.). $^{86}RbCl$ was purchased from the Radiochemical Centre (Amersham, Bucks, U.K.). When the effects of adrenaline were tested, the solutions were supplemented with 0.5 mM ascorbic acid to prevent oxidation of the catecholamine. The dihydropyridine, CGP 28392, was obtained from Ciba Geigy A.G. (Basle, Switzerland).

Results

Effects of clonidine and adrenaline on ^{86}Rb efflux

In the presence of a low concentration of glucose (1 mM), many ATP-sensitive K^+ channels in the β-cell membrane are open and the potassium permeability is high (Rorsman & Trube, 1985; Misler *et al.*, 1986; Ashcroft *et al.*, 1988). Under these conditions, the ^{86}Rb efflux rate is high and declines with time (Figure 1a). Addition of clonidine to the bathing medium reversibly inhibited ^{86}Rb efflux. A small but consistent decrease was observed at a clonidine concentration of 5 μM. The difference between test and control efflux rates ranged between 0.15 and 0.21% min^{-1} ($P < 0.05$ by paired t test). The inhibition by 100 μM clonidine was clearly larger (0.71% min^{-1}) and similar to that produced by 10 μM tolbutamide (0.66% min^{-1}), a specific blocker of ATP-sensitive K^+ channels (Figure 1b). In contrast, adrenaline (100 μM) was without effect on ^{86}Rb efflux under the same conditions. In 6 mM glucose, most ATP-sensitive K^+ channels are closed (Rorsman & Trube, 1985; Misler *et al.*, 1986; Ashcroft *et al.*, 1988) and ^{86}Rb efflux is low and stable. Diazoxide (100 μM), an opener of ATP-sensitive K^+ channels in β-cells (Trube *et al.*, 1986), caused a marked acceleration of ^{86}Rb efflux. Clonidine

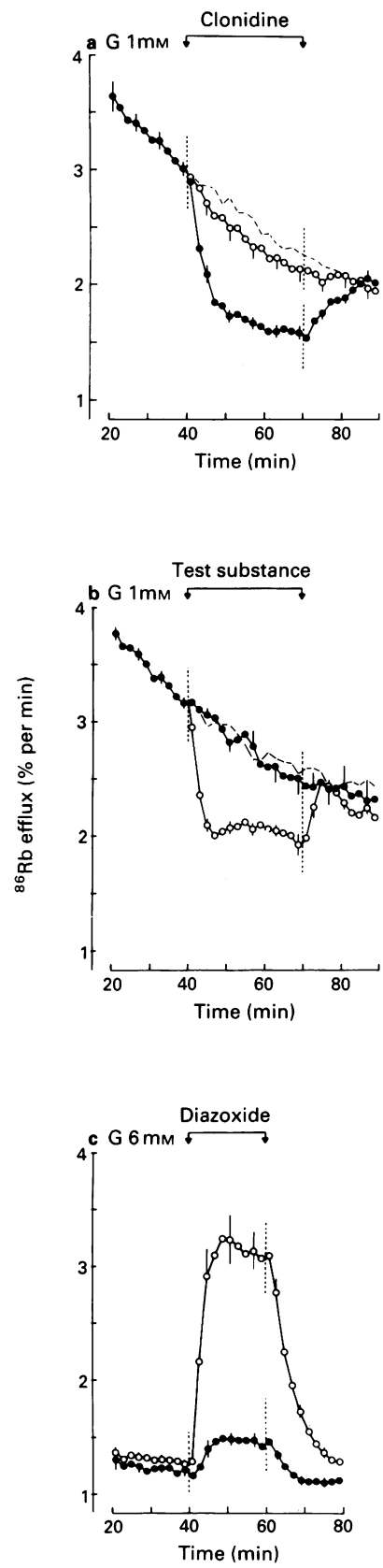

Figure 1 Effects of clonidine and adrenaline on ^{86}Rb efflux from mouse islets. (a) Clonidine was added at a concentration of 5 μM (○) or 100 μM (●) to a medium containing 1 mM glucose (G). (b) Adrenaline, at a concentration of 100 μM, (●), or tolbutamide, at a concentration of 10 μM, (○), was added to a medium containing 1 mM glucose. In (a) and (b), controls without the test substance, are shown by the broken line. (c) Diazoxide (100 μM) was added to a medium containing 6 mM glucose (G) alone (○), or 6 mM glucose plus 100 μM clonidine (●). Values are means for 4–5 experiments with s.e.mean shown by vertical lines.

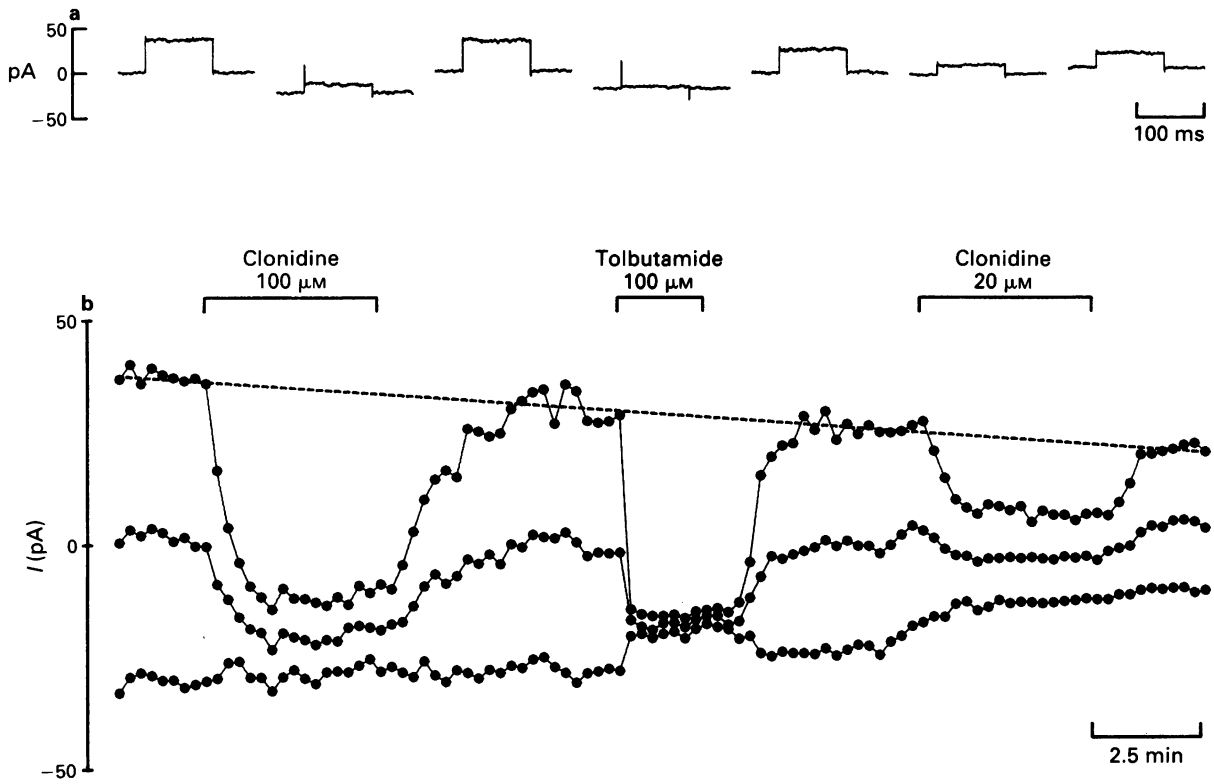

Figure 2 Inhibition of whole cell ATP-sensitive K^+ currents by clonidine. (a) Currents recorded during 100 ms pulses from −70 to −60 mV during the different phases of the experiment shown in (b). Outward currents are denoted positive and shown upwards. (b) Current values recorded at 15 s intervals at −60, −70 and −80 mV (upper, middle and lower traces respectively). The effects of 20 and 100 μM clonidine are compared to those of 100 μM tolbutamide. The time course of run-down of the ATP-sensitive K^+ current at −60 mV is indicated by the broken line. In this experiment, an inward current was observed following inhibition of the ATP-sensitive K^+ current. This probably resulted from leak currents between the pipette surface and the cell membrane.

(100 μM) markedly decreased the effect of diazoxide (Figure 1c).

Effects of clonidine and adrenaline on ATP-sensitive K^+ currents

At physiological ATP concentrations, the whole cell ATP-sensitive K^+ current is small because most of the channels are inhibited (Rorsman & Trube, 1985). When the cell is dialysed with a pipette solution containing a lower than physiological ATP concentration, ATP-sensitive K^+ currents activate with time following the establishment of the whole cell configuration and then decline at a rate dependent on the ATP concentration (Trube *et al.*, 1986; Garrino *et al.*, 1989). Drugs were applied during the approximately linear phase of run-down after the current had attained a maximum. Clonidine decreased the ATP-sensitive K^+ current in a dose-dependent manner (Figure 2). Effects of clonidine were visible starting at a concentration of 4 or 5 μM. The changes in current during the potential steps from −70 to −60 mV were used to quantify the effects of drugs. At a concentration of 100 μM, clonidine inhibited the current by 74.5 ± 2.2% (mean ± s.e.mean, $n = 9$) compared to the current before the addition of the drug. In comparison, tolbutamide (100 μM) applied after clonidine in five of these cells inhibited the current by 93.2 ± 0.8%. In the cell shown in Figure 2, 20 μM clonidine caused an inhibition of 56%. Adrenaline at concentrations up to 100 μM had no effect on the ATP-sensitive K^+ current in six cells (Figure 3).

The effects of clonidine (100 μM) on the responses to voltage ramps from −120 to +30 mV are shown in Figure 4 and compared to the effects of 100 μM tolbutamide. Under the conditions used, the response to voltage ramps has a number of components. Between −120 and −30 mV, the current is mainly ATP-sensitive K^+ current with a small contribution of leakage current. At more positive potentials, voltage-dependent K^+ currents are also activated. Tolbutamide a specific blocker of ATP-sensitive K^+ channels decreased the component between −120 and −30 mV without affecting the voltage-dependent current. Clonidine had similar, though less marked, effects to those of tolbutamide, but in addition appeared to decrease the voltage-dependent component slightly (see below).

Effects of clonidine on voltage-dependent K^+ currents

To determine whether the effects of clonidine are specific for ATP-sensitive K^+ currents, we directly tested the influence of the drug (20 and 100 μM) on the voltage-dependent K^+ current activated during pulses to 0 mV. At a concentration of 20 μM, clonidine was without effect (data not shown), whereas at 100 μM a slight inhibition of the current was observed in all cells studied (Figure 5). The mean inhibition compared to the current before the addition of the drug was 14.0 ± 1.5% ($n = 4$). The kinetics of the current were not markedly changed. Clonidine had no effect on the leakage current (data not shown).

Effects of clonidine on Ca^{2+} currents

Clonidine, at concentrations between 1 and 100 μM, did not have any effect on Ba^{2+} currents through voltage-sensitive Ca^{2+} channels, measured by the whole cell mode of the patch-clamp technique (data not shown). Owing to the rapid run-down of Ba^{2+} currents in many cells, small effects of clonidine may have been difficult to detect. We therefore measured Ca^{2+} currents by the perforated patch technique (Horn & Marty, 1988; Korn & Horn, 1989; Falke *et al.*, 1989; Smith *et*

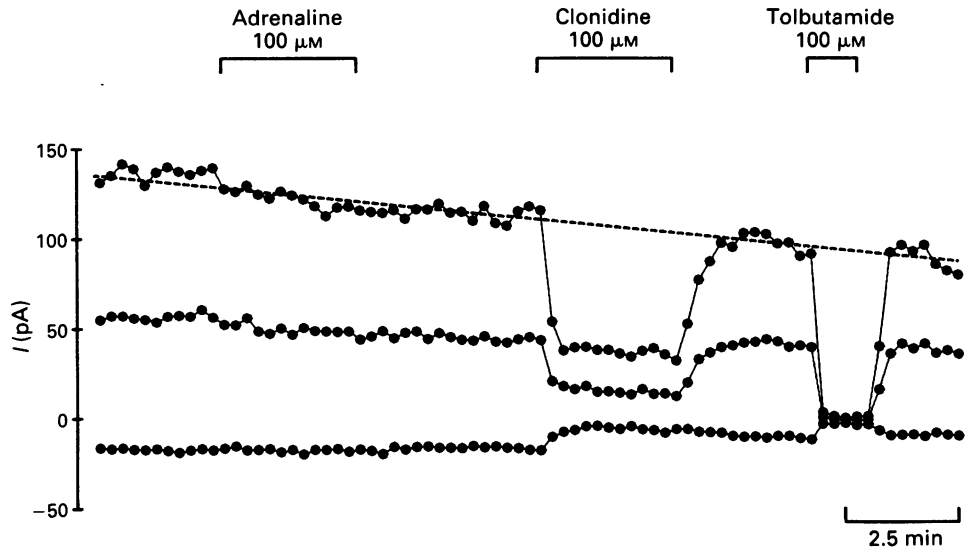

Figure 3 Effects of adrenaline on whole cell ATP-sensitive K^+ currents. Current values measured at −60, −70 and −80 mV (upper, middle and lower traces respectively). The effects of 100 μM adrenaline are compared to those of 100 μM clonidine and 100 μM tolbutamide. The broken line indicates the time course of run-down of the ATP-sensitive current at −60 mV.

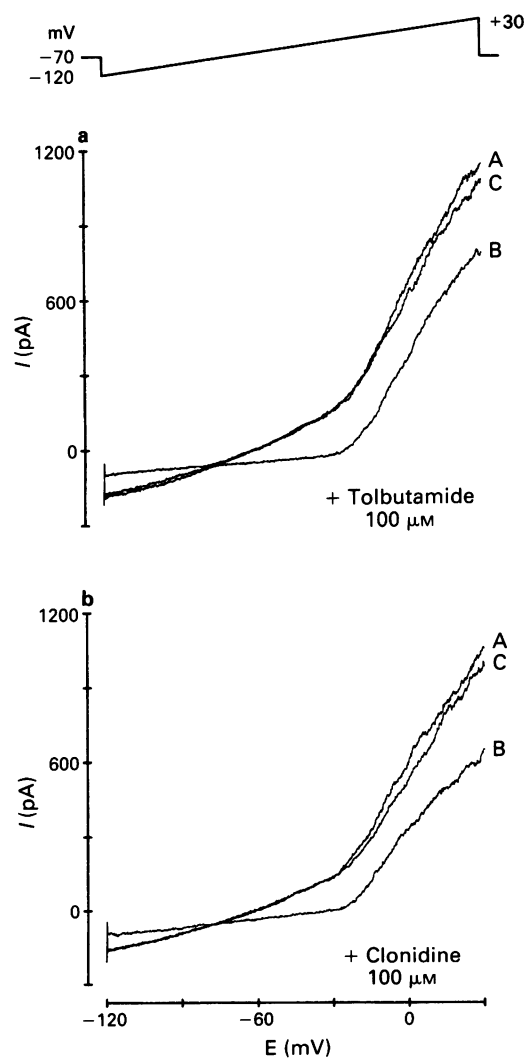

Figure 4 Effects of clonidine and tolbutamide on the current response to voltage ramps. (a) Responses to 480 ms ramps from −120 to +30 mV recorded before addition (A), in the presence (B), and after wash-out (C) of 100 μM tolbutamide. (b) Responses to ramps before addition (A), during the presence (B), and after wash-out (C) of 100 μM clonidine.

al., 1989). With this method calcium currents were much more stable than with the conventional whole cell technique (Plant, 1988), but did show a slow run-down in some cells. They were decreased by 100 μM Cd^{2+} and increased in amplitude by the dihydropyridine, CGP 28392, at a concentration of 5 μM (data not shown). As shown in Figure 6, clonidine (100 μM) had no effect on peak and steady-state Ca^{2+} currents recorded during voltage steps to −10 or 0 mV using this method.

Discussion

The evidence presented in this study shows that the α_2-adrenoceptor agonist clonidine, like the antagonists phentolamine and yohimbine (Plant & Henquin, 1990), inhibits ATP-sensitive K^+ channels in pancreatic β-cells. This is supported by three lines of evidence: (1) clonidine inhibits ^{86}Rb

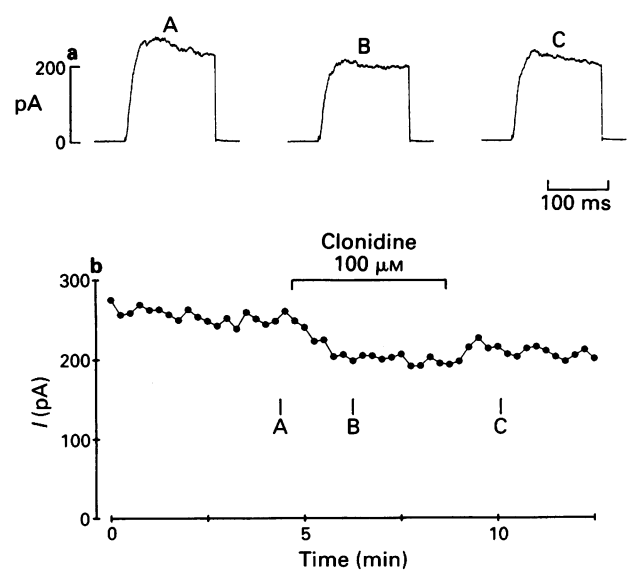

Figure 5 Effects of clonidine on voltage-dependent K^+ currents. (a) Currents recorded during 150 ms voltage steps from −70 to 0 mV at the times shown in (b). (b) Time-course of the effects of clonidine on the voltage-dependent K^+ current. Values of the maximum K^+ current were measured during pulses to 0 mV, applied at 15 s intervals, showing the effect of 100 μM clonidine. The bath solution contained tolbutamide (100 μM) to inhibit ATP-sensitive K^+ currents.

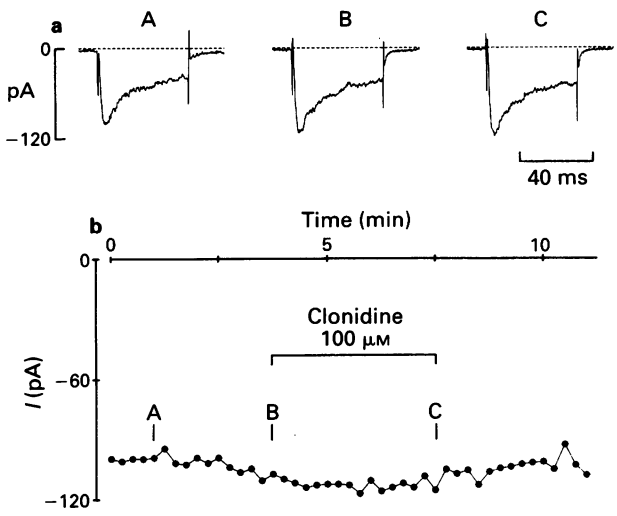

Figure 6 Effects of clonidine on Ca^{2+} currents recorded by the perforated patch technique. (a) Currents recorded in 10 mM Ca^{2+} during steps from −70 to 0 mV at the times shown in (b). (b) Peak Ca^{2+} current recorded at 15 s intervals plotted against time after the start of recording, showing the effect of the addition of 100 μM clonidine. To block K^+ currents the bath solution contained tetraethylammonium ions (20 mM) and tolbutamide (100 μM), and the pipette solution Cs^+ (140 mM).

efflux under conditions where only K^+ channels that are open in the β-cell membrane are the ATP-sensitive ones; (2) clonidine inhibits K^+ currents measured by the whole cell mode of the patch clamp technique, under conditions where almost all current is ATP-sensitive K^+ current, and (3) clonidine markedly decreases the acceleration of ^{86}Rb efflux induced by diazoxide, an opener of ATP-sensitive K^+ channels (Trube *et al.*, 1986). This closure of ATP-sensitive K^+ channels may explain the ability of clonidine to increase insulin release after irreversible blockade of α_2-adrenoceptors by benextramine (Schulz & Hasselblatt, 1989b).

The inhibitory effect of clonidine on the ATP-sensitive K^+ channels does not seem to be associated with the activation of α_2-adrenoceptors. Adrenaline, at concentrations up to 100 μM, was without effect on ATP-sensitive K^+ channels. Furthermore, clonidine acts on α_2-adrenoceptors to inhibit insulin release at much lower concentrations (1–100 nM) than those needed to affect the ATP-sensitive K^+ channels (Leclercq-Meyer *et al.*, 1980; Nakaki *et al.*, 1981; Langer *et al.*, 1983; Bertrand & Henquin, 1990). A similar dissociation exists between the dose-dependency of the effects of phentolamine or yohimbine on α_2-adrenoceptors and on ATP-sensitive K^+ channels (Plant & Henquin, 1990). It has been suggested that clonidine may exert non-specific membrane effects at high concentrations (Starke *et al.*, 1972). This trivial explanation does not hold here. Our results show that the effects of clonidine are selective for the ATP-sensitive K^+ channels. At high concentrations voltage-dependent K^+ channels are only slightly affected, while voltage-sensitive Ca^{2+} channels are unaffected.

The similarity of the effects of clonidine and phentolamine on ^{86}Rb fluxes and ATP-sensitive K^+ currents might indicate the involvement of an imidazoline binding site (Michel & Insel, 1989) in the regulation of ATP-sensitive K^+ channels in β-cells. This proposal would also be suggested by the observations that, like phentolamine (Henquin *et al.*, 1982), efaroxan antagonizes the inhibition of insulin release by diazoxide (Chan & Morgan, 1990), and that several other imidazoline derivatives increase glucose-induced insulin release (Schulz & Hasselblatt, 1989a). On the other hand, the fact that yohimbine, which is not recognized by the imidazoline receptors (Michel & Insel, 1989), also inhibits ATP-sensitive K^+ channels (Plant & Henquin, 1990) may cast doubt on this hypothesis. However, since these channels can be influenced by a host of chemically-unrelated substances (Cook & Quast, 1990; Henquin, 1990), it is possible that yohimbine and phentolamine fortuitously share the property of blocking both α_2-adrenoceptors and ATP-sensitive K^+ channels.

Clonidine also affects ionic currents in other tissues independently of an action on α_2-adrenoceptors. In bovine chromaffin cells, clonidine blocks the current through acetylcholine receptor channels, at similar concentrations to those at which it blocked the ATP-sensitive K^+ current in this study, whilst not affecting other voltage-dependent currents in these cells (Cull-Candy *et al.*, 1988). In vascular smooth muscle cells, however, clonidine does not share the property of phentolamine and some other imidazolines of preventing the hyperpolarization and relaxation induced by cromakalim, a putative opener of ATP-sensitive K^+ channels in this tissue (McPherson & Angus, 1989; Standen *et al.*, 1989).

Activation of α_2-adrenoceptors in β-cells results in a hyperpolarization of the membrane, associated with a decrease in electrical activity, and an inhibition of insulin release. The hyperpolarization of the membrane is accompanied by a decrease in Ca^{2+} entry through voltage-dependent Ca^{2+} channels. The decrease in Ca^{2+} entry could be either the primary effect resulting in the hyperpolarization, or occur as a result of the hyperpolarization caused by an increase in K^+ permeability. Most data obtained in normal β-cells support the latter hypothesis (Drews *et al.*, 1990; Rorsman *et al.*, 1991), but there is some evidence for a decrease in the Ca^{2+} current in the insulin-secreting HIT cell line (Keahey *et al.*, 1989). The present results from the whole cell and perforated patch experiments do not support the involvement of a receptor-mediated reduction in calcium current in the hyperpolarization induced by α_2-agonists.

In conclusion, high concentrations of the α_2-adrenoceptor agonist clonidine, but not of adrenaline, inhibit ATP-sensitive K^+ channels in pancreatic β-cells. Other ionic channels are only weakly affected or unaffected. The effect of clonidine resembles that of phentolamine. It is possible that an imidazoline-binding site is involved in the control of ATP-sensitive K^+ channels in β-cells.

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In vitro modulation of the eosinophil-dependent enhancement of the permeability of the bronchial mucosa

Carolyn A. Herbert, *David Edwards, *John R. Boot & [1]Clive Robinson

Immunopharmacology Group, Clinical Pharmacology, Southampton General Hospital, Southampton SO9 4XY and *Lilly Research Centre Ltd, Eli Lilly and Co., Erl Wood Manor, Windlesham, Surrey GU20 6PH

1 Basolateral to apical albumin flux has been measured in sheets of bovine bronchial and tracheal mucosa mounted *in vitro*.

2 Addition of guinea-pig peritoneal eosinophils or neutrophils to the basolateral side of such tissues had no significant influence on the transmucosal flux of albumin in either the bronchial or tracheal mucosa.

3 Stimulation of eosinophils or neutrophils by the calcium ionophore A23187, or by their presentation to an opsonized airways mucosa, resulted in a significant increase in the transbronchial flux of albumin. This effect was seen after only 60 min incubation of the leucocytes with the bronchial mucosa, and was no greater when the contact time was extended to 180 min. Incubation of bronchial mucosal tissues with 1 mg ml^{-1} polyarginine for 3 h produced a significant increase in albumin flux, but was ineffective at 0.5 mg ml^{-1}.

4 In contrast to the bronchial mucosa, the tracheal mucosa appeared resistant to the effects of stimulated eosinophils and neutrophils.

5 The lipoxygenase inhibitor AA-861 failed to influence the ability of eosinophils to augment the transmembrane flux of albumin. However, insertion of a Millipore filter mask between the eosinophils and the bronchial mucosa significantly inhibited the eosinophil-dependent enhancement of mucosal permeability.

6 The broad spectrum antiproteinase α_2-macroglobulin achieved almost total ablation of the action of stimulated eosinophils in the bronchial mucosa. These results suggest that proteinases may make a significant contribution to the genesis of epithelial injury, whereas leukotrienes do not.

Keywords: Eosinophil; neutrophil; airways epithelium; albumin; antiproteinase

Introduction

The infiltration of eosinophils into the bronchial sub-mucosa and epithelial injury are considered to be quintessential components of bronchial asthma (Naylor, 1962; Dunnill *et al.*, 1969; Filley *et al.*, 1982; Laitinen *et al.*, 1985; Elia *et al.*, 1988; Diaz *et al.*, 1989; Jeffery *et al.*, 1989). One consequence of this injury may be disruption of the normal barrier function of the airways epithelium, a process which has been postulated to be involved in the pathogenesis of bronchial hyperresponsiveness (Laitinen *et al.*, 1985; Robinson & Holgate, 1985; Gleich *et al.*, 1988; Vanhoutte, 1988; Wardlaw *et al.*, 1988; Jeffery *et al.*, 1989).

Normally, there is only limited transepithelial efflux of plasma proteins into the airway lumen (Webber & Widdicombe, 1989; Herbert *et al.*, 1990a,b,c). However, sustained plasma leakage precedes and is associated with late phase asthmatic responses (Lam *et al.*, 1985; Fabbri *et al.*, 1987), and elevated levels of plasma proteins are found in mucus plugs and sputum in patients with asthma (Ryley & Brogan, 1968; Lam *et al.*, 1985). These events can be modelled in experimental animals undergoing allergen challenge (Ranga *et al.*, 1983). Furthermore, in asthma there is a general increase in the permeability of the bronchial mucosa when compared with normal controls (Ilowite *et al.*, 1989). Together, these observations indicate a disease-related change in the effectiveness of the mucosal barrier, although the precise mechanism of this effect is unknown. Theoretically, such an increase in permeability might be driven by increased hydrostatic pressure in the submucosa, or in more extreme situations by physical disruption of the epithelial barrier (Persson, 1986a,b; 1988; Persson *et al.*, 1990).

When appropriately activated, eosinophils release a broad spectrum of mediators which could be involved in the genesis of mucosal injury (Gleich & Adolphson, 1987; Spry, 1988). These agents include eicosanoids (Sun *et al.*, 1989), oxygen-centred radicals (Agosti *et al.*, 1987; Ayars *et al.*, 1989) and polycations such as the major basic protein (MBP) and eosinophil cationic protein (ECP) (Frigas *et al.*, 1980; Hastie *et al.*, 1987; Ayars *et al.*, 1989; Motojima *et al.*, 1989) and are capable of interacting synergistically with other inflammatory cells to augment mediator release (Campbell *et al.*, 1989). In view of the association between eosinophil infiltration into the bronchial sub-mucosa in asthma, and the ability of these cells to liberate powerful tissue-damaging mediators, there is currently considerable interest in the pharmacological modulation of eosinophil differentiation and function (Sanjar *et al.*, 1989; 1990a,b).

As part of a programme of work which seeks to define the mechanisms of eosinophil-airway mucosal interaction, we have developed *in vitro* techniques to investigate the effect of inflammatory cells on the permeability of the tracheobronchial mucosa to albumin. We now describe investigations which define aspects of the interaction between leucocytes and the airways mucosa, and its modulation by selected manipulations. Some of this work has been presented in preliminary form to the British Pharmacological Society (Herbert *et al.*, 1990a,b,c) and the Sixth International Conference on Prostaglandins and Related Compounds.

Methods

Studies with tissue chambers

Fresh bovine lungs were obtained at a local abattoir. Upon arrival at the laboratory the lower trachea and first generation bronchi (1–2 cm internal diameter) were identified, opened by incision and the airways mucosa carefully removed by dissection. The tissue removed comprised the airways epithelium and basement membrane as judged by light and electron microscopy. A maximum of 6 pieces of tissue were obtained

[1] Author for correspondence at present address: Department of Pharmacology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE.

from adjacent areas of bronchus and 10 pieces from the trachea. Dissected tissue was placed in Eagle's Minimum Essential Medium (MEM) and washed extensively. The dissected mucosal tissue was cut into 1–2 cm^2 sized pieces and mounted between two perspex plates, each of which had an aperture of diameter 0.8 cm in the case of tracheal tissue or 0.5 cm in the case of bronchial mucosa. The tissue was sandwiched between the two plates by means of 4 mounting pins, and a leakproof seal ensured by a surrounding rubber O-ring on each of the plates. The assembled sandwiches were then mounted, by means of a further pair of O-ring seals, between two water jacketed half-chambers. This allowed the independent bathing of the apical and basolateral faces of the tissue with volumes ranging from 200 μl–2 ml depending on the nature of the experiment. The half-chambers were provided with luer fitting access ports which permitted the addition and removal of the chamber contents by syringe. The resulting sub-assembly was then mounted in a compression module which held the sandwiches securely between the two half-chambers. Both half-chambers were initially filled with MEM and maintained at 37°C. In routine experiments, up to ten of these chambers were operated simultaneously to enable control and test manipulations to be performed together. Bronchial and tracheal tissues demonstrated good viability as indicated by the release of 9.5 $\pm$ 2.5% and 7.7 $\pm$ 1.4% total cellular lactic dehydrogenase (LDH) after the longest experimental procedures (n = 6–10). In these experiments, LDH released into the chambers was measured by the Cabaud-Wroblewski method using kits purchased from Sigma (Poole, Dorset). Total tissue LDH was determined after homogenization and centrifugation of mucosal tissue.

After equilibration for 30 min, the serosal bathing solution was replaced with medium containing cells (1 ml) or mediator (2 ml). In the case of experiments with cells, maximum cell-mucosal tissue contact was assisted by mounting the chambers vertically on a metal rig. After appropriate periods of exposure (60 or 180 min) these solutions were removed and the chambers refilled with fresh cell- or mediator-free medium prior to the measurement of albumin flux.

Measurement of albumin flux

The half-chambers on the basolateral side of the tissue were loaded with 2 ml MEM containing 2 μCi ^{125}I-labelled bovine serum albumin (BSA, sp. act. 2.1–2.5 μCi μg^{-1}, NEN Research Products, Stevenage, Herts.). After addition of the tracer the chambers were agitated gently throughout the subsequent experimental period. At 0, 15, 30, 60, 90, 120, 150 and 180 min after addition, 25 μl samples of MEM were withdrawn by microsyringe from the opposite (apical) side of the airways mucosa. The amount of ^{125}I in each of these samples was quantified either by gamma counting (Canberra-Packard Cobra B5003) or liquid scintillation counting in a Canberra-Packard 2000CA liquid scintillation analyser after addition of 4 ml Opti-Fluor (Canberra-Packard, Caversham, Berks.). In both cases data were corrected for radioactive decay from the reference date of isotope preparation.

The amount of transepithelial flux which had occurred at each time point was calculated and plotted as a linear function of time to determine the net transmucosal flux (expressed in fmol albumin cm^{-2} membrane min^{-1}). In all the experiments described herein the vector of albumin flux was from the basolateral to the apical side of the epithelium. Our prior investigations demonstrated that tissues which were not mounted satisfactorily exhibited non-linear kinetics inconsistent with the normal diffusive flux of albumin across the membrane. Therefore criteria were established for the rejection of data where the correlation coefficients calculated for the relationship between BSA flux and time in each chamber were <0.95. Using this approach a rejection rate of 2.5% was obtained from 1149 chambers.

The molecular integrity of the albumin that traversed the epithelium was confirmed by radio-high performance liquid chromatography (h.p.l.c.) analysis of aliquots of chamber media on a Hamilton 5 μm PRP-1 column (15 x 0.46 cm). Unlabelled BSA (250 μg) was added as unlabelled carrier and to aid visualization by ultra-violet absorbance at 280 nm. Chromatography was performed with a 70% mixture of solvent A (acetonitrile:water:phosphoric acid (1:18.9:0.1 v:v)) and 30% solvent B (acetonitrile:water:phosphoric acid (16:3.9:0.1 v:v)) developed as a linear gradient over 9 min to a composition of 100% solvent B. Fractions of column effluent were automatically collected at 30 s intervals for 22 min and radioactivity content determined by scintillation counting.

Purification of eosinophils

A peritoneal eosinophila was established in guinea-pigs essentially as described by Pincus (1978). Male Dunkin-Hartley guinea-pigs were injected i.p. with 1 mg ml^{-1} polymyxin B in saline twice weekly for 4 weeks. At 24 h after the last injection, the animals were killed and the peritoneal cavity of each was washed with 50 ml Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium but containing 5 u ml^{-1} heparin (Paines & Byrne Ltd, Greenford, Middx). The lavage fluid was filtered through gauze and then centrifuged at 150 g for 10 min at room temperature. The pellet was then resuspended in Tyrode solution containing gelatin and deoxyribonuclease (DNase) for density gradient centrifugation.

Eosinophils were enriched by overlayering the washed lavage cells onto density gradients which consisted of a cushion of 2.5 ml Maxidens upon which a discontinuous gradient of 16, 18, 20, 22, 24, 26, 30% Metrizamide in Tyrode solution (supplemented with gelatin and DNase) had been prepared. The percentage composition and density of the Metrizamide solutions was confirmed with an Abbe refractometer. Each step in the gradient consisted of a volume of 2 ml and a maximum of 10^8 cells was loaded onto each gradient. Centrifugation was performed at 1200 g for 45 min at room temperature. The eosinophil-rich band at the 24–26% interface was carefully aspirated, washed in DPBS and the number and purity of cells determined by staining with eosin/pontamine sky blue. Viability was determined by exclusion of trypan blue. Preparations of $<80\%$ purity and $<90\%$ viability were discarded.

Purification of neutrophils

Guinea-pigs were given a single i.p. injection of 10 ml 2% casein in saline, killed 16–20 h later and peritoneal lavage performed as described above. The peritoneal cells were washed by centrifugation and resuspension. Erythrocytes were lysed in hypotonic saline and the neutrophil-rich cell preparation resuspended in MEM. Cells were counted as described above.

Stimulation of eosinophils and neutrophils

Two stimuli were employed to elicit mediator release from eosinophils and neutrophils. Calcium ionophore A23187 at a concentration of 5 μM was chosen as this was found to elicit a maximal release of both leukotriene B_4 (LTB_4), the major leukotriene from guinea-pig eosinophils and neutrophils (Sun *et al.*, 1989), and LTC_4 without having a measureable effect on epithelial permeability to albumin. For example, in eosinophils the net LTB_4 release obtained with 5 μM A23187 was 1.4 $\pm$ 0.5 ng per million cells compared with 107.7 $\pm$ 90.7 pg per million cells for LTC_4. These values were not significantly different from the release obtained with either 2.5 or 10 μM A23187. Ionophore was dissolved in dimethylsulphoxide (DMSO) and diluted in MEM for use. The final concentration of DMSO was 1% and a solution of 5 μM A23187 prepared in this way had no significant effect on epithelial function as judged by the release of prostaglandin E_2 (the major prostanoid released by this tissue), tissue morphology and measured albumin flux. In the chamber experiments described here, the

cells were activated by adding a suspension of them to chambers containing solutions of A23187 or DMSO to give the final concentrations described above.

For the second method of activating the cells we raised guinea-pig polyclonal antibodies against bovine airways mucosa in order to provide a means of promoting opsonic adherence of the leucocytes to the mucosa. Heat-inactivated serum was added to the basolateral side of each chamber assembly at a final dilution of 1:25 to opsonize the airways mucosa. After 30 min incubation, the chambers were washed with MEM and cells added as described above. The optimum dilution for opsonization, and confirmation of specific binding to the membrane was achieved by immunofluorescence microscopy. In these validation studies appropriate controls were incorporated by use of an irrelevant antibody.

Mediator assays

Radioimmunoassays for LTB_4 and LTC_4 were performed according to the standard protocols described by the supplier. Separation of bound from free ligand was achieved by use of dextran-coated charcoal and quantification of supernatant (bound) radioactivity was performed by liquid scintillation counting.

Materials

The following were purchased as indicated: Metrizamide and Maxidens (Nycomed, Birmingham); α_2-macroglobulin, ionophore A23187, polymyxin B sulphate, polyarginine, DMSO, DNase, FITC conjugated rabbit anti-guinea pig IgG, LDH assay reagents and Eagle's MEM (Sigma, Poole, Dorset); casein, heparin and general reagents for microscopy (BDH, Poole, Dorset); DPBS (Flow Laboratories, Rickmansworth, Herts.). Reagents for the radioimmunoassay of leukotriene B_4 and C_4 were purchased from Amersham International (Amersham, Bucks.). AA-861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecaiynyl)-1,4-benzoquinone) and synthetic leukotrienes were provided by the Department of Chemistry, Lilly Research Centre Ltd. All other standard laboratory reagents were obtained from BDH and were of the highest grade obtainable.

Statistics

Data are presented as mean ± s.e.mean of n experiments. Statistical comparisons were made with Student's t test for unpaired data. Probability values (P) <0.05 were considered statistically significant.

Results

The effect of eosinophils and neutrophils

Under control conditions the net basolateral-apical flux of BSA was 1.07 ± 0.04 ($n = 81$) and 2.19 ± 0.06 ($n = 143$) fmol cm^{-2} min^{-1} in tracheal and bronchial mucosal tissues respectively. The difference in these absolute values reflects the relative thickness of the tissues from the two sites which were determined by micrometry as being 0.1700 ± 0.0020 mm and 0.0840 ± 0.0004 mm in the tracheal and bronchial sheets ($n = 91$–199). Radio-h.p.l.c. analysis indicated that the albumin that traversed the epithelium was not subject to proteolysis resulting in the formation of smaller molecular radius fragments, nor was there any evidence of loss of specific activity of the tracer (data not shown).

Eosinophils obtained by peritoneal lavage were enriched to an average of $91.6 \pm 0.7\%$ purity ($n = 75$). The remaining contaminant cells comprised macrophages and few (<1%) neutrophils. The enriched eosinophils were challenged with 5 μM A23187 which elicits maximal release of superoxide anion and LTB_4. Peritoneal neutrophils were used at a final purity of $86.7 \pm 1.1\%$ ($n = 18$) and were similarly activated with A23187. The residual contaminant cells in this case were principally macrophages.

Initial experiments established that neither 5 μM A23187 nor its DMSO vehicle, at a final concentration of 1%, had any significant effect on transepithelial BSA flux when they were incubated with mucosal tissues for 60 min. In the tracheal mucosa the control net basolateral-apical flux of BSA in the presence of DMSO was 0.95 ± 0.04 fmol cm^{-2} min^{-1} ($n = 4$), compared to a value of 1.02 ± 0.11 ($n = 4$) in the presence of A23187. Corresponding values in the bronchial mucosa were 2.20 ± 0.22 ($n = 16$) and 2.17 ± 0.23 ($n = 17$). None of these values is significantly different from control rates measured in the absence of these substances using tissues derived from the same animals.

Table 1 illustrates the relationship between the number of eosinophils or neutrophils added to the basolateral side of bronchial or tracheal mucosa and their effect on the unidirectional flux of BSA measured after stimulation of the leucocytes with 5 μM A23187 for 60 min. None of the manipulations tested resulted in an alteration in tracheal permeability to BSA. However, incubation with both stimulated eosinophils and neutrophils resulted in significant increases in the transbronchial mucosa albumin flux after a threshold number of cells had been exceeded. All subsequent studies were performed with numbers of cells which consistently demonstrated clear enhancing effects in the bronchial mucosa; 3.3×10^6 eosinophils and 15×10^6 neutrophils respectively.

Exposure of tracheal and bronchial mucosal sheets to unstimulated eosinophils for either 60 or 180 min had no significant effects on the net flux of albumin measured in the absence of cells (Table 2). This was true whether the eosinophils were in the presence of the 1% DMSO vehicle used for the ionophore, or were incubated with pieces of mucosa which had undergone prior incubation with non-immune serum (sham opsonization). Moreover, the ionophore itself was without significant effect when incubated with the mucosal tissues alone (Table 2). However, there were significant increases in the net unidirectional flux of albumin measured after 60 or 180 min exposure of bronchial tissues to eosinophils stimulated with A23187 (Table 2). There was no significantly greater effect produced by the longer incubation period. In contrast to the results obtained with sheets of bronchial mucosa incubated with stimulated eosinophils, no effect was seen when tracheal mucosa was used.

A similar pattern of results was obtained when the eosinophils were activated by incubation with sheets of mucosal tissue which had been subjected to prior opsonization with antibodies raised against the airway mucosa. Exposure to activated eosinophils for 60 or 180 min resulted in significant

Table 1 Relationship between the number of leucocytes stimulated by 5 μM A23187 and their ability to promote increases in the basolateral-apical flux of albumin

Cells ($\times 10^{-6}$ ml^{-1})	*Net flux* (fmol cm^{-2} min^{-1}) *Bronchial*	*Tracheal*
Control	1.61 ± 0.14	0.82 ± 0.14
Eosinophils		
0.83	1.71 ± 0.26	0.80 ± 0.09
1.70	1.68 ± 0.33	0.88 ± 0.17
2.50	3.04 ± 0.50*	0.81 ± 0.18
3.33	5.85 ± 1.16*	0.82 ± 0.17
Neutrophils		
3.33	2.12 ± 0.44	—
7.50	2.96 ± 0.23*	—
11.25	3.45 ± 0.50*	—
15.00	4.61 ± 0.50*	—

Data are mean ± s.e.mean in 4 experiments. * $P < 0.05$ with respect to the cell-free control value. Leucocyte/mucosal contact time was 60 min.

Table 2 Effect of eosinophils stimulated by 5 μM A23187 or by opsonic adherence on the net basolateral-apical flux of albumin in bovine tracheo-bronchial mucosa

Conditions	*Net flux* (fmol cm^{-2} min^{-1}) *Bronchial*	*Tracheal*
60 min exposure		
Medium + DMSO	2.33 ± 0.32	0.95 ± 0.04
Medium + A23187	2.14 ± 0.20	1.02 ± 0.11
Eosinophils + DMSO	2.49 ± 0.23	1.04 ± 0.08
Eosinophils + A23187	5.77 ± 0.85*	1.07 ± 0.07
Medium + sham opsonization	2.43 ± 0.27	0.82 ± 0.09
Medium + opsonized tissue	2.44 ± 0.28	0.90 ± 0.16
Eosinophils + sham opsonization	2.03 ± 0.28	0.83 ± 0.11
Eosinophils + opsonized tissue	4.10 ± 0.31*	0.87 ± 0.15
180 min exposure		
Eosinophils + DMSO	2.55 ± 0.48	1.00 ± 0.09
Eosinophils + A23187	5.16 ± 0.98*	1.04 ± 0.30
Eosinophils + sham opsonization	2.13 ± 0.24	0.98 ± 0.15
Eosinophils + opsonized tissue	5.49 ± 0.99*	0.90 ± 0.18

Mucosal tissues were incubated with eosinophils or their relevant control for either 60 min or 180 min as described above before the net flux of albumin was determined. Eosinophils were used at a final cell number of 3.33×10^6 ml^{-1}. Details of the opsonization procedure are given in the Methods section. Data are mean ± s.e.mean in 4–8 experiments. * $P < 0.05$ with respect to the appropriate control (i.e. eosinophils + dimethylsulphoxide (DMSO) or eosinophils and sham opsonization).

increases in albumin flux in the bronchial, but not tracheal preparations (Table 2). Again, the extended incubation time did not produce an effect greater than the 60 min exposure period. Tissues which had been sham opsonized with irrelevant antibodies (from non-immune serum) failed to activate the eosinophils to produce alterations in permeability (Table 2).

When neutrophils were used in a similar series of experiments it was found that they too were capable of producing an increase in the transepithelial flux of albumin in response to stimulation by A23187 or an opsonized bronchial mucosa (Figure 1). The tracheal mucosa was again apparently resistant to the effects of unstimulated or stimulated cells.

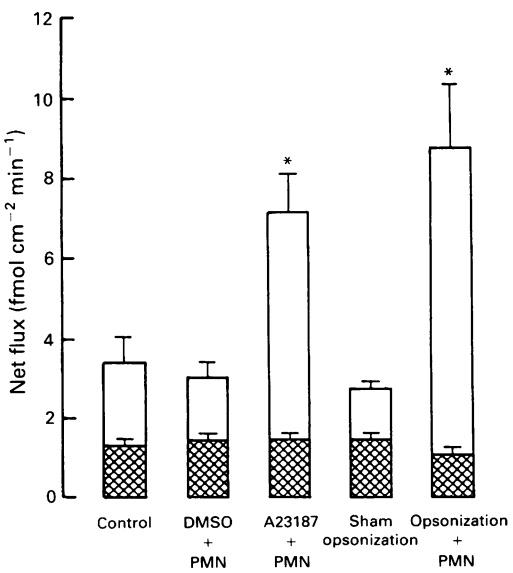

Figure 1 The effects of stimulated guinea-pig neutrophils on the transmucosal flux of albumin in bovine tracheal (hatched columns) and bronchial (open columns) mucosa. Asterisks indicate significant differences in bronchial tissues with respect to the appropriate controls: * $P < 0.05$.

Histological examination of bronchial mucosal tissues which had been exposed to stimulated eosinophils revealed significant changes to their normal morphology. Mucosal injury was apparent after 60 min exposure with evidence of relatively well-defined patches of epithelial cell exfoliation. In some instances it was possible to see groups of surface epithelial cells which had been detached from the basement membrane whilst retaining interepithelial adhesion. However, in most cases there was evidence also of considerable disruption and destruction of epithelial cells with retention of only basal cell attachment to the basement membrane (Figure 2). Tracheal tissues showed relatively little evidence of cellular injury, consistent with the tracer flux measurements.

Observations on the interaction between leucocytes and the airways mucosa

In order to investigate the characteristics of the reaction between eosinophils and neutrophils with the bronchial mucosa, we undertook further experiments in which we modified the incubation conditions. The interaction between the leucocytes and opsonized mucosal tissues is by definition a proximity-dependent interaction, involving the IgG Fc receptor-dependent binding of leucocytes to the Fc portion of IgG molecules localized to the mucosa through their specific Fab sites. However, we wished to establish whether there was a need for close proximity with the mucosa when the leucocytes were activated with calcium ionophore.

The results of these experiments are shown in Table 3. In order to prevent physical contact between eosinophils or neutrophils and the mucosal tissue, a Millipore type HA filter with a pore size of 450 nm (cf. molecular diameter of albumin, 8 nm) was interposed between the basolateral side of the mucosa and the added leucocytes. Two filter placement positions were investigated. Placement 1 kept the leucocytes at least 6 mm from the tissue, whilst placement 2 separated the leucocytes from the basolateral side of the bronchial mucosa by at least 0.148 mm (the thickness of the filters). Unlike place-

Table 3 The effect of preventing eosinophil-mucosal contact on the ability of stimulated eosinophils and neutrophils to augment the basolateral-apical flux of albumin in the bovine bronchial mucosa

Conditions	*Net transmucosal flux* (fmol cm^{-2} min^{-1})
No filter	
Eosinophils + DMSO	1.83 ± 0.29
Eosinophils + A23187	5.99 ± 1.01*
Neutrophils + DMSO	1.26 ± 0.13
Neutrophils + A23187	4.10 ± 0.75*
Filter placement 1	
Eosinophils + DMSO	2.86 ± 0.28**
Eosinophils + A23187	2.37 ± 0.21**
Neutrophils + DMSO	1.23 ± 0.05
Neutrophils + A23187	1.31 ± 0.12**
Filter placement 2	
Eosinophils + DMSO	1.39 ± 0.26
Eosinophils + A23187	2.62 ± 0.60**

Data are mean ± s.e.mean values from 4–5 separate experiments in each case. * $P < 0.05$ with respect to the appropriate value with unstimulated cells (no filter) or ** with respect to the filter-free ionophore data in the case of chambers containing the filters. Placement 1 positioned the filter 6 mm from the mucosa, whereas in placement 2 the filter was juxtaposed against the basolateral surface of the piece of mucosa. The control basolateral-apical flux of albumin in the absence of leucocytes, but with a filter in position 1 or 2 was 2.07 ± 0.26 fmol cm^{-2} min^{-1}. Eosinophils were used at a final cell number of 3.33×10^6 ml^{-1}, whereas neutrophils were used at 15×10^6 ml^{-1}. Both cell types were stimulated with 5 μM A23187.

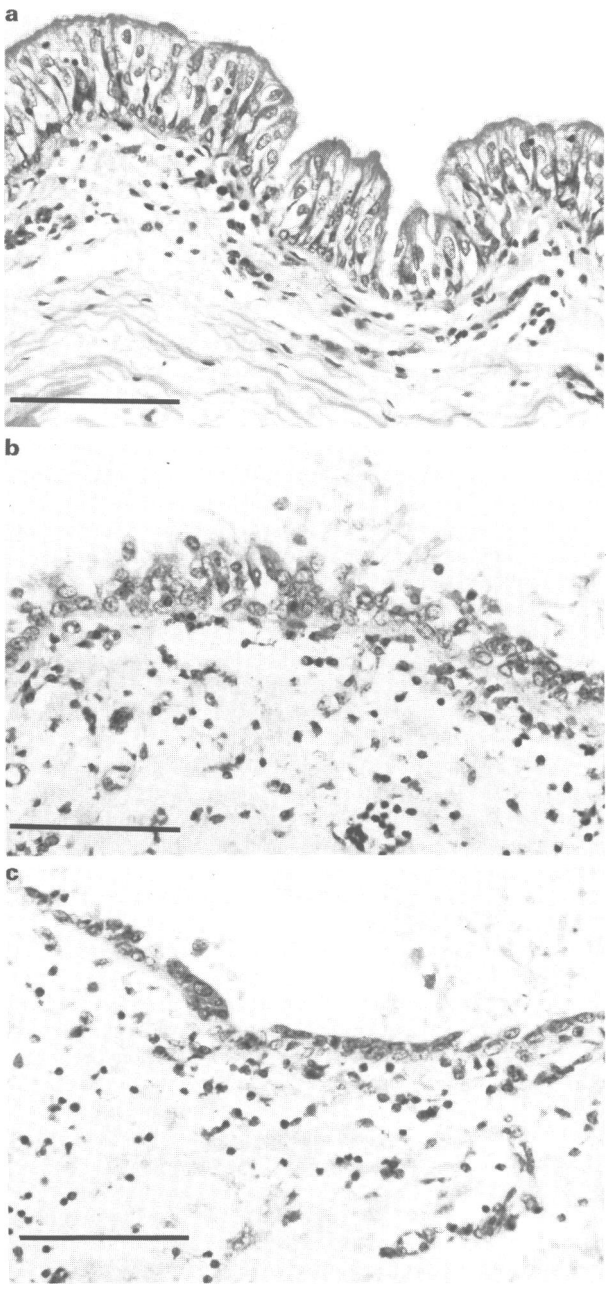

Figure 2 Light photomicrographs of sections from bovine bronchial mucosal sheets which had been mounted in the bathing chambers and exposed to different treatments. Photograph (a) shows a tissue which was incubated with cell-free buffer. Photographs (b, c) are of a tissue exposed to ionophore-activated eosinophils for 60 min and illustrates the evidence of extensive cellular damage and detachment of epithelial cells from the basal cell layer and basement membrane. Tissues were stained with haematoxylin and eosin. The scale bar represents 100 μm.

ment 1, the positioning of the filter in the second type of experiment meant that the filter itself was in contact with the mucosa. Both arrangements of the filters produced a significant inhibition of the increase in permeability elicited by stimulated eosinophils. Furthermore, although position 2 was not tested, placement 1 was effective at inhibiting the action of stimulated neutrophils.

In order to establish whether products of the 5-lipoxygenase pathway were responsible in any way for the increased bronchial epithelial flux of albumin resulting from the exposure to stimulated eosinophils, a series of experiments was performed using the 5-lipoxygenase inhibitor AA-861 (Ashida *et al.*, 1983). After preincubation of the eosinophils with AA-861 for 5 min the cells were added to the chamber together with A23187 or DMSO and the incubation continued for a further 60 min. These experiments showed that this 5-lipoxygenase inhibitor was without significant effect on the action of stimulated eosinophils to increase bronchial mucosal albumin flux

Table 4 Lack of effect of cytochalasin b and the lipoxygenase inhibitor AA-861 on the eosinophil-dependent augmentation of the basolateral-apical flux of albumin in bovine bronchial mucosa

	Net flux (fmol cm^{-2} min^{-1})
Control*	1.67 ± 0.12
Eosinophils + A23187	4.13 ± 0.68†
Eosinophils + A23187 + AA-861	4.00 ± 0.41
Eosinophils + A23187 + cytochalasin	2.49 ± 0.73

Data are mean ± s.e.mean in 4 experiments. * For clarity the control value is a composite figure based on values obtained from chambers containing medium alone or with 10 μM AA-861 and 1 μM cytochalasin b. † Indicates ionophore value significantly different from control. There was no significant inhibition produced by either AA-861 or cytochalasin b. Tissue contact time with the eosinophils was 60 min.

Table 5 Effect of α_2-macroglobulin (α_2M) on the eosinophil-dependent increase in the net basolateral-apical flux of albumin in bovine bronchial mucosa

Conditions	*Net flux* (fmol cm^{-2} min^{-1})
Control†	1.60 ± 0.09 (19)
Eosinophils + A23187	2.93 ± 0.34 (10)*
Eosinophils + A23187 + 100 μg ml^{-1} α_2M	1.74 ± 0.19 (6)**
Eosinophils + A23187 + 400 μg ml^{-1} α_2M	1.61 ± 0.18 (4)**

Data are mean ± s.e.mean values of (n) experiments. * $P < 0.001$ with respect to the control data. ** $P < 0.01$ compared to the results for ionophore-stimulated eosinophils. † The control value is, for reasons of clarity, a composite figure derived from data for medium alone, medium and α_2M, eosinophils and dimethylsulphoxide (DMSO) and eosinophils, DMSO and α_2M. None of these separate controls were significantly different from each other. Eosinophils were used at a final cell number of 3.33 × 10^6 ml^{-1} and were stimulated with 5 μM A23187. Eosinophil-mucosal contact time was 60 min.

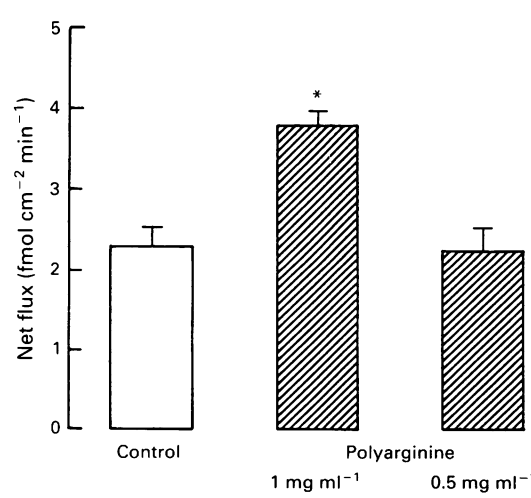

Figure 3 The effect of polyarginine on the net transmucosal flux of albumin in bovine bronchial mucosa. A contact time between polyarginine and the mucosa of 180 min was used before measurement of permeability. Asterisk indicates significantly different with respect to the buffer control: * $P < 0.05$.

(Table 4). This lack of effect was not due to failure of the compound to behave as an inhibitor of 5-lipoxygenase because assay of incubation medium for LTB_4 indicated $89.0 \pm 4.9\%$ ($n = 4$, $P < 0.001$) inhibition of the normal formation of $18.6 \pm 8.1\,ng\,ml^{-1}$ of this compound under these challenge conditions. Furthermore, in 4 separate experiments we confirmed that LTB_4, LTC_4, LTD_4 and LTE_4 were without any significant permeability promoting effects in this preparation when tested at a concentration of $0.1\,\mu M$ for 180 min in the presence of the labelled albumin (data not shown).

Incubation of eosinophils for 5 min with $1\,\mu M$ cytochalasin b resulted in a partial to complete reduction of the effect of stimulated eosinophils in 3 out of 4 experiments. However, the overall effect under these conditions was not statistically significant (Table 4). This concentration of cytochalasin b was chosen on the basis of its ability to produce 70–80% inhibition of the eosinophil-dependent lysis of IgG-coated sheep erythrocytes (our unpublished observations). It is clear from the present experiments that the activity of cytochalasin b is lower than anticipated, suggesting that erythrocyte lysis may not be an accurate predictor for inhibitory activity in more complex systems. However, it is possible that higher concentrations of cytochalasin b would produce a more effective inhibition.

The broad spectrum antiproteinase α_2-macroglobulin was an effective inhibitor of the increased transmucosal flux of albumin elicited by ionophore stimulated eosinophils. Significant degrees of inhibition were seen when initial concentrations of α_2-macroglobulin of $100\,\mu g\,ml^{-1}$ and $400\,\mu g\,ml^{-1}$ were preincubated with the eosinophils for 5 min prior to addition of the ionophore (Table 5). Control preparations demonstrated that neither concentration of α_2-macroglobulin had any significant effect on the net flux of albumin measured after exposure to medium alone. Furthermore, the antiproteinase was not an inhibitor of eosinophil activation as judged by the release of LTB_4 ($9.53 \pm 4.4\,ng\,ml^{-1}$ in the absence of the inhibitor, and $24.97 \pm 10.33\,ng\,ml^{-1}$ in the presence of $400\,\mu g\,ml^{-1}$ α_2-macroglobulin).

Exposure of bronchial mucosal tissues to polyarginine, a basic amino acid polymer, at a concentration of $1\,mg\,ml^{-1}$ for 3 h resulted in a significant potentiation of transmucosal albumin flux (Figure 3). However, a lower concentration ($0.5\,mg\,ml^{-1}$) was without significant effect.

Discussion

In this study we have shown that eosinophils stimulated either by the calcium ionophore A23187 or by opsonic adherence are capable of producing cellular disruption of the bronchial epithelium. This is accompanied by an increase in the permeability of the tissue to albumin. In contrast, the tracheal mucosa was resistant to the effects of eosinophils, this protection most probably being conferred by the greater thickness of the tracheal mucosa. In this context it is interesting to note that, in another study, ciliostasis and epithelial injury induced by PAF-stimulated guinea-pig eosinophils required 14–24 h of exposure before significant effects were noted in rings of guinea-pig trachea (Yukawa *et al.*, 1990).

A notable feature of our experiments is the relatively modest number of eosinophils required to produce these effects, together with the rapid onset of the associated tissue damage. Indeed, extension of the eosinophil-mucosal contact time to 180 min did not produce a significant increase in albumin flux when compared with the results obtained with 60 min exposure. This finding is unlikely to be due to the permeability increase being a maximum response of the bronchial tissue because we have observed larger fluxes after exposure of bronchial mucosa preparations of collagenases or the house dust mite allergen *Der p* I (our unpublished observations).

When activated by either stimulus, neutrophils were capable also of augmenting the net unidirectional flux of albumin in the bronchial, but not tracheal, mucosa. However, compared with eosinophils, larger numbers of cells were required to produce similar effects. This disparity may result from differences in the priming state of the two types of cell or their profile and extent of mediator release.

Morphological examination of mucosal tissues exposed to stimulated inflammatory cells revealed evidence of extensive disruption of the epithelium. Areas of complete exfoliation were clearly evident, together with cellular debris indicative of cytolysis. In places, it was interesting to note that apparently normal basal cell-matrix attachment was also evident, suggesting that columnar cell–basal cell attachment may be more susceptible to injury processes. It therefore seems reasonable to conclude that the increase in albumin permeability results from the loss of epithelial integrity, in particular the interepithelial tight junctions which provide the greatest resistance to the paracellular flow of solutes in the airways epithelium. The presence of exfoliation, together with debris indicative of cytolytic damage of cells retaining attachment to the basement membrane points to several mechanisms contributing to the changes seen (e.g. cytotoxic proteins, oxidants and enzymatic activities). The pattern of epithelial disruption and injury seen in this model system is strikingly reminiscent of the appearance of the airways mucosa in asthma (Naylor, 1962; Dunnill *et al.*, 1969; Elia *et al.*, 1988; Jeffery *et al.*, 1989). Thus it may be an appropriate system with which to investigate the mechanism(s) of eosinophil-dependent injury in this disease, and also to investigate pharmacological manipulations to promote salvage of the airways mucosa.

We have not made a systematic study of all of the possible leucocyte-derived mediators and their effects on epithelial protein flux. Our principal aim here was to establish whether this *in vitro* system could be used as a model of airway mucosal injury. Nevertheless, we took the opportunity to investigate certain characteristics of the interaction between leucocytes and the epithelium.

Our experiments with the antioxidant 5-lipoxygenase inhibitor AA-861 (Ashida *et al.*, 1983) indicate that leukotrienes are unlikely to play a significant role in the augmentation of epithelial permeability associated with eosinophil-dependent cellular injury. However, it seems plausible that they might contribute to enhanced epithelial permeability *in vivo* by promoting transendothelial plasma leakage, sub-mucosal oedema and an increase in subepithelial hydrostatic pressure capable of uncoupling epithelial tight junctions (Persson, 1986a,b; 1988; Persson *et al.*, 1990). Although the antioxidant properties of AA-861 suggest that oxidants may not be involved in this model of cell injury, it is clear that a rigorous appraisal should investigate their putative involvement as mediators of mucosal injury. In particular, it would be of importance to assess their involvement in the activation of proteinases (see later).

The filter masking studies indicate that close proximity between eosinophils and their cellular targets may be necessary to initiate cellular injury to the epithelium and permit an increase in the permeability to albumin. This is a potentially attractive mechanism as it might account for the apparent integrity of the bronchial epithelium adjacent to activated eosinophils in the sub-mucosa of bronchial biopsies taken from patients with mild asthma (Djukanovic *et al.*, 1990). If cell-cell contact is necessary for injury to occur then this is similar to the mechanism of eosinophil-dependent parasite killing in which major basic protein and free radicals are believed to be the principal mediators (Butterworth *et al.*, 1979). However, we cannot fully exclude the possibility that one effect of the filters is to bind nonspecifically a mediator released from the eosinophils. Further experiments will be necessary to investigate this potentiality.

Eosinophil-derived major basic and cationic proteins have been incriminated as major effectors of epithelial injury in asthma. Whilst the purified proteins derived from human eosinophils are certainly capable of reproducing some of the effects of activated eosinophils, these actions are seen at concentrations of $30–200\,\mu g\,ml^{-1}$ and require several hours expo-

sure for their onset (Frigas *et al.*, 1980; Hastie *et al.*, 1987; Flavahan *et al.*, 1988; Motojima *et al.*, 1989). Assuming a concentration of 10 pg of these proteins per cell (Ayars *et al.*, 1989) it is clear that a substantial number of eosinophils activated for maximum degranulation would be necessary for these basic proteins to reproduce the effects seen in our experiments. This view is supported by the investigations performed with the basic amino acid polymer polyarginine (M_r 11 600) which was used as a model of the arginine-rich proteins of eosinophil granules. A significant increase in albumin flux was seen when tissues were exposed to a concentration of $1\,mg\,ml^{-1}$ for 180 min. However, no effect was seen when a lower concentration of polyarginine was used. Furthermore, the duplication of the eosinophil-dependent effect by neutrophils (which do not contain MBP) also argues against the view that MBP is uniquely responsible for epithelial injury.

At both of the concentrations tested the broad spectrum antiproteinase α_2-macroglobulin was an effective inhibitor of the increased bronchial permeability evoked by eosinophils. This effect was not due to prevention of eosinophil activation, indicating the most likely mechanism of action is that of inhibition of a released mediator. It is important to note that the starting concentrations of α_2-macroglobulin used may be significantly decreased after incubation with the eosinophils owing to the radical-catalysed inactivation of the antiproteinase (Reddy *et al.*, 1989). At present we have no means of knowing what was the actual effective concentration of the inhibitor. The extent of the inhibitory effect of α_2-macroglobulin suggests that a significant mechanism for the eosinophil-dependent enhancement of albumin flux may involve the expression of proteinases, although other contributory effects such as oxidant scavenging remain to be formally excluded. Guinea-pig and human eosinophils express a classical vertebrate collagenase which has specificity for types I and III (i.e. interstitial) collagen (Davis *et al.*, 1984). However, unlike neutrophils they do not express an elastase or a non-specific proteinase. Therefore, an objective of our future experiments will be to establish exactly which proteinase(s) may be a target for inhibition by α_2-macroglobulin. It will also be of interest to establish whether antiproteinases offer a new pharmacological approach to salvage of the airways mucosa in asthma.

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Adenosine receptors and nucleoside transport sites in cardiac cells

[1]F.E. Parkinson & [2]A.S. Clanachan

Department of Pharmacology, 9-70 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

1 Potential mechanisms responsible for the prominent depression of atrioventricular conduction by adenosine have been investigated in guinea-pig heart.

2 Adenosine A_1 receptors and nucleoside transport (NT) sites were identified and enumerated in cardiac myocytes, atrioventricular conduction cells and coronary endothelial cells in 10 μm sections by autoradiographical analysis of the binding of the A_1 selective antagonist 8-cyclopentyl-1,3-[^{3}H]-dipropylxanthine ([^{3}H]-DPCPX) and the NT ligand [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR), respectively.

3 Atrioventricular conduction cells were identified by acetylcholinesterase histochemistry and endothelial cells by von Willebrand factor immunohistochemistry.

4 Site-specific binding of [^{3}H]-DPCPX, when expressed as grains per cell nucleus was significantly higher (30 fold) in conduction cells than in surrounding myocytes. [^{3}H]-DPCPX site density on endothelial cells in adjacent coronary vessels was not significantly different from myocytes.

5 In contrast, autoradiography of [^{3}H]-NBMPR sites in these areas indicated that, relative to myocytes, conduction cells and endothelial cells were significantly enriched (2 fold and 4.5 fold, respectively) in NT sites.

6 The pronounced dromotropic effect of adenosine in guinea-pig heart is correlated with a higher density of adenosine A_1 receptors in atrioventricular conduction cells than in myocytes. The NT capacity of these cells, as estimated by [^{3}H]-NBMPR binding site density, is not increased in proportion to A_1 receptors.

Keywords: Adenosine receptors; cardiac myocytes; conduction cells; endothelial cells; nucleoside transport sites; acetylcholinesterase (AChE); cyclopentyldipropylxanthine (DPCPX); nitrobenzylthioinosine (NBMPR)

Introduction

As early as 1929, it was observed that adenosine has negative chronotropic and dromotropic effects in heart; of the species studied, the effect on the sinoatrial node was predominant in dog, cat and rabbit while the atrioventricular nodal depression was predominant in guinea-pig (Drury & Szent-Gyorgi, 1929). In man, both sinus rate and atrioventricular conduction are depressed by the intravenous administration of adenosine and this is of therapeutic benefit in some forms of paroxysmal supraventricular tachycardia (DiMarco *et al.*, 1983). Additionally, it has been proposed that conduction delays, seen in cases of ischaemia and hypoxia, may be due to adenosine released from cardiac cells under hypoxic stress (Belardinelli *et al.*, 1980; West & Belardinelli, 1985; Clemo & Belardinelli, 1986a,b; Wesley & Belardinelli, 1989).

Adenosine is able to eliminate action potentials in sinoatrial and atrioventricular nodal cells (West & Belardinelli, 1985; Clemo & Belardinelli, 1986a). In contrast, adenosine shortens the action potential duration in atrial myocytes (Belardinelli *et al.*, 1983b), and, in the presence of compounds which increase intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) such as isoprenaline, in ventricular myocytes (Belardinelli *et al.*, 1984). These effects of adenosine are mediated by cell surface receptors of the A_1 subtype (Clemo & Belardinelli, 1986a); one resultant of stimulation of these receptors in atrial cells is an increase in potassium conductance that produces hyperpolarization (Sperelakis, 1987). Nucleoside transport inhibitors, such as dipyridamole and nitrobenzylthioinosine (NBMPR), potentiate, whereas the adenosine receptor antagonist, aminophylline, inhibits the cardiovascular actions of adenosine (Belardinelli *et al.*, 1980; 1982).

Adenosine A_1 receptors are present in low density in cardiac tissue. Estimates of the maximum site density are about 15–30 fmol mg^{-1} protein in rat (Linden *et al.*, 1985; Martens *et al.*, 1987), guinea-pig (Wu *et al.*, 1989; Froldi & Belardinelli, 1990), bovine (Lohse *et al.*, 1985), pig (Leid *et al.*, 1988) and diseased human (Böhm *et al.*, 1989) cardiac preparations. Since the density of A_1 receptors has not been determined in cells of the impulse conduction system, it is possible that increased receptor density on these cells may account for the predominant chronotropic and dromotropic effects of adenosine in heart.

Another possibility is that altered adenosine metabolism, for example decreased transport of adenosine into conduction cells, may allow increased adenosine-mediated effects in these areas. In support of this idea, we have shown in previous studies that nucleoside transport sites identified with [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR) have a greater site density in coronary endothelial cells than in ventricular cardiac myocytes (Parkinson & Clanachan, 1989a,b). Other cell types within heart may also exhibit differences in transporter density.

Other possible factors that could contribute to the enhanced sensitivity of conduction cells to adenosine include increased receptor affinity for adenosine, increased coupling efficiency between receptors and transduction mechanisms, or the dependence of conduction cells upon slow Na^+-Ca^{2+} channels for depolarization.

In this study, we examined whether guinea-pig atrioventricular conduction cells had altered receptor or nucleoside transporter densities relative to other cardiac myocytes. At present, it is not possible to purify conduction cells, in amounts sufficient for binding analysis, separate from the other cellular components of heart. Therefore these cell types were identified histologically and autoradiography was performed with the A_1 receptor antagonist, 8-cyclopentyl-1,3-[^{3}H]-dipropylxanthine ([^{3}H]-DPCPX) and the nucleoside transport inhibitor [^{3}H]-NBMPR. In this way, site densities of receptors and

[1] Present address: Department of Pharmacology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.
[2] Author for correspondence.

transport inhibitory sites in conduction cells and endothelial cells relative to other cardiac myocytes were determined.

Methods

Guinea-pig hearts were rapidly removed, and from each heart the area of right atrium containing coronary sinus and inferior vena cava, plus the interatrial septum and the upper part of the interventricular septum was isolated as this preparation contains atrioventricular conduction cells (Clemo & Belardinelli, 1986a). The tissues were embedded in sectioning media (20 parts OCT Compound: 10 parts distilled water: 7 parts tragacanth gum), frozen in isopentane cooled with liquid nitrogen and stored at −70°C for up to two weeks. Sections (10 μm) were cut (−22°C) parallel to the long axis of the heart with a cryostat (International Equipment Company model CT1), mounted onto gelatin-coated slides, desiccated overnight at 4°C and then used immediately or stored at −70°C for up to two weeks.

Acetylcholinesterase histochemistry

Cardiac conduction cells can be reliably identified by positive staining for acetylcholinesterase (AChE) (Bojsen-Møller & Tranum-Jensen, 1971; Anderson, 1972). Vagal innervation of cardiac tissue is concentrated on impulse conducting cells within the atria, and both the nerves and the innervated cells are characterized by having large quantities of AChE. The procedure used for detecting AChE in these cells was that described by El-Badawi & Schenk (1969).

Von Willebrand factor immunohistochemistry

Von Willebrand factor is a component of clotting factor VIII and is a reliable marker for endothelial cells (Jaffe, 1984). Sections were first preincubated for 5 min in ice-cold physiological salt solution (PSS) of the following composition (mM): NaCl 137, Na_2HPO_4 6, KCl 2.7, KH_2PO_4 1.5, $CaCl_2$ 0.9 and $MgCl_2$ 0.5; pH 7.4. This was followed by 10 min in 0.4% formaldehyde in ice-cold PSS and two subsequent 5 min washes in ice-cold PSS. Sections were then incubated in presoak buffer (5% bovine serum albumin (BSA), 0.05% NaN_3, 0.3% Triton X 100 in PSS) for 1 h at 20°C. Washes, at this and later steps, were performed with 1% BSA and 0.05% NaN_3 in PSS, 2 × 5 min at 20°C. Sections were then incubated with the primary antibody, rabbit polyclonal antibody to human von Willebrand factor (1:1600 dilution in 1% BSA, 0.05% NaN_3, 0.3% Triton X 100 in PSS), for 1 h at 20°C. Sections were washed then incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (1 : 400 dilution in the same buffer used for primary antibody), for 1 h at 20°C. Sections were washed again then incubated with horseradish peroxidase avidin D (1:100 dilution in the same buffer used for washes), for 1 h at 20°C. Sections were washed again then incubated with 0.05% diaminobenzidine and 0.001% H_2O_2 in PSS, for 5 min at 20°C in the dark. Sections were washed again, then dehydrated and mounted.

[^{3}H]-nitrobenzylthioinosine binding

Sections were preincubated in ice-cold PSS for 5 min, then incubated in 10 nM [^{3}H]-NBMPR for 30 min at 20°C. Fixation of the tissue sections (10 min) resulted from the addition of neutral buffered formaldehyde (final conc. 0.4%) to the incubation medium for 10 min. Sections were washed twice for 5 min with ice-cold PSS and rinsed three times with ice-cold distilled water. Sections were blown dry with cold air and desiccated overnight. The amount of [^{3}H]-NBMPR that bound in the presence of 30 μM dipyridamole was termed the non-specific binding component.

8-Cyclopentyl-1,3-[^{3}H]-dipropylxanthine binding

Sections were preincubated in PSS, 4°C for 5 min, then incubated in 5 nM [^{3}H]-DPCPX for 2 h at 20°C. The results of preliminary experiments indicated that [^{3}H]-DPCPX bound to guinea-pig cardiac membranes with a K_D value of 1.5 nM (0.6–3.9). Tissue sections were then processed as described for [^{3}H]-NBMPR binding assays. Total [^{3}H]-DPCPX binding was defined as that which occurred in the presence of 5 nM [^{3}H]-DPCPX, 0.01% CHAPS and 5.5 units/assay adenosine deaminase. Non-specific binding was defined as the amount of [^{3}H]-DPCPX that remained bound in the presence of 2-chloroadenosine (100 μM).

Autoradiography

The autoradiographical technique used was modified from that described by Young & Kuhar (1979). Coverslips were prepared by dipping into Kodak NTB2 emulsion (diluted 1:1 with distilled water; 43°C) and stored over desiccant, protected from light, for 12–48 h. The emulsion-coated coverslips were glued at one end to slides containing tissue sections previously exposed to [^{3}H]-NBMPR or [^{3}H]-DPCPX as described above. The slides and coverslips were held tightly together with squares of teflon and binder clips and kept at 4°C for 2–3 ([^{3}H]-NBMPR) or 5–6 ([^{3}H]-DPCPX) weeks. This hinged coverslip method avoided the immersion of the sections in emulsion and the associated risk of diffusion of the ligand from the section prior to the generation of the autoradiogram.

To develop the coverslips the binder clips and teflon squares were removed, and the coverslips were gently propped up from the tissue sections. The emulsions were developed in Dektol Developer (diluted 1:1 with water) for 2 min, then rinsed in water for 10 s, fixed in Kodak Fixer for 5 min and washed in water for 5 min. The sections were then stained with hematoxylin and eosin and the coverslips were permanently mounted. With this technique correct alignment of the emulsion with the tissue sections was maintained.

Quantitative autoradiography

Site densities for [^{3}H]-NBMPR and [^{3}H]-DPCPX were quantitated by counting silver grains in small areas (700 μm^2) on each cardiac section. Sections were randomized and counted by an independent observer who was blind to the experimental treatment of the section (NBMPR or DPCPX; total or nonspecific binding condition). For comparison of conduction cells with other cardiac myocytes, silver grains were counted in three areas of each cell type and three areas of slide background (non-tissue). Background grains were subtracted from tissue values; specific binding was then calculated as the difference between total binding and nonspecific binding in adjacent sections. In order to account for potential cell size differences between areas, cell nuclei in each chosen field were also counted; specific binding site densities were then calculated as grains per cell nucleus. The small difference between grains per nucleus and grains per cell due to the small percentage of bi-nucleated, and larger, cells was not considered significant. Similar methods were used to compare binding site densities on myocytes and coronary endothelial cells. The significance of the difference in grain densities between cell types was determined by two-tailed Student's paired t test and judged to be statistically significant when $P < 0.05$.

Materials

Compounds and radioligands for these studies were obtained from the following sources. [^{3}H]-nitrobenzylthioinosine (Sp. Act. 37 Ci mmol^{-1}), from Moravek Biochemicals, CA, U.S.A. and repurified before use by high performance liquid chroma-

tography (h.p.l.c.); 8-cyclopentyl-1,3-[^{3}H]-dipropylxanthine (Sp. Act. 120 Ci mmol^{-1}), from New England Nuclear, Boston, MA, U.S.A.; rabbit polyclonal anti-human von Willebrand factor, biotinylated anti-rabbit IgG and horseradish peroxidase avidin D, from Dimension Laboratories Inc., Mississauga, Ont., Canada; dipyridamole, diaminobenzidine, CHAPS(3 - [(3 - cholamidopropyl) dimethyl - ammonio] - 1 - propane-sulphonate), and adenosine deaminase type VII, from Sigma Chemical Co., St. Louis, MO, U.S.A.; 2-chloroadenosine, from Research Biochemicals Inc., Natick, MA, U.S.A.; glue (Super Bonder 495), from Loctite Corporation, Newington, CT, U.S.A.; Kodak NTB2 nuclear track emulsion, Dektol Developer and Kodak Fixer, from Calgary Photo, Edmonton, Alta., Canada; coverslips (25 × 60 mm, Corning No. 1), OCT compound and tragacanth gum from Fisher Scientific, Edmonton, Alta., Canada.

Results

Acetylcholinesterase histochemistry

Conduction cells, cholinergic nerves and cholinergic ganglion cells were clearly identified by positive staining for AChE (Figure 1a, 2a). Also, conduction cells were not as tightly packed as myocytes. Conduction cells, including atrioventricular node, His bundle and left and right bundle branches,

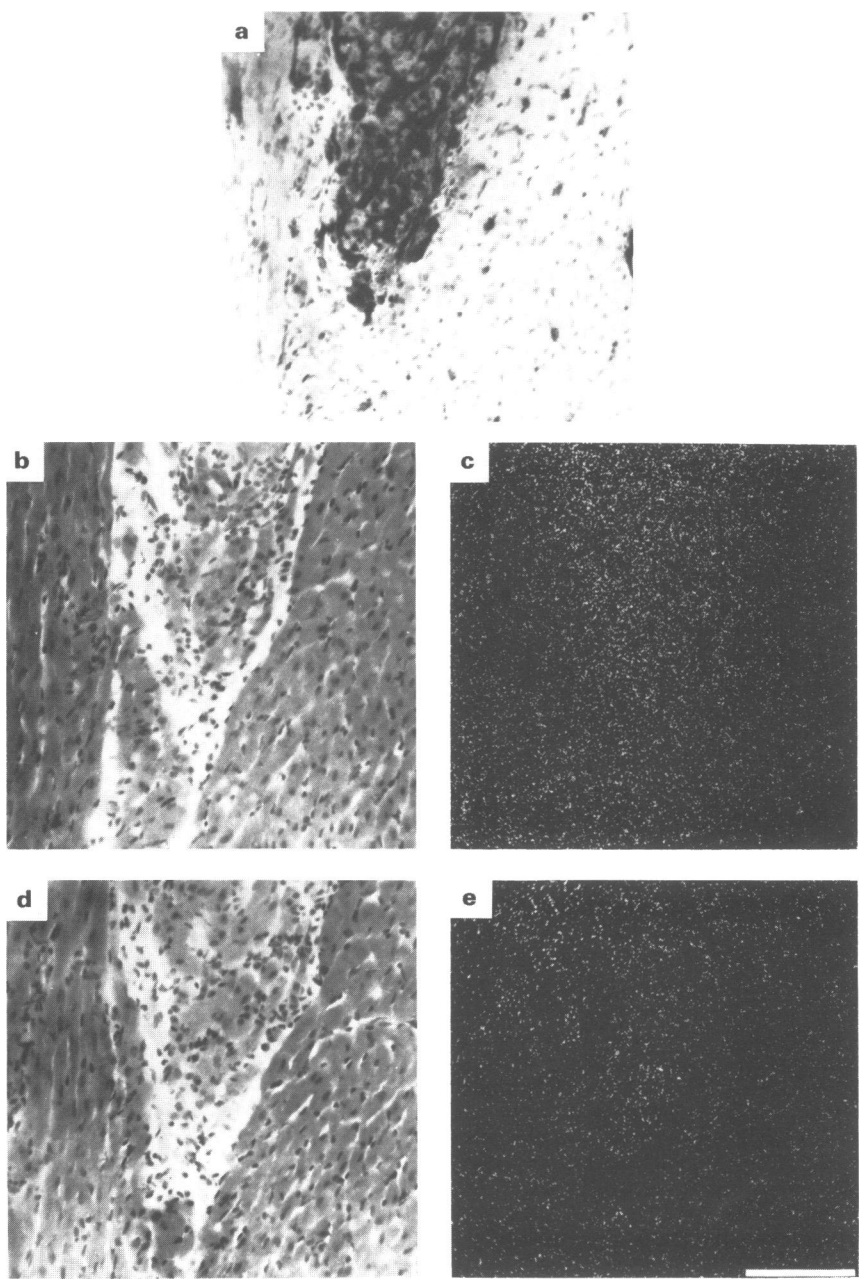

Figure 1 Autoradiographical localization of [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR) binding to atrioventricular conduction cells from guinea-pig. (a) Photomicrograph of conduction cells, identified by dark staining for acetylcholinesterase (AChE). An adjacent section stained for hematoxylin and eosin is shown in (b). A dark field photomicrograph of the same section, (c), shows the distribution of total [^{3}H]-NBMPR binding sites. The density of silver grains over conduction cells and ventricular myocytes appears similar. However, quantitative autoradiography indicated a 2 fold higher density over conduction cells compared to myocytes. An adjacent section, stained for hematoxylin and eosin is shown in (d). This section was used to determine the distribution of silver grains in the non-specific [^{3}H]-NBMPR binding condition and the dark field photomicrograph is shown in (e). A uniform distribution of silver grains is evident over the two cell types. Bar represents 100 μm.

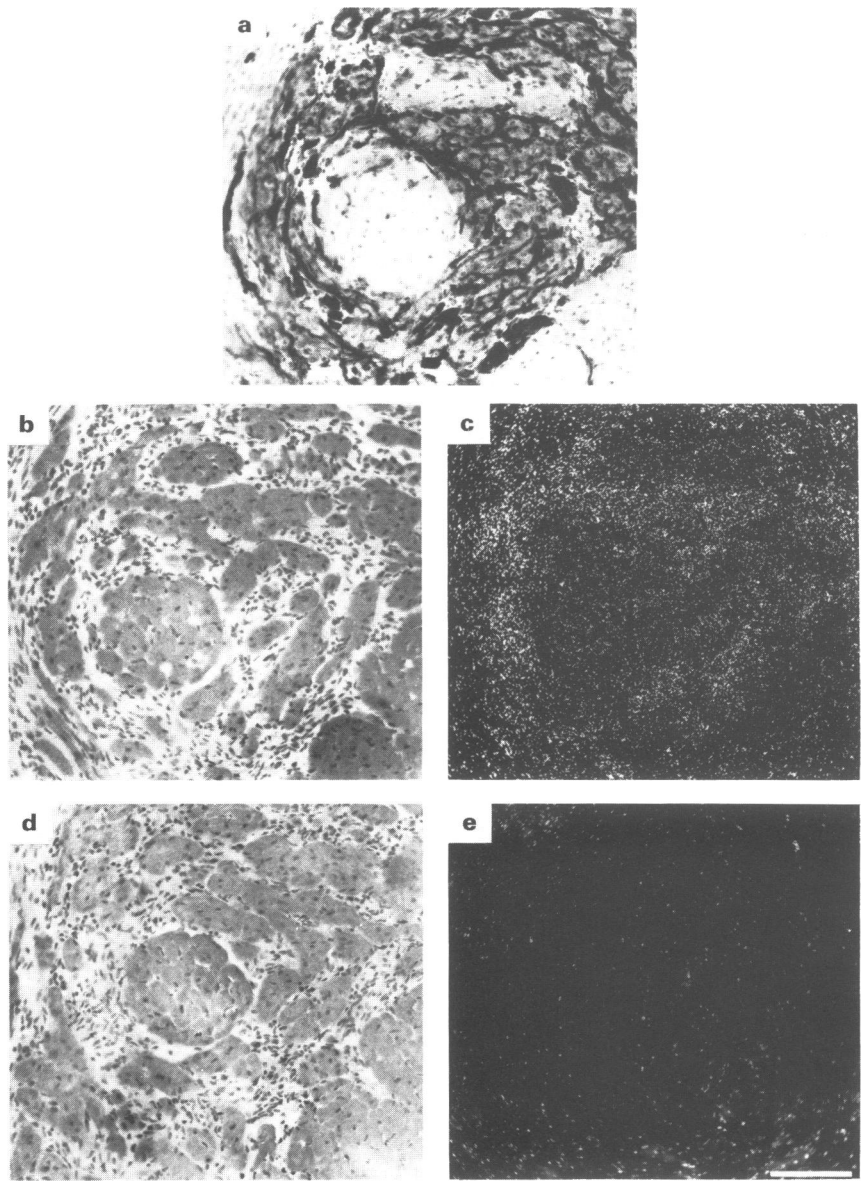

Figure 2 Autoradiographical localization of 8-cyclopentyl-1,3-[^{3}H]-dipropylxanthine ([^{3}H]-DPCPX) binding sites in atrioventricular conduction cells from guinea-pig heart. (a) Photomicrograph of a section stained for acetylcholinesterase; darkly stained cells are conduction cells. An adjacent section, stained with hematoxylin and eosin, is shown in (b). This section was used to determine the distribution of the total [^{3}H]-DPCPX binding sites and the dark field photomicrograph of this section is shown in (c). The grain density is greater over the conduction cells than over ventricular myocytes. An adjacent section, used for the non-specific [^{3}H]-DPCPX binding condition, is shown stained with hematoxylin and eosin in (d). The dark field photomicrograph of this section is shown in (e). The grain density is uniform over conduction cells and ventricular myocytes. Bar represents 100 μm.

were present in approximately 100 sections. The atrioventricular node was often not clearly evident due to the difficulty in obtaining the optimal plane of sectioning. The His bundle was clearly identified in 70% or more of positively staining sections depending on the plane of sectioning.

Von Willebrand factor immunohistochemistry

Immunoperoxidase staining for von Willebrand factor was positive for endothelial cells of blood vessels and the endocardial lining of heart (Figure 3c,f,i).

[^{3}H]-nitrobenzylthioinosine binding

Specific [^{3}H]-NBMPR binding, represented by the difference in density (grains/cell nucleus) of silver grains between total (Figure 1c) and nonspecific (Figure 1e) binding autoradio-

Table 1 Comparison of the site-specific binding of [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR) and 8-cyclopentyl-1,3-[^{3}H]-dipropylxanthine ([^{3}H]-DPCPX) to cardiac myoctyes (Myo) and atroventricular conduction cells (CC) in 10 μm sections of guinea-pig heart

	n	*Myocytes*	*Conduction cells*	*CC/Myo ratio*	P
[^{3}H]-NBMPR	10	11.9 ± 5.1	24.5 ± 5.5	2	0.024
[^{3}H]-DPCPX	19	0.7 ± 3.0	20.3 ± 7	30	0.0002

Site densities are expressed as the number of silver grains divided by the number of cell nuclei. Data are means ± s.e.mean of grain densities in triplicate areas from *n* sections from 5 hearts. *P* values for the significance of the differences between cell types were calculated by Student's paired *t* test.

grams, was slightly higher (2 fold; $P = 0.024$, Table 1) over atrioventricular conduction cells, identified in adjacent sections by heavy staining for AChE (Figure 1a), than over cardiac myocytes. Endothelial cells, identified by immunoperoxidase staining for von Willebrand factor (Figure 3c,f,i), have a much greater grain density, and hence [^{3}H]-NBMPR binding site density (Figure 3b, e, h), than cardiac myocytes (4.5 fold; $P = 0.003$) (Table 2). The high density of [^{3}H]-NBMPR binding sites appeared to be associated with endothelial cells of the endocardium, venules, arterioles, and the aorta, but was not associated with aortic smooth muscle cells, cholinergic nerves, or adipose.

8-Cyclopentyl-1,3-[^{3}H]-dipropylxanthine binding

The distribution of silver grains in the total binding autoradiograms (Figure 2c) was non-uniform in contrast to their

Table 2 Comparison of the densities of site-specific binding of [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR) and 8-cyclopentyl-1,3-[^{3}H]-dipropylxanthine ([^{3}H]-DPCPX) to cardiac myocytes (Myo) and coronary endothelial cells (Endo) in 10 μm sections of guinea-pig heart

	n	*Myocytes*	*Endothelial cells*	*Endo/Myo ratio*	P
[^{3}H]-NBMPR	8	25.1 ± 5.4	114.8 ± 18.2	4.6	0.003
[^{3}H]-DPCPX	6	6.7 ± 2.1	14.1 ± 7.6	2.1	0.270

Site densities are expressed as the number of silver grains divided by the number of cell nuclei. Data are means ± s.e.mean of grain densities in triplicate areas from *n* sections from 4 hearts. *P* values for the significance of the differences between cell types were calculated by Student's paired *t* test.

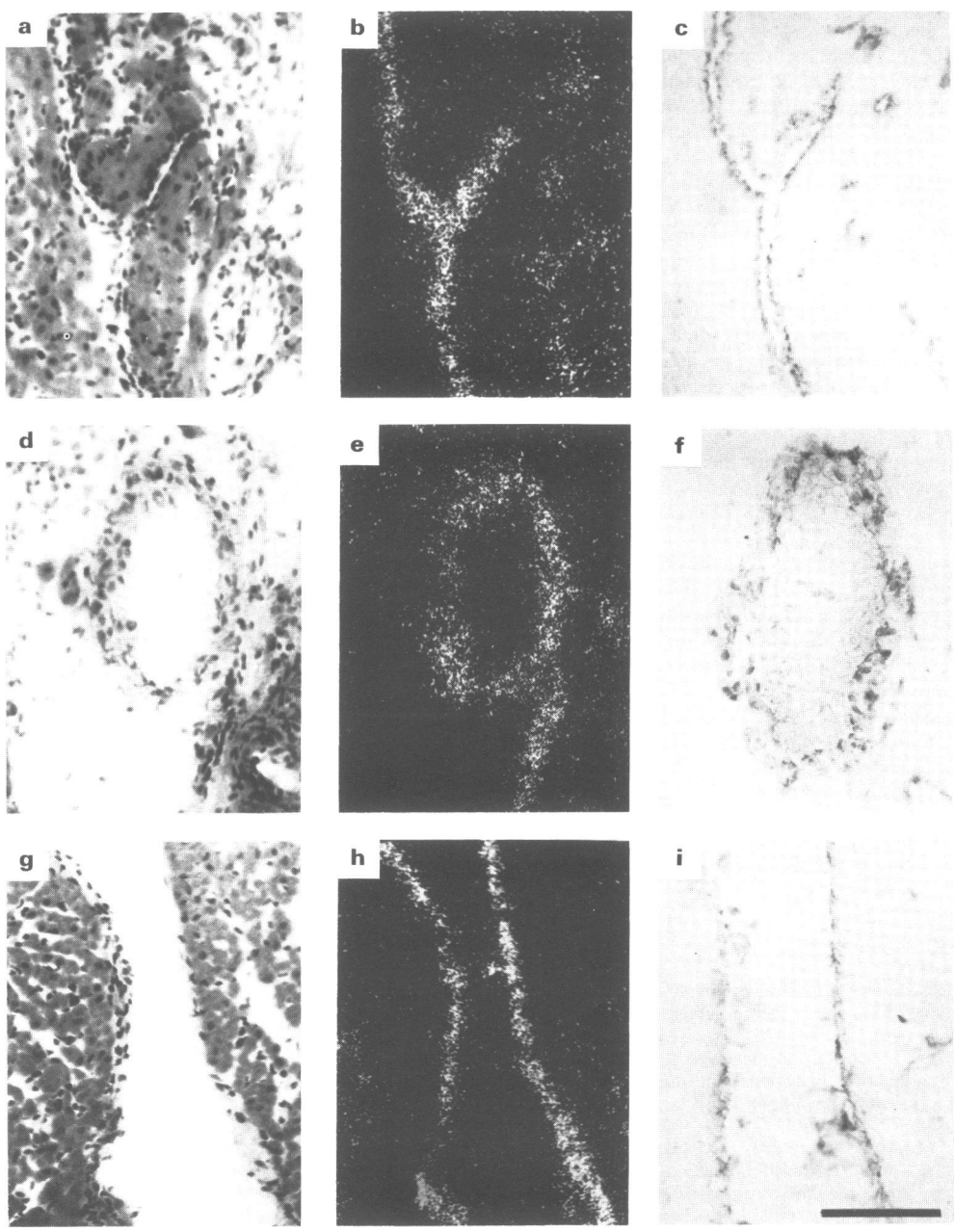

Figure 3 Autoradiographical localization of [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR) binding to cardiac endothelial cells. Bright field photomicrographs of sections stained with hematoxylin and eosin show a blood vessel cut in longitudinal section (a), a blood vessel cut in cross section (d), or endocardial lining (g). Dark field photomicrographs of the same sections show the distributions of total [^{3}H]-NBMPR binding sites associated with these structures (b, e, h). Adjacent sections were used to identify endothelial cells by use of immunoperoxidase staining for von Willebrand factor (c, f, i). Endothelial cells associated with these structures have high densities of [^{3}H]-NBMPR binding sites. Sections used to determine non-specific binding showed uniform grain distributions. Bar represents 100 μm.

distribution in the nonspecific binding condition (Figure 2e). The density of [^{3}H]-DPCPX binding sites was significantly greater in conduction cells (30 fold increase in grain density; $P = 0.0002$, Table 1) identified by dark staining regions for AChE in adjacent sections (Figure 2a), than in cardiac myocytes. The highest density of binding sites for the adenosine A_1 receptor antagonist was associated with conduction cells. No other areas of high site-specific binding of [^{3}H]-DPCPX were observed; the binding to endothelial cells was not significantly different from cardiac myocytes.

Discussion

The main finding of this study is that a higher density of adenosine A_1 receptors exists on atrioventricular conduction cells, indicated by a 30 fold increase in autoradiographic silver grain density, than on cardiac myocytes. Additionally, there is a significantly higher density of NT sites in atrioventricular conduction cells (2 fold increase in grain density) and endothelial cells (4.5 fold increase in grain density) relative to myocytes. The latter finding agrees with our previous low resolution Ultrofilm ^{3}H studies (Parkinson & Clanachan, 1989a,b).

For the localization of adenosine receptors in this study, the radioligand [^{3}H]-DPCPX was used as it is considered relatively selective for adenosine A_1 receptors. It has approximately 500 fold greater affinity for A_1 receptors than for A_2 receptors (Lee & Reddington, 1986; Bruns *et al.*, 1987; Lohse *et al.*, 1987). At the concentration of [^{3}H]-DPCPX used (5 nM), only A_1 receptor binding was detectable and these receptors should have been almost saturated. Therefore our finding of very low silver grain density associated with cardiac myocytes, considering the longer exposure time and the higher specific activity of the ligand relative to [^{3}H]-NBMPR, is in agreement with the low A_1 receptor density found in other cardiac preparations (Linden *et al.*, 1985; Lohse *et al.*, 1985; Martens *et al.*, 1987; Leid *et al.*, 1988; Wu *et al.*, 1989; Froldi & Belardinelli, 1990).

Early work by Belardinelli and co-workers (Belardinelli *et al.*, 1980) showed that the conduction delay produced by adenosine was specific to the atria-His bundle interval, while the His bundle-ventricles interval was unchanged. Subsequently, Clemo & Belardinelli (1986a) found that the effects of adenosine on impulse conduction were predominantly associated with a particular subset of cells (N cells) of the atrioventricular node. Our results, primarily with His bundle and branching bundle cells, show these cells to have increased numbers of A_1 receptors in comparison to atrial and ventricular myocytes and it is possible that N cells have an even greater A_1 receptor density.

In the last few years, several nucleoside transport (NT) systems have been identified (Paterson *et al.*, 1987). [^{3}H]-NBMPR is a useful ligand for the facilitated diffusion system that has high sensitivity to NBMPR. Transport studies with guinea-pig isolated ventricular myocytes have demonstrated that this system is a major component of nucleoside entry in these cells (Heaton & Clanachan, 1987). However, it is possible that other cell types in heart may have additional transport systems, not demonstrable by low nanomolar concentrations of NBMPR.

We have shown that putative NBMPR-sensitive transporters are found in the greatest density in endothelial cells associated with coronary vessels and the endocardial lining. Guinea-pig coronary endothelial cells possess a barrier function to the movement of adenosine between interstitial and intravascular spaces (Nees *et al.*, 1985); this appears due to the high density of nucleoside transporters in these cells in addition to their high catabolic activity for adenosine. Thus, they may be important both in protecting cardiac myocytes from the depressant effects of circulating adenosine and in scavenging adenosine that is released from cardiac myocytes during hypoxic stress.

Previous studies have shown that nucleoside transport inhibitors, such as dipyridamole and NBMPR, potentiate the conduction delay produced by adenosine (Belardinelli & Isenberg, 1983a). From the present autoradiographical analysis of NBMPR binding sites it would appear that this is, in part, due to inhibition of adenosine transport into conduction cells. However, given the higher density of binding sites on endothelial cells, it is likely that these cells also have a significant role. Inhibition of adenosine transport into nearby endothelial cells may contribute to the potentiation of the effects of exogenous adenosine on conduction cells that is seen with these compounds.

In conclusion, the results of this study, showing greatly increased A_1 receptor density and a small increase in nucleoside transporter density, are consistent with increased effects of adenosine in conduction cells versus other cardiac myocytes. However, it is important to recognize that action potentials of nodal cells are different from those of other cardiac myocytes. Therefore, the increased adenosine-mediated effects in conduction cells may be due to the different ionic basis of the action potentials in conjunction with increased receptor density.

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Neuroprotective activity of chlormethiazole following transient forebrain ischaemia in the gerbil

[1]Alan J. Cross, Julie A. Jones, Helen A. Baldwin & A. Richard Green

Astra Neuroscience Research Unit, 1 Wakefield Street, London WC1N 1PJ

1 The effect of chlormethiazole, and other drugs which potentiate γ-aminobutyric acid (GABA) function on delayed neuronal death in the hippocampus has been examined in the gerbil.

2 Chlormethiazole (100 mg kg^{-1}, i.p.) and two other drugs previously reported to be neuroprotective (dizocilpine, 3 mg kg^{-1}, i.p. and ifenprodil, 4 mg kg^{-1}, i.p.) were all found to prevent neurodegeneration of CA1/CA2 neurones in the hippocampus when given 30 min before a 5 min episode of bilateral carotid artery occlusion.

3 Chlormethiazole (100 mg kg^{-1}) was neuroprotective when given up to 3 h, after the ischaemic episode.

4 Given 1 h after the cartoid artery occlusion, chlormethiazole produced significant protection against hippocampal neurodegeneration at a dose of 50 mg kg^{-1}, but not at 25 mg kg^{-1}.

5 Phenobarbitone (100 mg kg^{-1}, i.p.) and Saffan (alphaxalone, 45 mg kg^{-1} plus alphadalone, 15 mg kg^{-1}, i.p.) were not protective when given 1 h after the ischaemic episode while pentobarbitone (30 mg kg^{-1}, i.p.) had a modest protective effect.

6 Evidence is presented to show that neither the operating procedure nor the chlormethiazole administration lowered rectal or cerebral temperature.

7 The data suggest that chlormethiazole may be a useful treatment in the prevention of neurodegeneration following stroke or cardiac arrest.

Keywords: Ischaemia; neuroprotection; chlormethiazole; hippocampal neurodegeneration; barbiturates; dizocilpine; ifenprodil

Introduction

Transient forebrain ischaemia can be induced in the Mongolian gerbil by bilateral occlusion of the common cartoid arteries (Crockard *et al.*, 1980). Following a brief episode of ischaemia a characteristic and highly specific pattern of neuronal degeneration is observed, with the large neurones of the hippocampal CA1/CA2 subfields being particularly sensitive (Brown *et al.*, 1979). The degeneration of these neurones develops following a period of 24 h during which no obvious morphological changes are apparent, a process which has been termed 'delayed neuronal death' (Kirino, 1982).

A large number of studies have examined the efficacy of various pharmacological agents in preventing the delayed degeneration of hippocampal CA1 neurones. Most prominent amongst these agents are antagonists of excitatory amino acid receptors, particularly the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) preferring subtypes (see Meldrum, 1990 for review). It is clear that NMDA receptor antagonists exert a powerful neuroprotective action, consistent with the concept that release of glutamic acid (or another excitatory amino acid) mediates the delayed neuronal death. It is thought likely that glutamic acid initiates an 'excitotoxic' process (Olney, 1988) involving excessive membrane depolarization and pathological rises in internal calcium ion concentrations (Simon *et al.*, 1984).

Many other drugs have been examined in the gerbil model of transient forebrain ischaemia. Several compounds which enhance inhibitory processes in the hippocampus have been shown to have some neuroprotective effect, particularly when treatment is initiated around the time of the ischaemic episode (Kirino *et al.*, 1986; De Leo *et al.*, 1988; Alps *et al.*, 1988; Taft *et al.*, 1989). Chlormethiazole is an anticonvulsant, sedative and hypnotic drug (Ogren, 1986; Green & Murray, 1989), which acts at the γ-aminobutyric acid (GABA) receptor both to potentiate the effects of GABA and to modulate channel opening directly (Harrison & Simmonds, 1983; Moody & Skolnick, 1989; Cross *et al.*, 1989). In the present study we have examined the effects of chlormethiazole in the gerbil model of transient forebrain ischaemia, and compared these effects with those of other compounds which modulate $GABA_A$ receptor function.

Some of these studies have been described in a communication to the British Pharmacological Society (Cross *et al.*, 1991).

Methods

Animals and operative procedures

Male Mongolian gerbils weighing 60–80 g were used. They were maintained on a 12 h light/12 h dark cycle (lights on: 07 h 00 min–19 h 00 min) and given food and water *ad libitum*.

Animals were anaesthetized with Saffan (alphaxalone 45 mg kg^{-1} plus alphadalone 15 mg kg^{-1}, i.p.) placed on an electrically heated mat and operated on while under heating lamps to maintain body temperature at 37°C (continuously monitored by rectal probe). The carotid arteries were exposed by cervical midline incision, separated from the vagus nerve and occluded for 5 min with aneurism clips, the effectiveness of the occlusion being confirmed visually.

Following the period of ischaemia the clips were removed and the incision sutured. Sham operated animals had their arteries exposed but not occluded.

In some experiments halothane anaesthesia was employed. In this case anaesthesia was induced with 5% halothane in 70% N_2O/30% O_2, and maintained with 1.5% halothane in 70% N_2O/30% O_2.

Measurement of hippocampal damage

Four days following carotid artery occlusion the animals were anaesthetized with pentobarbitone (300 mg kg^{-1}, i.p.) and perfused transcardially with 10% formaldehyde in 5% sucrose. Brains were removed and placed in 5% formaldehyde in 30%

[1] Author for correspondence.

sucrose at 4°C for 2 days. Coronal 20 μm sections were cut through the hippocampus with a cryostat and stained with cresyl violet. Sections from each brain taken at the level of the hippocampus (analogous to level A 3290 μm of the rat according to the atlas of Konig & Klippel, 1963) were used to assess the extent of damage to hippocampal neurones. All assessments of histological sections were made by an observer who was unaware of the drug treatment of the animals.

The extent of neurodegeneration in the hippocampal CA1/CA2 region was routinely determined by a method similar to that described by Gill *et al.* (1987). One section from each brain was photographed and measurements taken from both hippocampi. The total length of the CA1/CA2 region containing degenerated neurones was measured. This was possible as the degeneration occurred in discrete patches (see Gill *et al.*, 1987). The length of the degenerated CA1/CA2 region was expressed as a percentage of the entire CA1/CA2 region (i.e. % degeneration), in this way correcting for any minor differences in the level of sectioning through the hippocampus.

Brain temperature measurement

Brain temperature was measured with a thermocouple probe (29 gauge, Model 3AT-12, Physitemp). Gerbils were anaesthetized and a 7.5 mm stainless steel cannula (23 gauge) implanted above the hippocampus (stereotaxic coordinates from bregma; 0 mm anterior, 2 mm lateral, 1 mm ventral). The following day the gerbils were anaesthetized with Saffan and the temperature probe inserted into the cannula. Bilateral carotid artery occlusion was performed as above, and 1 h post-occlusion the animals received chlormethiazole (100 mg kg^{-1}, i.p.). Throughout the experiment (i.e. 2 h) rectal temperature was maintained at 37°C, thus mimicking exactly the neuroprotection study.

Drugs

Drugs were obtained from the following sources (in parentheses): Saffan, (Veterinary Drug Co, U.K.), sodium pentobarbitone (Sagatal, RMD Animal Health Ltd, U.K.), phenobarbitone (Sigma London Ltd, U.K.), dichlormethiazole ethane disulphonate (Astra Research Centre, Sodertalje, Sweden). Ifenprodil and dizocilpine (MK-801) were generous gifts from Synthelabo Recherche, France and Merck, Sharp & Dohme, U.K. respectively. Drugs were administered intraperitoneally in isotonic saline, and all doses refer to the free base. Control animals received saline injections.

Statistics

Each set of data was evaluated by Student's t test or one way analysis of variance as appropriate. In the latter case, individual group differences were determined by use of adjusted t-tests with significance levels corrected by the Bonferroni method.

Results

Pretreatment with chlormethiazole and other drugs

Transient occlusion of the carotid arteries of the gerbil for 5 min resulted in marked degeneration of hippocampal CA1/CA2 neurones 4 days post-ischaemia (Figure 1). This damage did not extend into the CA3 region or the dentate gyrus. Pretreatment with dizocilpine (3 mg kg^{-1}, i.p.) or ifenprodil (4 mg kg^{-1}, i.p.) resulted in a significant reduction in the extent of damage to the CA1/CA2 pyramidal neurones (Figure 2). In most cases the CA1 region of animals pretreated with

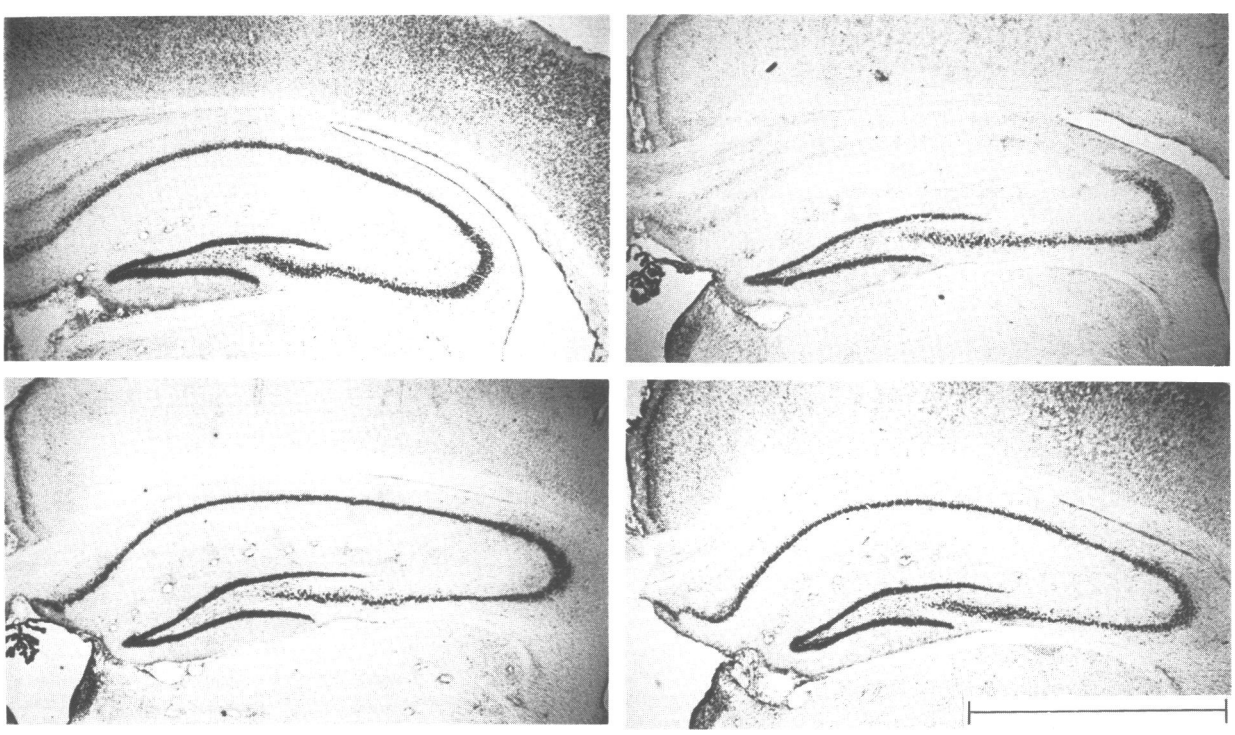

Figure 1 Neuroprotective effect of dizocilpine (3 mg kg^{-1}) and chlormethiazole (100 mg kg^{-1}) when given 30 min before 5 min bilateral carotid artery occlusion. Micrographs of the hippocampus (20 μm coronal sections) stained with Cresyl Violet, 4 days post-ischaemia. Upper left: control; upper right: ischaemia; lower right: chlormethiazole/ischaemia; lower left: dizocilpine/ischaemia. Note almost complete destruction of CA1/CA2 region in ischaemic hippocampus, and protection of this region by both chlormethiazole and dizocilpine. Scale bar = 2 mm.

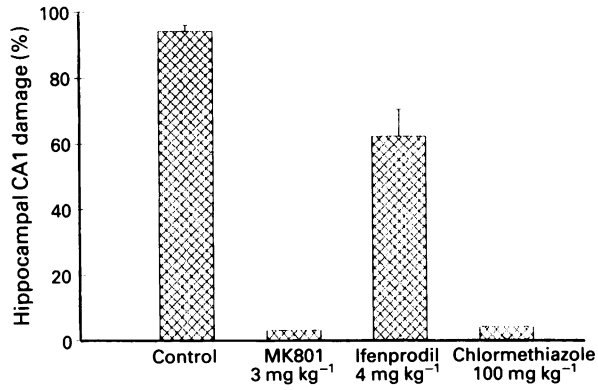

Figure 2 The effect of chlormethiazole, dizocilpine (MK-801) and ifenprodil on hippocampal CA1/CA2 degeneration following 5 min transient ischaemia. All drugs were administered as i.p. injections 30 min before bilateral carotid artery occlusion; controls received saline. Columns represent mean with s.e.mean shown by vertical bars; $n = 6$–10. ANOVA $F = 143$, d.f. = 3, 26, $P < 0.01$. All treatment groups are significantly different from control ($P < 0.01$).

dizocilpine (30 min) showed no obvious damage (Figure 1). Pretreatment with chlormethiazole (100 mg kg^{-1}, i.p.) also resulted in marked protection of hippocampal CA1/CA2 neurones (Figure 2) and again no obvious histological damage

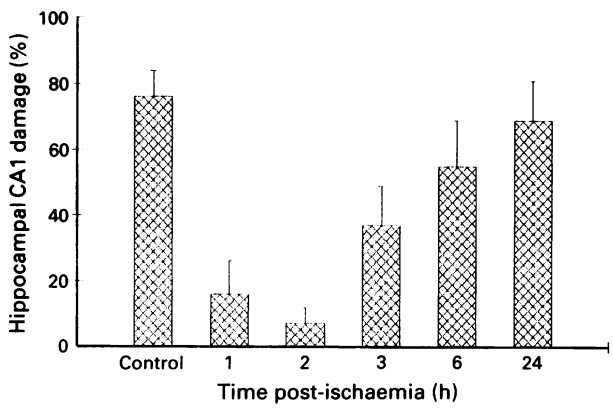

Figure 3 The effect of chlormethiazole on hippocampal CA1/CA2 pyramidal cell degeneration when administered post-ischaemia. Chlormethiazole (100 mg kg^{-1}) was administered i.p. in saline at the indicated times after 5 min bilateral carotid artery occlusion, controls received saline. Columns represent mean with s.e.mean shown by vertical bars, $n = 8$–24. ANOVA $F = 7.08$, d.f. = 5, 62, $P < 0.01$. Treatment groups at 1 h and 2 h ($P < 0.01$) and 3 h ($P < 0.05$) were significantly different from control.

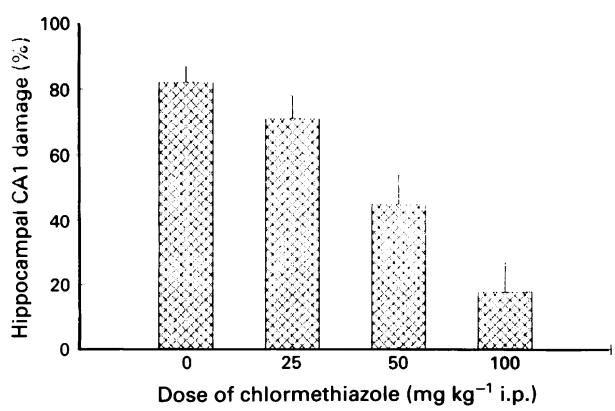

Figure 4 The effect of various doses of chlormethiazole on hippocampal CA1/CA2 degeneration following 5 min ischaemia. Chlormethiazole was administered by i.p. injection in saline 1 h post-ischaemia, controls received saline. Columns represent mean with s.e.mean shown by vertical bars; $n = 11$–12. ANOVA $F = 16.3$, d.f. = 3, 43, $P < 0.01$. Treatment with 50 and 100 mg kg^{-1} chlormethiazole produced significantly less damage ($P < 0.01$).

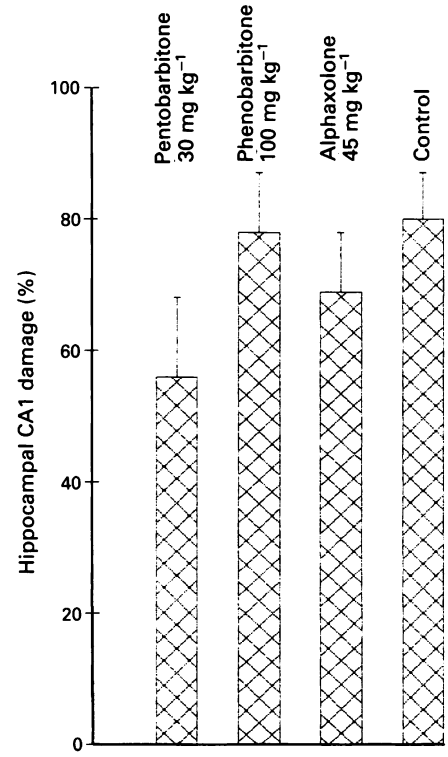

Figure 5 The effect of γ-aminobutyric acid (GABA)-potentiating drugs on hippocampal CA1/CA2 degeneration following 5 min ischaemia. Drugs were given i.p. in saline 1 h post-ischaemia, controls received saline. Columns represent mean with s.e.mean shown by vertical bars; $n = 6$–24. ANOVA $F = 3.06$, d.f. = 3, 56, $P < 0.05$. Treatment with phenobarbitone (100 mg kg^{-1}) or alphaxolone (45 mg kg^{-1} + 15 mg kg^{-1} alphadalone) did not significantly reduce hippocampal damage. Treatment with pentobarbitone (30 mg kg^{-1}) resulted in significantly less damage ($P < 0.05$).

was present in the hippocampus of most chlormethizole treated animals (Figure 1). This dose of chlormethiazole induced sedation but not loss of the righting reflex.

Treatment with chlormethiazole post-ischaemia

Chlormethiazole (100 mg kg^{-1}) produced a marked neuroprotective effect when given up to 3 h after the carotid artery

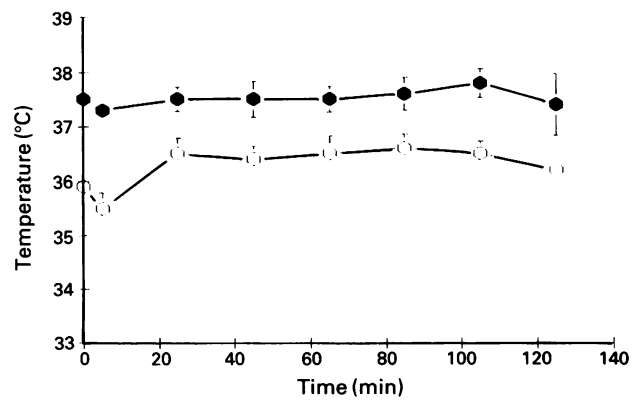

Figure 6 Brain and rectal temperature following transient forebrain ischaemia and subsequent chlormethiazole treatment. Rectal temperature (●) and hippocampal temperature (○) were measured as described in the text. Bilateral carotid artery occlusion was performed immediately after the first temperature measurement (t = 0) for 5 min; 60 min later (t = 65) animals received chlormethiazole (100 mg kg^{-1} i.p.). Rectal temperature was maintained between 37–38°C with heated mats and lamps. Symbols represent mean with s.e.mean shown by vertical bars; $n = 4$.

occlusion (Figure 3). The degree of protection seen when the drug was given 6 h after the ischaemic episode failed to reach statistical significance and no protection was observed when the drug was given 24 h after the insult (Figure 3).

Various doses of chlormethiazole were examined for their protective effect when given 1 h after the ischaemic episode (Figure 4). Whilst 50 mg kg^{-1} chlormethiazole produced significant protection, this was not apparent following a dose of 25 mg kg^{-1}. A log dose versus % protection plot was linear (not shown), suggesting that a protection of 50% neurone loss would be achieved with a dose of chlormethiazole of 55 mg kg^{-1}.

A series of experiments were performed with halothane anaesthesia. In these experiments the duration of occlusion was limited to 4 min, to produce the same degree of neuronal damage as under Saffan anaesthesia. When chlormethiazole (100 mg kg^{-1}) was given 1 h after the ischaemic episode, a marked neuroprotective effect was still observed (saline: 72 ± 10, $n = 12$, chlormethiazole: 37 ± 7, $n = 12$, $P < 0.01$).

The effects of GABA potentiating drugs

The effect of administering 3 other GABA potentiating drugs after the ischaemic episode was also investigated. Saffan had no significant effect on CA1 neurone degeneration and phenobarbitone (100 mg kg^{-1}, i.p.) was also without effect (Figure 5). A sedative dose of pentobarbitone (30 mg kg^{-1} i.p.) did produce a small but significant reduction of the damage to CA1/CA2 neurones (Figure 5).

The effects of chlormethiazole on brain temperature

Brain and rectal temperatures were recorded in 4 gerbils during carotid artery occlusion and subsequent chlormethiazole treatment. Following the protocol described, rectal temperature was successfully maintained between 37–38°C (Figure 6). While the temperature of the hippocampus did not decrease during the occlusion there was a small but statistically significant rise afterwards (Figure 6). Administration of chlormethiazole did not alter cerebral temperature (Figure 6).

Discussion

The present study clearly demonstrates that chlormethiazole prevents the degeneration of hippocampal CA1/CA2 pyramidal neurones following transient forebrain ischaemia in the gerbil. There are, however, a couple of factors which need to be considered when comparing the results of the present study with other published studies.

The current study generally used the injectable anaesthetic Saffan rather than an inhalant anaesthetic such as the commonly used halothane. It has previously been shown that the severity of hippocampal damage following ischaemia varies with the anaesthetic agent (Lightfoote *et al.*, 1977; Kuroiwa *et al.*, 1989). However, chlormethiazole was protective when either Saffan or halothane had been used. Furthermore, dizocilpine was protective in Saffan-anaesthetized animals (this study) to a similar degree to that seen in halothane anaesthetized animals (Gill *et al.*, 1987). Additionally, ifenprodil, which is neuroprotective in the rat and cat (Gotti *et al.*, 1988) was shown to be neuroprotective in our experiments in the gerbil. We do not feel therefore that the choice of anaesthetic influenced the results obtained. This view is supported by the failure of a second anaesthetic dose of Saffan to reduce hippocampal damage following ischaemia. We would also suggest that Saffan is easier to use than an inhalant anaesthetic in the operative procedure.

A second point is that chlormethiazole administration produces hypothermia in gerbils (unpublished observations) and it is well established that hypothermia can be neuroprotective (Busto *et al.*, 1987). Care was therefore taken to maintain body temperature during the ischaemic episode and subsequent drug treatment. The success was evidenced by the observation that brain as well as body temperature was unaltered by drug treatment, rendering unlikely any proposal that chlormethiazole was neuroprotective because it produced hypothermia.

In common with several other anticonvulsant drugs with diverse mechanisms of action, including phenytoin, barbiturates, diazepam and etomidate (Taft *et al.*, 1989; Sternau *et al.*, 1989; Hermans *et al.*, 1983), chlormethiazole was an effective neuroprotective drug when given just prior to the ischaemic episode. What distinguishes chlormethiazole from these other drugs is its high degree of efficacy as a neuroprotectant when given up to several hours after the ischaemic insult. It has previously been shown that phenytoin, diazepam and etomidate are ineffective when given after ischaemia (Sternau *et al.*, 1989) and the current study demonstrated a lack of effect of phenobarbitone. Only pentobarbitone had any effect when given 60 min after the ischaemic episode, and this was marginal.

While it is possible therefore that the neuroprotective effect of chlormethiazole is related to its anticonvulsant activity when given before the ischaemic episode, the failure of other anticonvulsants to exert neuroprotection when given post-ischaemia suggests a different mechanism of action is required to explain its efficacy when given 2–3 h after the ischaemic insult.

Electrophysiological and biochemical experiments have demonstrated that whilst chlormethiazole can potentiate the action of GABA acting at the $GABA_A$ receptor, chlormethiazole can also directly modulate $GABA_A$ receptor function independent of agonist (Harrison & Simmonds, 1983; Moody & Skolnick, 1989). In contrast to benzodiazepines and barbiturates, this action may occur at lower concentrations of chlormethiazole than those required to potentiate GABA agonists (Simmonds & Turner, 1987). It may well be that this distinct profile of action accounts for the neuroprotective effect of chlormethiazole during the post-ischaemic period. It is clear that during ischaemia and immediately following reperfusion there is a massive efflux of neuroactive amino acids, including GABA and glutamate (Benveniste *et al.*, 1984; Hagberg *et al.*, 1985; Globus *et al.*, 1988). However, extracellular concentrations of GABA and glutamate return to normal shortly after reperfusion. Whilst GABA-containing interneurones in the hippocampus have generally been found to be remarkably resistant to ischaemic damage (Francis & Pulsinelli, 1982; Johansen *et al.*, 1983), there is some evidence to suggest that a reversible functional deficit may occur shortly after ischaemia (Johansen *et al.*, 1987; Grimaldi *et al.*, 1990). It may well be that the ability of chlormethiazole to activate postsynaptic $GABA_A$ receptors in the absence of sufficient endogenous GABA, explains its efficacy.

Following an intraperitoneal dose of 100 mg kg^{-1}, the peak plasma concentration of chlormethiazole in the gerbil is around 200 nmol ml^{-1} (unpublished observations). In man, similar plasma levels have been observed during clinical use of the drug (Christensen *et al.*, 1983). This concentration of chlormethiazole corresponds well with that required for inhibition of [^{35}S]-t-butylbicyclophosphorothionate ([^{35}S]-TBPS) binding to the GABA receptor (IC_{50} 140 μM, Cross *et al.*, 1989).

As has been discussed by Meldrum (1990), the excitotoxic process probably depends on a balance between excitatory and inhibitory mechanisms. Within the hippocampus of the rat the majority of inhibitory interneurones use GABA as transmitter, and $GABA_A$ receptors are present on hippocampal pyramidal neurones (Bowery *et al.*, 1987). Thus any imbalance leading to a net overactivity of excitatory amino acid systems may be counteracted by increasing the activity of the major inhibitory system mediated by GABA. In this respect it is interesting to note that chlormethiazole is an effective inhibitor of the tonic convulsions induced by peripherally administered NMDLA (unpublished data).

Chlormethiazole was effective in reducing the extent of hip-

pocampal damage administered at least 3 h post-ischaemia. Although many compounds have been shown to reduce ischaemic damage in the hippocampus when administered during the ischaemic period, few are effective when administered post-ischaemia. Prominent amongst these are excitatory amino acid antagonists, acting at NMDA (Boast *et al.*, 1987; Gill *et al.*, 1988) or AMPA (Sheardown *et al.*, 1990) subtypes of receptor. However, there are problems with the use of the non-competitive NMDA antagonists since in rodents they have been found to induce a complex motor syndrome and ataxia (See Tricklebank *et al.*, 1989) and can also produce transient pathological changes in the morphology of specific populations of brain neurones (Olney *et al.*, 1989). Both these observations raise questions as to the safety of such drugs in clinical use. No evidence has been obtained for chlormethiazole acting at the glutamate, glycine or polyamine sites of the NMDA receptor complex or at the AMPA receptor (Addae & Stone, 1988; Cross *et al.*, unpublished observations). It therefore seems likely that the neuroprotective action of chlormethiazole results from the known interaction of the drug with the GABA receptor complex (Cross *et al.*, 1989; Moody & Skolnick, 1989; Vincens *et al.*, 1989).

Chlormethiazole has been used clinically as a sedative, hypnotic and anticonvulsant drug for over 25 years (Evans *et al.*, 1986). A safe approach to the treatment of ischaemic stroke involving GABAergic mechanisms can therefore be proposed.

In conclusion, the present results demonstrate that following transient forebrain ischaemia a single dose of chlormethiazole prevents the degeneration of hippocampal pyramidal neurones. Chlormethiazole is effective when administered at least 3 h after ischaemia, suggesting that it may be useful clinically in attenuating the neurodegeneration following stroke or cardiac arrest.

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The sources of calcium for carbachol-induced contraction in the circular smooth muscle of guinea-pig stomach

[1]Anant B. Parekh & Alison F. Brading

University Department of Pharmacology, South Parks Road, Oxford OX1 3QT

1 The action of carbachol on the mechanical activity of circular muscle from guinea-pig upper stomach was studied. High concentrations of carbachol (e.g. 10^{-4} M) produced a rapid phasic contraction followed by a smaller, sustained tonic contraction. Low concentrations (e.g. 10^{-7} M) caused a contraction which did not generally show marked distinction between phasic and tonic components.

2 The response to 10^{-7} M carbachol was very sensitive to 10^{-5} M nifedipine as was the phasic response to 10^{-4} M carbachol. The tonic contraction to the latter, however, was only slightly reduced by nifedipine.

3 The carbachol-induced contractions remaining in the presence of nifedipine were dose-related and very dependent on the presence of external calcium.

4 Carbachol, 10^{-7} M, did not produce a contraction after 4 min exposure to calcium-free solution whereas 10^{-4} M carbachol did and this was phasic in nature but much reduced relative to the control in normal Ca.

5 A phasic followed by a small tonic contraction to 10^{-4} M carbachol was seen superimposed on the K contracture in tissues depolarized with 100 mM K, whereas only a small tonic response occurred for 10^{-7} M carbachol.

6 In the absence of a functional carbachol-sensitive intracellular store, 10^{-4} M carbachol was unable to trigger a contraction in calcium-free solution. However, when calcium was simultaneously readmitted with carbachol after exposure to calcium-free solution, a contraction occurred.

7 Carbachol, 10^{-7} M, did not significantly increase inositol polyphosphate levels, whereas 10^{-4} M carbachol did. The increase with 10^{-4} M occurred rapidly peaking within 2 min and was undetectable after 5 min, in the absence of lithium.

8 It is concluded that low concentrations of muscarinic agonist trigger a contraction predominantly through a nifedipine-sensitive route whereas higher concentrations further utilize intracellular calcium release and a receptor-operated extracellular calcium-dependent pathway. The former is probably associated with the phasic component and the latter with the tonic one.

Keywords: Guinea-pig stomach; smooth muscle; calcium stores; carbachol; nifedipine; inositol polyphosphates

Introduction

Muscarinic receptors mediate several distinct responses in various tissues including smooth muscles (Bolton, 1979). For example, in the longitudinal smooth muscle of guinea-pig ileum, muscarinic receptor activation increases spike frequency and depolarizes the plasma membrane (Bolton, 1972), stimulates inositol polyphosphate production (Best *et al.*, 1985) and increases guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Lee *et al.*, 1972). This diversity presumably results either from several distinct subtypes of muscarinic receptor present or linkage of one receptor to several different transduction mechanisms. The existence of subtypes was suggested by binding studies with the antagonist pirenzepine which revealed the existence of binding sites with different affinities (Hammer *et al.*, 1980). Recent progress in molecular biology using receptor cloning techniques has demonstrated the presence of several muscarinic subtypes and it appears that different muscarinic subtypes are encoded by separate genes (Bonner *et al.*, 1987). These subtypes are linked to different transduction mechanisms although there is some overlap (Lechleiter *et al.*, 1989). In the guinea-pig stomach, the muscarinic agonist carbachol produces a contraction consisting of an initial phasic and a late tonic component. From the sensitivities of these components to nifedipine, Golenhofen (1976) postulated two mechanisms of contraction in smooth muscle namely the P and T systems. The phasic (initial component) was nifedipine-sensitive whereas the tonic (later) component was nifedipine-resistant. However, this generalization seems a little too simplistic. Carbachol can mobilize intracellular calcium stores in visceral smooth muscle (Brading & Sneddon, 1980) and the role of this in the P and T system is not clear.

The aim of this study was to investigate the sources of calcium mobilized by different concentrations of carbachol in guinea-pig stomach and how these contractions were affected by nifedipine. We conclude that high concentrations of carbachol trigger and maintain contraction using mechanisms in addition to those used by low concentrations.

Methods

Isometric contraction

Guinea-pigs of either sex were killed by stunning and bleeding. Whole stomachs were rapidly removed, opened along the greater curvature and the contents washed out in physiological saline solution (PSS, composition described below). Both halves of the stomach wall were then incubated in PSS for about 10 min at 37°C before the mucosa was removed completely under an optical microscope. Muscle strips (about 1 mm wide and 10 mm long) from the fundus were cut along the direction of the circular fibres and then mounted for isometric tension as previously described (Syed *et al.*, 1990; Brading & Sneddon, 1980). The bath temperature was maintained at 35–38°C.

Inositol polyphosphates

Dissected tissues were finely minced with microscissors and then loaded with 30 μCi [^{3}H]-myoinositol for 1.5 h. The suspension was then washed several times in warm PSS.

[1] Author for correspondence.

In some experiments (see below) 10 mM Li-containing PSS was added to inhibit inositol-1-monophosphatase (Berridge *et al.*, 1982). The labelled tissue suspension was taken and added to tubes containing PSS with vehicle or agonist. Reactions were terminated after different times (see text) with 940 μl of chloroform: methanol: HCl (50 : 100 : 1 v/v), 310 μl chloroform and 310 μl distilled water, vortexed and then centrifuged at 1000 *g* for 5 min; 800 μl of the upper (cytoplasmic) phase was diluted with 1 ml water and then passed through a Dowex column (50% slurry). Inositol was eluted from the column by applying 60 mM ammonium formate/5 mM sodium tetraborate. Total [^{3}H]-inositol phosphates (IP_1, IP_2 and IP_3) were then eluted with 0.8 M ammonium formate/0.1 M formic acid (Guard *et al.*, 1988). This fraction was collected in scintillation vials and then counted for radioactivity after addition of scintillation fluid.

Of the lower organic lipid phase, 200 ml was removed and counted for radioactivity as above. This was done to normalize each assay with respect to the amount of tissue, assuming that the amount of lipid is proportional to the amount of tissue.

Solutions

The composition of the PSS was (mM): NaCl 130, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 5, glucose 11, HEPES 5; pH was adjusted to 7.35–7.40 with Tris base. The solutions were gassed with 100% oxygen. In experiments where high K was used, NaCl was replaced isosmotically with KCl to the desired concentration. Calcium-free solutions contained 1 mM EGTA and Ca was isosmotically replaced with Mg.

Drugs used were carbachol chloride, propranolol hydrochloride, caffeine, procaine, and nifedipine (all from Sigma).

Statistics

Results are expressed as the mean ± s.e.mean. The significance was assessed by a paired *t* test when responses were compared in the same strip and an unpaired *t* test when the inositol polyphosphates were analysed. Values of *P* less than 0.05 are shown with a single * and less than 0.001 with two **.

Results

Responses in the presence of extracellular calcium

General patterns of response Application of carbachol resulted in a concentration-dependent contraction, the pattern of which depended on the concentration of agonist. A maximal dose of carbachol (10^{-4} M) resulted in a rapid phasic (initial) contraction followed by a maintained tonic (later) component which was 51.9 ± 2.8% of the peak phasic contraction ($P < 0.001$, $n = 48$ strips from 10 animals). For a low concentration of agonist (10^{-7} M, the threshold for a detectable response being about 10^{-8} M), there was generally no clear demarcation between phasic and tonic components. In Figure 1e, dose-response curves are shown for the contraction after 2 and 15 min applications of carbachol. There was a significant difference between the response to 10^{-4} and 10^{-7} M carbachol after a 2 min application ($P < 0.05$).

Sensitivity of contractions to nifedipine The 1,4 dihydropyridine, nifedipine, blocks the L-type calcium channel in smooth muscle. Nifedipine concentration-dependently reduced both the phasic (initial) (measured after 2 min) and tonic (later) responses to a low dose of carbachol (10^{-7} M) but was able to attenuate markedly only the phasic response to a high concentration of agonist (10^{-4} M). The tonic component produced by 10^{-7} M carbachol was more-or-less totally abolished by 10^{-5} M nifedipine whereas the tonic component produced by

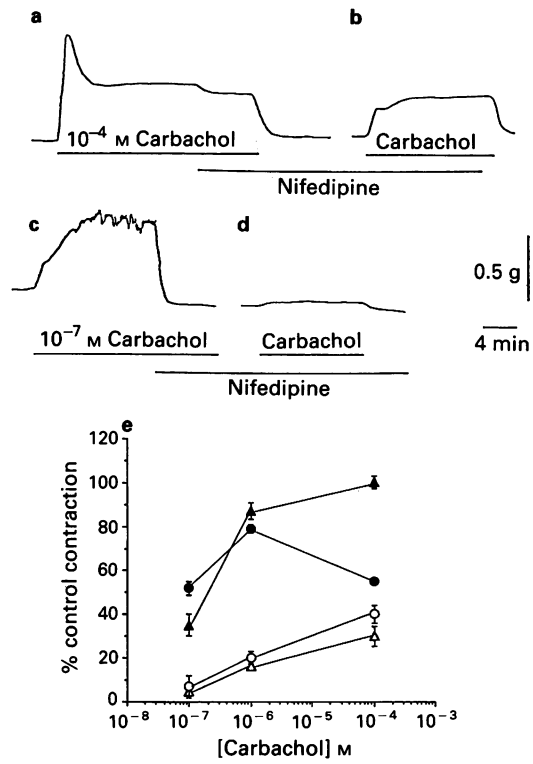

Figure 1 Effects of 10^{-5} M nifedipine on carbachol contractions. Nifedipine applied during contraction to (a) 10^{-4} M and (c) 10^{-7} M carbachol. Responses in the presence of nifedipine after 15 min pretreatment to (b) 10^{-4} M and (d) 10^{-7} M carbachol. (e) Concentration-response curves after 2 min exposure to carbachol in the absence (▲) and presence (△) of nifedipine (10 strips from 2 animals) and responses after 15 min application of carbachol in the absence (●) and presence (○) of nifedipine (20 strips from 4 animals).

10^{-4} M carbachol was only slightly but significantly reduced from 58.8 ± 5.9% to 41.1 ± 4.6% ($P < 0.05$). Typical results are shown in Figure 1. Nifedipine was applied during the tonic component of the contraction to a high (10^{-4} M) and low (10^{-7} M) concentration of carbachol (Figure 1a and c respectively). The contraction to 10^{-4} M carbachol was slightly reduced whereas nifedipine almost totally abolished the tonic contraction to 10^{-7} M carbachol. In another set of experiments, preparations were exposed to nifedipine for at least 15 min before carbachol was applied in the maintained presence of nifedipine. As is evident from Figure 1, the contraction after 2 min exposure to agonist was greatly reduced for both a high and a low concentration of carbachol (Figure 1b and d respectively) whereas the tonic component to a low concentration was more sensitive to nifedipine.

Dependence of the nifedipine-insensitive component on extracellular calcium concentration The size of the tonic (later) component of contraction to 10^{-4} M carbachol in the presence of nifedipine was dependent on the concentration of extracellular calcium (Figure 2a and b). Similar results were obtained for both 10^{-7} M and 10^{-6} M carbachol (8 strips from 2 animals, data not shown). La, 1 mM, which blocks the calcium-dependent contraction to noradrenaline in rat vas deferens (Swamy *et al.*, 1976), rapidly abolished the tonic response to 10^{-4} M carbachol (5 strips from 1 animal, data not shown).

Responses in Ca-free solution

To study the role of intracellular calcium stores in the response to carbachol, external calcium was removed by perfusion with calcium-free solution. Such a method has been

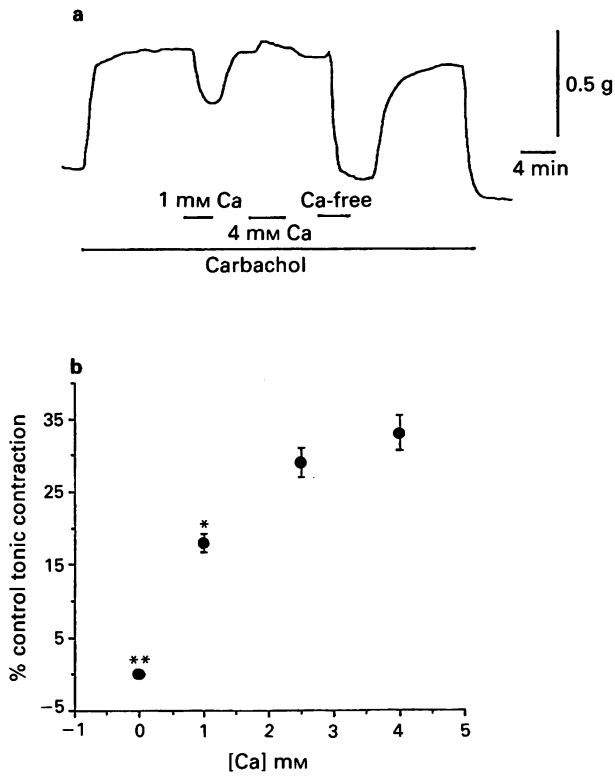

Figure 2 Dependency of tonic contraction on external calcium. (a) Changing extracellular calcium produced changes in the tonic contraction to carbachol (10^{-4} M) in the presence of nifedipine (10^{-5} M). (b) Graphical representation of the data, given as mean of 12 strips from 2 animals with s.e.mean shown by vertical bars. The tonic response in 2.5 mM Ca in the absence of nifedipine was taken as 100%. The significance of changing extracellular calcium on steady-state tone was determined relative to the contraction to 2.5 mM Ca in the presence of nifedipine.

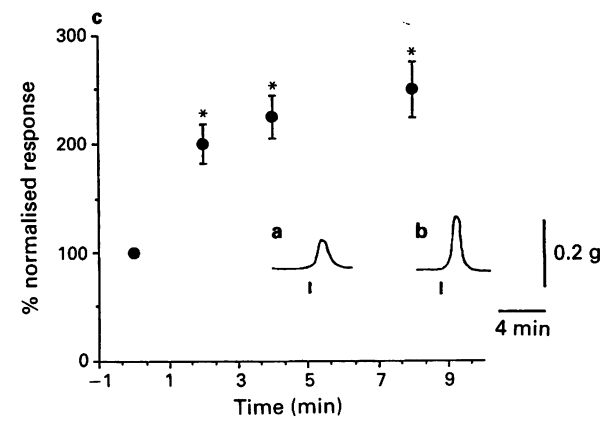

Figure 3 Effect of pretreatment with 40 mM K solution on the contractile response to 10^{-4} M carbachol in Ca-free solution. Tissues were exposed to high K for the times indicated (c) and then superfused with Ca-free (5.9 mM K) solution for 4 min before exposure to carbachol in Ca-free solution for 2 min. Points are mean of 31 strips from 6 animals; s.e.mean shown by vertical bars. Response in the absence of pretreatment was taken as 100%, and significance was determined relative to this. Inset: (a) contraction to carbachol without 40 mM K pretreatment; (b) contraction after 4 min 40 mM K pretreatment.

widely used in smooth muscle (Casteels & Raeymakers, 1979; Brading & Sneddon, 1980; Ashoori & Tomita, 1983; Lalanne *et al.*, 1984).

After exposure to calcium-free solution for 4 min, high K calcium-free solution did not produce a contraction (13 strips from 3 animals). This suggests that, after this time, most of the external calcium had been removed. Under these conditions, 10^{-7} M carbachol failed to produce a contraction but 10^{-4} M carbachol produced a phasic response which was much smaller than the phasic contraction in the presence of calcium (Figure 3a); the tonic component was negligible.

For ease of analysis, the size of the carbachol-induced contraction in calcium-free solution was elevated by imposing a calcium load on the tissue. This was achieved by exposure to 2.5 mM calcium in 40 mM K solution (this contained 0.1 μg ml^{-1} tetrodotoxin to abolish action potential-mediated release of endogenous neurotransmitters) for different times followed by calcium-free 1 mM EGTA solution for 4 min then agonist challenge in calcium-free solution. Under these conditions the 10^{-4} M carbachol-induced contraction was increased 2.2 ± 0.2 fold (27 of 31 strips from 6 animals, Figure 3b), whereas 40 mM K in calcium-free solution still did not produce a contraction (10 strips from 2 animals). This is probably a consequence of increased calcium uptake into the carbachol-sensitive intracellular store. The contraction to carbachol increased as a function of exposure time to 40 mM K solution as shown in Figure 3c and as observed by Casteels & Raeymakers (1979) in guinea-pig taenia. For subsequent experiments, 4 min high K pretreatment was used. Carbachol, 10^{-7} M, did not trigger a contraction in calcium-free solution even if a calcium load had been imposed (10 strips from 2 animals).

The carbachol response was lost rapidly after stimulation. After a contraction to 10^{-4} M carbachol had been obtained in calcium-free solution, preparations were maintained in calcium-free solution for a further 4 min and then challenged again with 10^{-4} M carbachol. No contraction occurred. This is unlikely to reflect receptor desensitization because 10^{-4} M carbachol still did not produce a contraction in calcium-free solution if the initial concentration of carbachol applied was lower (10^{-5} M, 10 strips from 2 animals). This suggests that the calcium released by carbachol is not taken back up into the store from which it was released.

Effects of caffeine and procaine Agents known to interfere with calcium release from internal stores were studied in calcium-free solution.

Procaine is thought to inhibit calcium-induced calcium release (Endo, 1977). It also produced a dose-dependent reduction of the 10^{-4} M carbachol-induced contraction in a concentration-dependent way with an IC_{50} of 0.1 mM (Figure 4a; 14 strips from 3 animals).

To enhance the effects of the methylxanthine, caffeine, on calcium release from the sarcoplasmic reticulum (Iino, 1989) experiments were conducted at room temperature (20–24°C) as the contractile response is increased in this temperature range (Endo, 1977). Caffeine produced a small, transient contraction (about 30% of the carbachol-induced contraction in calcium-free solution) at concentrations > 1 mM and this was enhanced after a calcium load (data not shown). Caffeine pretreatment resulted in a concentration-dependent reduction in the carbachol contraction, with an IC_{50} of 1 mM (Figure 4b; 14 strips from 3 animals).

Effects of nifedipine on store refilling in the presence of extracellular calcium

After prolonged treatment with nifedipine the small contraction to carbachol in calcium-free solution was reduced (15 strips from 3 animals). This probably did not reflect non-specific rundown of the tissues because these contractile responses in control preparations were not reduced with time. It was difficult to quantify these contractions as they were small if a calcium load was not imposed as discussed above (e.g. with high K, but this potentiation is nifedipine-sensitive). This raises the key question of the role of the nifedipine-sensitive route in the response to carbachol. It is possible that

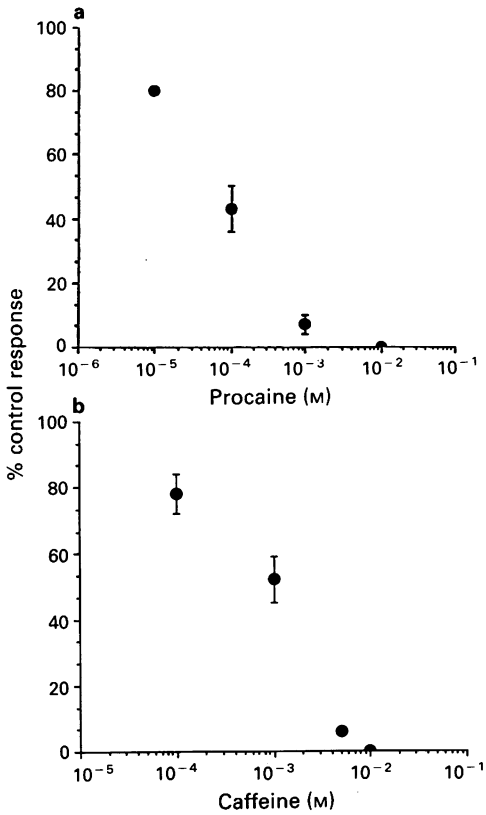

Figure 4 Effects of agents interfering with intracellular calcium release on the contraction to 10^{-4} M carbachol. After high K pretreatment, tissues were exposed to Ca-free solution and drug for 4 min before 10^{-4} M carbachol was applied in the maintained presence of these drugs: (a) procaine; (b) caffeine. Points are mean of 14 strips from 3 animals each; vertical bars show s.e.mean. The contraction after high K treatment was taken as 100%.

calcium through this route could directly contribute to activation of the contractile system or that this calcium first passes into the carbachol-sensitive store and is then released by the agonist or triggers a form of calcium-induced calcium release. The following experiments were designed to gain some information on the contribution of the nifedipine-sensitive route and intracellular calcium release on the phasic component of the carbachol contraction.

Phasic (initial) response in the absence of the nifedipine-sensitive route

As mentioned above, we believe the carbachol-sensitive store is at least partly refilled through a nifedipine-sensitive route. To study the effects of nifedipine on store release, we applied 10^{-5} M nifedipine for 10 s before giving 10^{-4} M carbachol. This short time was chosen because it did not seem to affect calcium store filling (as judged by the contraction to carbachol in calcium-free solution) yet the time was sufficient to block the 100 mM K-induced contraction (in the presence of nifedipine, 2 out of 2 experiments, data not shown). Under these conditions, the phasic response was significantly reduced to $67.8 \pm 6.1\%$ ($P = 0.002$) and also declined more rapidly. This phasic contraction was larger, however, than the early peak response to carbachol obtained after prolonged nifedipine treatment (data not shown).

Responses in depolarized tissues

The nifedipine-sensitive calcium channel in other smooth muscles shows both voltage- and calcium-dependent inactivation (Ohya *et al.*, 1988; Bean, 1989). Therefore in the presence of high external K (which would both depolarize and increase cytoplasmic free calcium) one would anticipate some inactivation and therefore reduced availability of the nifedipine-sensitive channel for contribution to the phasic response of carbachol. Thus preparations were depolarized by exposure to 100 mM K which resulted in a phasic followed by a tonic contraction (Figure 5a). Once a steady-state had been reached, application of 10^{-4} M carbachol resulted in a rapid phasic contraction and this was followed by a slowly developing tonic component superimposed on the tonic component of 100 mM K (Figure 5b). For 10^{-7} M carbachol, only a slowly developing tonic component was observed without any phasic response (10 strips from 2 animals, Figure 5c). This suggests that the phasic response to 10^{-4} M carbachol under these conditions is due to calcium release from well-loaded intracellular stores (due to the high K) while the lack of a phasic response to 10^{-7} M carbachol is due to an inability to release calcium from this store.

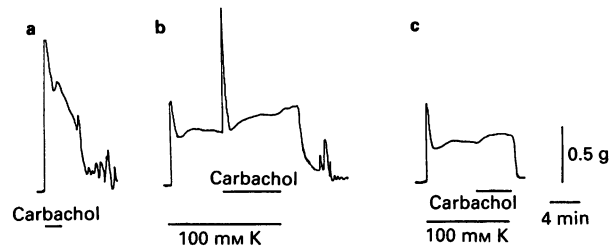

Figure 5 Responses to carbachol in the presence of 100 mM K. (a) Control 10^{-4} M carbachol response in normal solution; (b) in response to 10^{-4} M and (c) to 10^{-7} M carbachol in high K.

Responses in the absence of a functional intracellular store

Preparations were exposed to calcium-free 1 mM EGTA solution for 10 min and then calcium-free solution in the absence of EGTA for 5 min. Subsequent application of 10^{-4} M carbachol (in calcium-free solution) did not produce a contraction. This indicates functional loss of the carbachol-sensitive intracellular calcium pool. If, after the calcium-free perfusion described above, 10^{-4} M carbachol was applied simultaneously with 2.5 mM Ca for 10 s before returning the preparations to calcium-free 1 mM EGTA, a transient phasic contraction occurred (Figure 6) and this was $28.7 \pm 5.1\%$ of the control phasic response (10 strips from 2 animals). If 10^{-5} M nifedipine was added during the calcium-free 1 mM EGTA perfusion and then maintained, the response to simul-

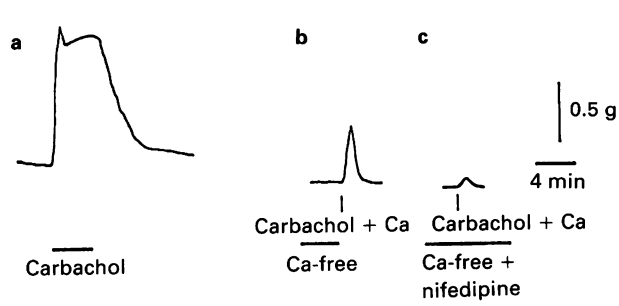

Figure 6 Responses to 10^{-4} M carbachol in the absence of its functional intracellular store. (a) Control contraction. After Ca-free exposure (see text) carbachol was applied with 2.5 mM Ca for 10 s in the absence (b) or presence (c) of nifedipine. Nifedipine was applied 10 min before carbachol application. Calcium readmission alone for 10 s did not produce a contraction.

taneous carbachol and calcium application was significantly reduced to 7.9 ± 3.2% ($P = 0.001$, 10 strips from 2 animals). A 10 s application of calcium alone did not result in contraction. This suggests that (1) a phasic nifedipine-sensitive contraction can occur in the absence of a functional carbachol-sensitive intracellular pool and (2) this contraction is not a consequence of calcium-induced calcium release.

We cannot discriminate between direct activation of the contractile system by this calcium influx or whether it passes first into an intracellular pool and is then released by a second messenger. From the results described below with inositol polyphosphate measurements, we favour the former possibility.

Effects on inositol polyphosphate production

The effects of carbachol on total inositol phosphate levels (IP_{1-3}) were studied. Carbachol, 10^{-7} M, did not produce a significant increase in IP_{1-3} levels whereas 10^{-6} and 10^{-4} M carbachol triggered dose-dependent increases. The results are depicted graphically in Figure 7. In two larger animals it was possible to compare different doses of carbachol. Here 10^{-7} M carbachol still did not cause a significant increase, yet, in the same animal, 10^{-6} M did. There was some variation in the IP_{1-3} response to 10^{-7} M carbachol and the range was 90–130% of the mean. In 11 out of 28 assays from 7 animals, application of 10^{-7} M carbachol resulted in no increase relative to control. In those 17 assays where an increase was observed, the range was 105–127% (mean 117 ± 4%, control 100%). In those animals that produced a large response to 10^{-4} M carbachol, the tendency was that a small increase was seen with 10^{-7} M. This suggests that 10^{-7} M carbachol may be the critical concentration such that beyond this increases in inositol polyphosphates are obtained.

These results were obtained by exposing the preparations to agonist for 15 min in the presence of 10 mM Li to inhibit inositol-1-monophosphatase (Berridge *et al.*, 1982) in order to amplify and maintain increases. To gain more insight into the role of IP_{1-3} in contraction it was therefore necessary to measure the time-course of change in IP_{1-3}.

Time-course of change in IP_{1-3}

To monitor the rate of change of IP_{1-3} levels, carbachol was applied for different times. As seen in the experiment shown in Figure 8, the production of IP_{1-3} occurred rapidly and there was no further significant increase between 2 and 15 min. This suggests that IPs are rapidly produced and, in the absence of their breakdown, the rate of synthesis returns to basal levels despite the maintained presence of agonist. In longitudinal muscle of ileum, however, IP_{1-3} production increases linearly over 20 min (Best *et al.*, 1985). It is not clear why gastric fundus is atypical, but one possibility may be that phosphatidyl inositol 4,5, bisphosphate is substrate limiting.

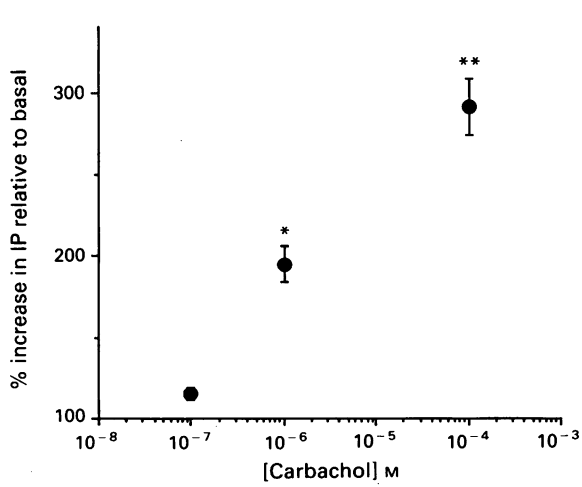

Figure 7 The effect of carbachol on inositol polyphosphate production. The total polyphosphates produced during 15 min exposure to carbachol are expressed relative to basal production. Points are the means of 5 experiments with s.e.mean shown by vertical bars.

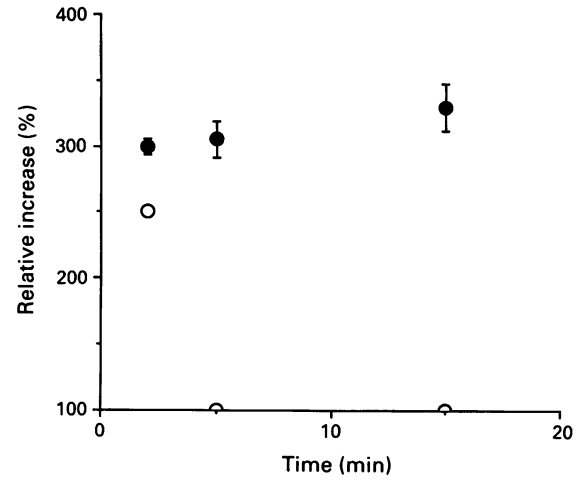

Figure 8 Time course of inositol polyphosphate production. Preparations were exposed to 10^{-4} M carbachol for the times indicated in the presence (●) or absence (○) of Li. Results are expressed relative to basal production.

To see if the rate of synthesis fell if breakdown of inositol polyphosphates can occur, experiments were performed in the absence of Li and the IP_{1-3} levels observed after different agonist exposure times. In all experiments (11 assays from 3 animals) 10^{-4} M carbachol did not produce a significant increase in IP_{1-3} relative to control after 15 min ($P > 0.05$; Figure 8). These results suggest that IP_{1-3} production has returned to basal values before 15 min.

Discussion

The main finding of this study is that there are at least three sources of calcium mobilized by carbachol for contraction. These are: (1) a nifedipine-sensitive component, (2) a nifedipine-insensitive but extracellular calcium-dependent component and (3) a carbachol-sensitive intracellular store. We have found that different concentrations of carbachol can activate these components differentially.

Low concentrations of carbachol

Low concentrations of carbachol did not elicit a contraction under calcium-free conditions suggesting weak mobilization of an intracellular store. Consistent with this was the finding that such concentrations did not significantly elevate inositol polyphosphates, which would be presumed to mobilize such stores (Streb *et al.*, 1983). The methodology for inositol polyphosphate measurements, however, is very crude in that it does not allow localized changes to be resolved. Only net inositol phosphate changes in the whole cytoplasm are detected. As there are reports of superficial intracellular calcium stores in rapid exchange with the extracellular solution (e.g. Brading & Sneddon, 1980; Casteels & Droogmans, 1981), it is possible that low concentrations of carbachol mobilize these stores under normal conditions through a localized increase in inositol polyphosphates. Dilution with the cytoplasm may render this increase undetectable.

The contraction to low concentrations of carbachol was very sensitive to nifedipine. Both the early portion as well as

the tonic component were significantly reduced. The small contraction remaining in the presence of nifedipine was dependent on external calcium. This suggests that the main source of calcium for low concentrations of carbachol is through a nifedipine-sensitive route but there is also a small contribution during the tonic phase through a nifedipine-insensitive route. This is similar to the observations of Cauvin *et al.* (1984) in rabbit mesenteric resistance vessels. These workers found that low concentrations of noradrenaline did not release calcium from intracellular stores but utilized a diltiazem-sensitive route for contraction. As in other gastrointestinal smooth muscle (e.g. ileum, Bolton, 1972), 10^{-7} M carbachol can produce significant depolarization in gastric fundus (unpublished observation). It seems reasonable to suppose that low concentrations of carbachol act largely through electromechanical coupling.

High concentrations of carbachol

The contractions to higher concentrations of carbachol were complex and consisted of an initial phasic component which rapidly declined to a later tonic phase. For analytical convenience let us consider each component in turn.

The tonic component was only slightly reduced by nifedipine treatment but was critically dependent on the presence of external calcium. These results are consistent with the ^{45}Ca uptake data of Lammel (1977) who found both a nifedipine-sensitive and a nifedipine-insensitive component of calcium uptake in guinea-pig gastric fundus in response to a high muscarinic agonist concentration. Recently, Lammel *et al.* (1991) have shown acetylcholine depolarizes single gastric fundus smooth muscle cells by inhibiting an outward I_K, thus depolarizing the cells, and presumably increasing L-type calcium channel activity. A role for non-selective cation channels was not revealed in this study. Gastric fundus circular muscle is therefore different from longitudinal muscle of ileum where muscarinic receptors activate non-selective cation channels which are permeable to calcium (Inoue & Isenberg, 1990) and hence may contribute to a nifedipine-insensitive contraction. It seems reasonable to suppose that the nifedipine-insensitive tonic response is due to a receptor-operated calcium influx pathway (Bolton, 1979), although we cannot determine whether this calcium activates the contractile system directly or indirectly via activation of some mediating intracellular factor. As the inositol polyphosphates declined rapidly after stimulation, it seems unlikely that intracellular calcium mobilization by these messengers is involved in the tonic response.

The phasic response was more complex. As it was severely attenuated by prolonged pretreatment with nifedipine, it is tempting to attribute this to calcium influx through a nifedipine-sensitive route. However this may be too simplistic. We found that nifedipine, applied in the presence of extracellular calcium, was able to reduce the subsequent carbachol contraction in calcium-free solution suggesting that this store refills, at least in part, through a nifedipine-sensitive route. This is similar to observations in guinea-pig coronary artery where the related drug D-600 reduced responses dependent on intracellular calcium release at rest (Ganitkevich & Isenberg, 1990). This raises the possibility that nifedipine block of the phasic response is due indirectly to its ability to reduce store refilling and that intracellular calcium release is the cause of the phasic response. It was therefore necessary to determine whether the nifedipine-sensitive route could directly contribute to the phasic response. We think it can for the following reasons. First, exposure to nifedipine for a very short time (10 s, a time too short to compromise nifedipine-dependent refilling of the intracellular store) was sufficient to block the high K contraction. This duration of exposure did not completely block but significantly reduced the phasic response to carbachol. As the high K response was lost using this procedure, it seems likely that there is block of the L-type calcium channel which is widely thought to be the chemically sensitive site of nifedipine (Tsien, 1983; Bean, 1989 for reviews), and hence this channel is involved directly in the phasic contraction.

Second, after functional loss of the carbachol-sensitive intracellular pool by a long exposure to calcium-free solution, application of carbachol and calcium for a very short time resulted in a phasic contraction and this was much reduced by nifedipine. This may represent calcium influx through the L-type channel. We think it unlikely that this calcium first passes into an intracellular store (and is then mobilized) because a low concentration of carbachol did not increase inositol polyphosphates yet the contraction was very sensitive to the blocking action of nifedipine (see above), but we cannot exclude the possibility.

It seems probable that intracellular calcium release also contributes to the phasic response because short prior application of nifedipine reduced but did not abolish the phasic response. Furthermore, in depolarized tissues in which some of the calcium channels should be inactivated, only high concentrations of carbachol produced a phasic response and these concentrations also increased inositol polyphosphates (we are assuming inositol trisphosphate is the main mediator of intracellular calcium release; to our knowledge there is no other viable candidate released on muscarinic stimulation). Finally, only high concentrations of carbachol produced a small contraction in calcium-free solution and this was also phasic in nature and sensitive to agents that interfere with intracellular calcium store homeostasis.

Taken together we propose that intracellular calcium release and calcium influx through a nifedipine-sensitive route both contribute directly to the phasic component. These pathways interact in that intracellular calcium store requires, at least partly, the nifedipine-sensitive route for filling. The tonic component is a result of calcium influx through both a nifedipine-sensitive and a nifedipine-insensitive route and there is probably little contribution from IP_3-releasable stores.

Extreme caution must be applied to the interpretation of results when one agonist appears to utilize different mechanisms to elicit a response in a given tissue. Activation of the mechanisms may not be independent but rather one may be caused by another. For example, the transfected M_1 receptor-subtype can increase both IP_3 and cyclic AMP levels but the latter seems to be due to calcium-calmodulin activation of adenylate cyclase, the calcium arising from IP_3-induced store release (Felder *et al.*, 1989; Buck & Fraser, 1990). We believe the different transduction mechanisms in this paper are independent. Clearly, inositol polyphosphate production cannot be the cause of activation of the nifedipine-sensitive route because the latter was utilized by a low concentration of carbachol which did not affect inositol polyphosphate levels. Similarly, the nifedipine-sensitive route cannot be the mechanism of production of inositol polyphosphates because pretreatment with 10^{-5} M nifedipine did not affect inositol polyphosphate production by 10^{-4} M carbachol (data not shown).

It is therefore possible that each transduction mechanism is linked to a specific muscarinic receptor subtype, each subtype having different sensitivity to carbachol. If this is so, then it would appear that the subtype linked to the nifedipine-sensitive pathway has the highest relative sensitivity whereas that linked to inositol polyphosphate production has the lowest. Another possibility, however, is that one muscarinic receptor-type is linked to several different transduction pathways but with different efficacies. Hence the activated agonist-receptor complex would have high efficacy in activating the nifedipine-sensitive component but a low efficacy for inositol polyphosphate production. From studies on the effects of the transfected M_2 receptor subtype on IP_3 production and cyclic AMP inhibition, Ashkenazi *et al.* (1987) concluded that this subtype is linked to two different transduction mechanisms.

As the amount of acetylcholine released from a parasympathetic nerve terminal varies with the frequency of stimulation (Straughan, 1960), the concentration of acetylcholine to

which a tissue is exposed is variable. The physiological significance of this study then, may be as follows. Low sustained activity of parasympathetic nerves (presumably maintaining a low acetylcholine concentration in the synaptic cleft) can maintain a large contraction in the smooth muscle and an increase in nerve activity can induce a rapid further phasic contraction arising from a different transduction pathway.

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Discrepancy between the short and long term effects of ouabain on the sodium pumps of human cells grown in culture

Nina M. Griffiths, Patricia H. Ogden, *Richard Cormack & [1]J.F. Lamb

Departments of Biology & Preclinical Medicine, and of *Statistics, University of St Andrews, Fife KY16 9TS

1 Human cells (HeLa) were cultured for periods up to 48 h in growth medium in the absence or presence of a range of concentrations of cardiac glycosides. In some experiments the potassium concentration of the medium was varied between 0.3 mM and the usual 5 mM.

2 For periods up to 2 h in ouabain the association and dissociation rate constants were measured and the equilibrium binding constant (K_D) calculated; the apparent equilibrium binding constant (K'_D) was measured after 1–2 days growth in ouabain.

3 Ouabain had a K'_D after 2 days of 2–6 nM in 5 mM K^+ growth medium, a 4 fold greater blocking effect on sodium pumps after 2 days than expected from the association and dissociation rate constants measured in untreated or previously ouabain-treated cells.

4 This effect was: (a) approximately the same over a range of external potassium concentrations from 0.3 to 5 mM, although the absolute effect of ouabain over this range of potassium was much different; (b) probably not due to different isoforms of pumps in cells grown in ouabain compared to untreated cells; (c) apparently not a consequence of internalisation of pump-glycoside complexes.

5 We conclude that ouabain has only a limited access to sodium pumps in whole cells; this could be because sodium pumps cycle continuously through an inaccessible region of the plasma membrane. This effect needs to be considered both in the assessment of the magnitude of the long term effects of cardiac glycosides on cells, and in the measurement of the glycoside affinities of various isoforms of the pump.

Keywords: Sodium pumps; ouabain; Na^+, K^+-ATPase; cardiac glycosides; internalisation

Introduction

In cultured human cells, as in most other animal cells, active sodium and potassium ion transport is mediated by a Na^+, K^+-ATPase (the sodium pump) situated in the plasma membrane, which is specifically inhibited by the cardiac glycosides. The sodium pump has two types of protein subunit, alpha and beta; the alpha subunit (112 kDa) contains the binding sites for adenosine 5'-triphosphate (ATP) and cardiac glycosides, and is often loosely known as the 'catalytic subunit'. The beta subunit (35 kDa) is a glycoprotein that seems to be required for enzymatic activity, but its exact function is unknown. It was initially thought that the sodium pumps from all tissues and species were the same, only varying in their sensitivity to cardiac glycosides, but in the last few years it has become apparent that there are at least three different molecular forms of the alpha subunit, now called α1, α2 and α3 (Sweadner, 1989).

Considerable efforts are now being made to identify the distribution of these isozymes in various species and tissues by use of cDNA probes to identify their mRNA, and antibodies to demonstrate and measure their quantities in the plasma membranes. Concomitant with these studies the kinetic and functional differences of these isozymes are being re-measured to relate the molecular form of the isoform to its pharmacological properties.

HeLa cells, an epithelial cell line derived from human cervix, have only one alpha isoform of around 3.7 kb (Cutler *et al.* 1988), probably equivalent to α1 in rat (Emanuel *et al.*, 1986), so that the pharmacological properties of the glycoside-Na^+, K^+-ATPase interaction of these cells can be unambiguously related to this isoform. It is of particular interest to study this isoform for we had long noticed that the effect of cardiac glycosides on these and other cultured human cells under equilibrium conditions (see e.g. Lamb & McCall, 1972) seemed to be much larger than either that reported in the literature or that expected from the usual short term measurements. We describe here a detailed study of this effect.

[1] Author for correspondence.

Methods

Cell culture

Outline: HeLa (human epitheloid carcinoma cervix) cells were cultured routinely in tissue culture flasks (Sterilin) in Minimum Essential Medium (MEM) with Earle's salts containing 10% newborn calf serum, 1% non-essential amino acids and kanamycin 50 μg/ml. For experiments, cells were subcultured into 12 or 24 well multiwell plates (some earlier experiments used 3 cm diameter petri dishes) at typical seeding densities of 3–4 × 10^4 cells cm^{-2}. The plates were incubated at 37°C in an atmosphere of 95% air/5% CO_2. Cells were grown for 2–4 days until near confluence; the medium was then aspirated and replaced with fresh normal or experimental medium for the next 0.5 to 2 days. A stock of HeLa cells was also kept in liquid N_2 to ensure continuity of the cell line in case of infection.

Measurement of numbers of sodium pumps

Sodium pump numbers were estimated by measuring the specific [^{3}H]-ouabain binding. Cells were incubated for 20 min at 37°C in 200 nM [^{3}H]-ouabain in a modified Krebs-Hensleit solution containing either no potassium (0 K^+), or 15 mM potassium (15 K^+) or 1 mM unlabelled ouabain. The specific binding was taken as the difference between the total (0 mM K^+) and non-specific (15 mM K^+ or 1 mM ouabain) binding. Usually the 'non-specific' binding in excess ouabain was about half that in 15 mM potassium, but as the non-specific binding was much less than 10% of the total binding, this difference did not affect the conclusions drawn. The [^{3}H]-ouabain bound in 15 mM K^+ solution was directly related to the total binding, whereas that in excess ouabain was not, possibly indicating that 15 mM K^+ did not block all the [^{3}H]-ouabain binding to the pump. After incubation with [^{3}H]-ouabain, the plates were rinsed with ice-cold Krebs solution to remove extracellular isotope and the cells were then detached with trypsin/EDTA (0.25% trypsin, 2 mM EDTA in basal salt solution without Ca^{2+} or Mg^{2+}) to obtain a single cell suspension. Cell numbers and volume were then measured

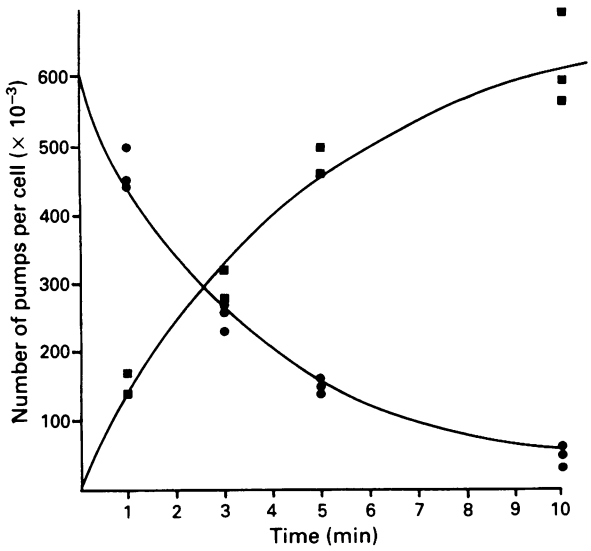

Figure 1 Direct and indirect measures of sodium pumps with [^{3}H]-ouabain. The ordinate scale shows the number of sodium pumps occupied by [^{3}H]-ouabain directly (■), or the disappearance of the unoccupied pumps (●) with time; the indirect measure. The first order rate equation fitted to the direct measure had a rate of association of 0.209 ± 0.033 min^{-1} and a maximum value of $704 \pm 55 \times 10^3$ pumps per cell; the indirect measure gave values of 0.276 ± 0.014 and 597 ± 19 respectively. As neither the rate nor maximum are significantly different the mean values are 0.243 min^{-1} (i.e. a $t_{1/2}$ of 2.9 min) and maximum pump numbers of 650×10^3 per cell.

with a Coulter Counter (Model ZM) and channelyser (Model 256). The amount of isotope was determined with a liquid scintillation Spectrometer (Packard Tricarb).

In some conditions it was not possible to measure the total number of sodium pumps directly, so an indirect method was used. In this we first bound non-radioactive ouabain or other glycosides to the cells and then estimated the unoccupied pumps by binding [^{3}H]-ouabain to them; the original number of pumps occupied by the non-radioactive ouabain (or other glycoside) was then calculated by measuring the total pumps in control cells. In cells treated chronically with glycosides this method is only applicable if the total number of pumps does not change much; this has been found to be so by Pollack *et al.* (1981). Figure 1 shows that in untreated cells direct and indirect procedures both give the same total number of pumps and the same initial rate of binding. The mean density of sodium pumps in untreated cells was 726,000 ± 36,000 (mean ± s.e.mean, $n = 25$) per cell.

Measurement of pump activity and cell sodium and potassium

Pump activity was assessed by measuring the ouabain-sensitive potassium influx (with ^{86}Rb as the tracer) for 5 min. This influx time gave sufficient counts and was short in comparison to the equilibrium time (> 1 h). We took account of our earlier evidence that ouabain sometimes stimulates co-transport, and so included bumetanide in the influx solutions to block this effect (Bakker-Grunwald *et al.*, 1982).

To measure cell sodium and potassium, plates were rinsed with an ice-cold isotonic sorbitol solution (275 mM sorbitol, 15 mM Tris-HCl; pH 7.4 at 4°C) and then lysed with ion-free distilled water for 1 h or more. The sodium and potassium concentrations were then measured by flame photometry. The cell water was obtained by multiplying the total cell volume by 0.8. Much previous work by us on these cells has shown that the Coulter system used, as described, gives a good measure of the cell volume; a direct measure of the cell water with labelled 3-O-methyl [^{14}C]-glucose sugars confirmed this method (see Aiton & Simmons, 1983).

Equilibrium constant for ouabain binding

In the absence of internalisation the dissociation constant, K_D, for the ouabain-ATPase complex formation is given by k_{-1}/k_1 where k_1 and k_{-1} are the association and dissociation rate constants respectively. The sodium pumps of HeLa cells, however, are being continuously turned over by the process of internalisation of membrane pumps and synthesis of new ones (Cook *et al.*, 1982), so this needs to be considered when measuring the binding of ouabain and other ligands. The apparent dissociation constant, K'_D, for this process is given by $(k_{-1} + k_{int})/k_1$ where k_{int} is the rate constant of internalisation. Substituting for k_1 yields the expression $K'_D = K_D (1 + k_{int})/k_{-1}$. Thus it follows that internalisation always increases the apparent equilibrium constant (i.e. decreases the apparent affinity; Klausner *et al.*, 1985). In almost all the experiments described in this paper we measure $(k_{-1} + k_{int})$ and not k_{-1} alone.

This equation assumes that all the pumps which are internalised are returned to the membrane, either by new synthesis or by recycling, so that the total density of the pumps on the membrane is constant over time. We tested this directly by measuring the rate of recovery of unoccupied pumps after a digoxin block and comparing it with the loss of digoxin from the cells (Algharably *et al.*, 1986). The rate of recovery of unoccupied pumps was $7.7 \pm 0.6\%$ h^{-1}, not different from the mean rate of loss of digoxin (which measures both internalisation and dissociation, see later) from the cells.

Plasma membrane isolation

Cells were grown on microcarrier beads, and the membranes prepared as follows. A 10 ml sample from the culture was collected in a sterile universal container. This sample was washed 4 times with cold 2-amino-2-hydroxymethyl-propan-1,3-diol (Tris) buffered saline (0.9% NaCl, 5 mM Tris) at pH 8. Between each wash the microcarrier beads were allowed to settle out before the supernatant was removed (around 3 min). The sample was incubated with ice-cold hypotonic solution (1 mM Tris-HCl, pH 8.0) for 30 min and then washed twice in ice-cold 10 mM Tris-HCl solution, pH 8.0. Then 10 mM Tris was added, the cells vortexed for 10 s to complete cell lysis. The beads with the attached membranes were then washed twice more, resuspended in 10 mM Tris-HCl pH 8.0 to give a 50% suspension. This was then sonicated for 10 s at 20 W and washed twice in ice-cold buffer. Analysis of isolated membranes showed that there was about a 25% recovery of membrane markers, with a purity similar to other methods (see Algharably *et al.*, 1986 for other details).

Experimental design and analysis of results

Experiments were designed to reduce the inter-cell differences as far as possible. With multi-well plates it was possible to examine several conditions on the same plate; if this was not possible then we handled different plates in as similar a way as possible. All comparisons between different conditions were made with the same batch of cells in the same experiment. The commercial package 'Enzfitter' was used on a desktop computer to fit curves and compute errors. This package fits equations to the raw data without any transformation, using a 'non-linear regression' method. It allows the error structure to be varied to check the 'robustness' of the conclusion for different assumptions about variability. Little difference was found; the curves shown were those fitted with constant variance (no weighting). The rectangular hyperbolae shown have a linear X-axis rather than the more common log axis. For simple t tests the commercial package 'Minitab' was used.

Drugs and materials

Tissue culture plastic ware, i.e. flasks, Petri dishes and 12 or 24 multiwell plates, were supplied either by Sterilin or Flow

Laboratories (Linbro). Culture media were obtained from Life Technologies. Cardiac glycosides and the Cytodex Microcarrier beads were bought from Sigma Chemical Co. [21, 22-^{3}H]-ouabain and ^{86}RbCl were bought from Amersham International. All other chemicals used were of Analar grade from BDH.

Solutions

The modified Krebs solution contained (mM): NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, Tris 15, HCl 12 and glucose 10 (pH 7.4). For 0 mM K^+ Krebs, K^+ salts were omitted and replaced by Na^+ salts.

Results

Steady state effects of cardiac glycosides

Figure 2 shows an experiment in which the effects of exposure of cells to various cardiac glycosides were studied over 48 h in the same batch of cells. The order of potency was ouabain, digitoxin, digoxin, acetylstrophanthidine and dihydro-ouabain. For a detailed comparison of short and long term exposure we used ouabain. Figure 3 shows that exposure of cells to a ouabain concentration of a few nM for 48 h reduces the unoccupied pump numbers to half; the best-fit equilibrium binding constant (K'_D) being 2.6 ± 0.7 nM. In two of the experiments in which there were sufficient data points the K'_D values were 2.5 and 2.9 nM, so that amalgamating the data did not distort the result. At higher ouabain concentrations than shown (> 27 nM) the cells died, and even at 27 nM they did not divide; this may explain the poorish fit of the curves at these higher concentrations. When experiments were done with a single concentration of ouabain, we used 10 nM as a compromise between seeing an effect and not causing too much cell deterioration.

In some experiments cells were only grown for 24 h in ouabain, and in these the best-fit K'_D was around 10 nM so that the steady state had not been reached in this time. In Appendix 1 we show that this time discrepancy would be expected to have only a minor effect.

Effect of serum and the potassium concentration

The serum concentration of the growth medium affects the density of sodium pumps (Aiton & Lamb, 1984), so we examined the effects of growth in ouabain at different serum concentrations. Over 48 h, raising serum from 0.1% to 20% increased pump density by 30% but 10 nM ouabain produced the same block (66%) at both serum concentrations, with no interaction (by ANOVA). Similar observations were made in 2 experiments in which cells were grown for 48 h in digoxin.

A low concentration of potassium in the growth medium upregulates the density of sodium pumps (Boardman *et al.*, 1974) in HeLa, and most cultured cells. Figure 4 shows that over 24 h a low potassium concentration greatly increased the absolute ouabain binding as well as upregulating pump density, so that ouabain had a much larger absolute and proportional effect at low potassium concentrations than at high ones.

Short term experiments with ouabain

In order to check if the expression for K'_D (see Methods) is a good description of the steady state binding of ouabain to the

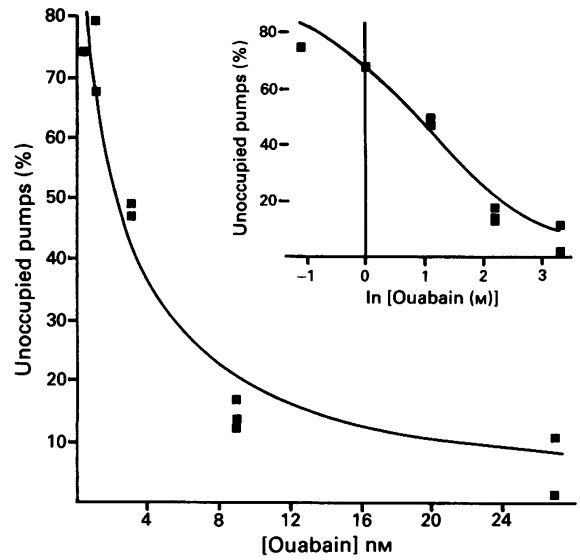

Figure 3 The effect of growing HeLa cells for 48 h in a range of ouabain concentrations on unoccupied pump numbers. After 4 days of growth in normal medium the cells were transferred to growth medium containing ouabain; 48 h later the unoccupied pumps were measured with 200 nM [^{3}H]-ouabain. The hyperbolic curve shown has a K_m of 2.5 ± 0.7 nM.

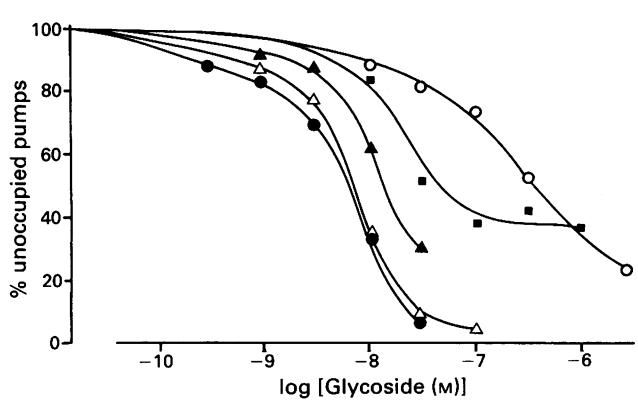

Figure 2 Effect of growth in the presence of various cardiac glycosides on unoccupied sodium pumps. HeLa cells were incubated in normal growth medium containing a range of concentrations of ouabain (●); dihydro-ouabain (○); digoxin (▲); digitoxin (△); or acetylstrophanthidin (■) for 48 h. The unoccupied pumps were estimated by incubation with 200 nM [^{3}H]-ouabain. The results are expressed as a % of the control value. Each point is the mean of 3 measurements. The digitoxin concentration is corrected for protein binding. All measurements were made on the same batch of cells. Lines fitted by eye; the s.e.mean was less than 5% of the mean.

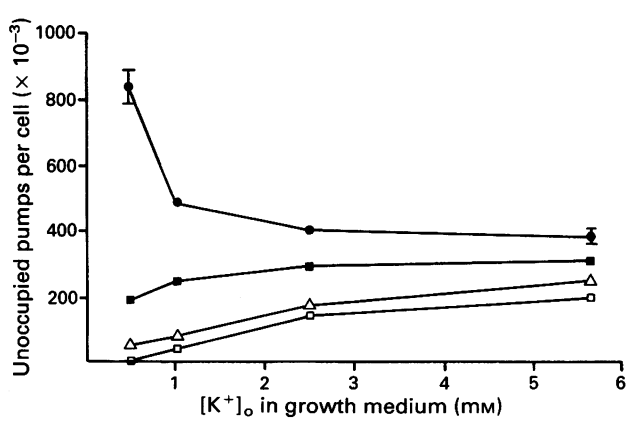

Figure 4 Effect of the potassium concentration of the medium on the density of unoccupied sodium pumps on HeLa cells in the presence and absence of various ouabain concentrations. Cells were grown for 4 days in normal medium and then transferred for 24 h to media containing various combinations of potassium shown; the ouabain concentrations in the growth medium were 0 (●); 2 (■); 5 (△) and 10 (□) nM. The unoccupied sodium pumps were then measured by exposure to [^{3}H]-ouabain. Each point is the mean of 3 replicate observations: typical s.e.means are shown; others were less than 5% of the mean.

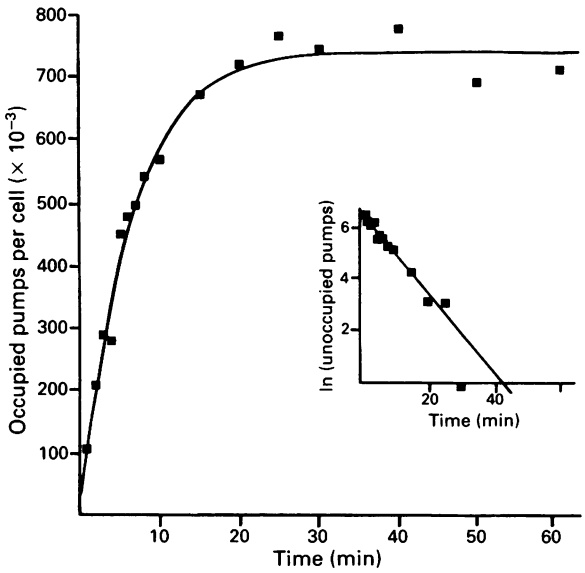

Figure 5 [^{3}H]-ouabain binding to untreated HeLa cells from a potassium-free medium. The first order rate equation fitted gives a V_{max} of $746 \pm 13 \times 10^3$ sites per cell and an association rate of 0.159 ± 0.009 min^{-1}; a $t_{1/2}$ of 4.4 min. The inset shows a log transformation of the data.

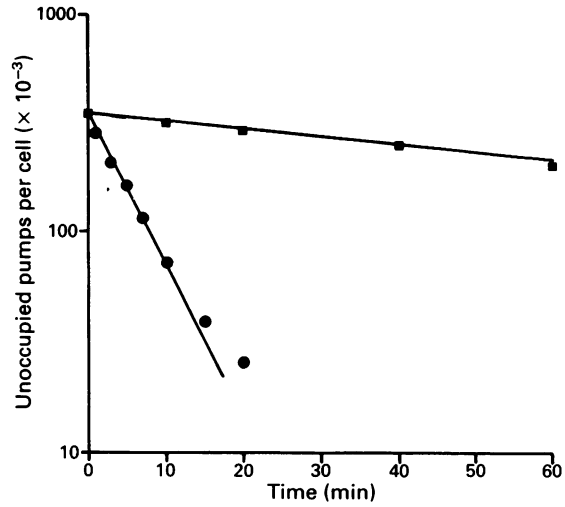

Figure 6 [^{3}H]-ouabain binding to HeLa cells grown in ouabain. The cells were grown for 3 days in normal medium and then for 2 days in medium containing 10 nM ouabain. [^{3}H]-ouabain binding was then measured as in the legend to Figure 5 both in potassium-free and in 5 mM potassium containing Krebs. In potassium-free Krebs (●) V_{max} is $353 \pm 5 \times 10^3$ sites per cell and the rate constant is 0.159 ± 0.01 min^{-1}, and in 5 mM potassium (■) V_{max} is $385 \pm 118 \times 10^3$ and the rate constant is 0.008 ± 0.003 min^{-1}. The lines drawn are best-fit lines to the rate equations (Enzfitter).

sodium pumps of HeLa cells we needed to measure the association rate constant ($k_1 = \ln 2/t_{1/2}$), the sum of internalisation and dissociation rates constants ($k_{-1} + k_{int}$), and the apparent equilibrium binding constant (K'_D), as has been done for the sodium pumps of red blood cells (Furukawa *et al.*, 1980). However, the apparent dissociation of ouabain from the pumps of human cultured cells is very slow, so that it takes many hours to reach equilibrium. At equilibrium the cell sodium is high and the potassium low, so that the cells are no longer in a normal state. The best that can be done is to measure the association and dissociation rates as usual, and the apparent binding constant under non-equilibrium conditions.

The rate of association of ouabain with the sodium pump is first order with respect to the ouabain concentration (Baker & Willis, 1970; Furukawa *et al.*, 1980; a point we also checked) and, in HeLa cells at least, is very sensitive to the concentration of potassium in the bathing medium, a condition which is used to estimate the non-specific binding. We made measurements of the rate of association in zero and 5 mM potassium both in untreated cells and in cells that had been grown in ouabain for 24–48 h.

Figure 5 shows the time course of ouabain binding to untreated cells in a nominally potassium free medium with 200 nM [^{3}H]-ouabain; the inset shows that the binding follows a log curve. The $t_{1/2}$ of this was 4.4 min, giving an association rate constant of 4.7×10^7 h^{-1} M^{-1}. Figure 6 shows the rates of binding in 0 and 5 mM potassium of 200 nM [^{3}H]-ouabain in cells which had been grown for 48 h in 10 nM ouabain. The $t_{1/2}$ values are around 4 and 86 min respectively (association rate constants of 4.8×10^7 h^{-1} M^{-1} and 2.4×10^6 h^{-1} M^{-1}; a potassium effect of 20 fold). We concluded from these experiments that there was no difference in the association rate constants of the ouabain-ATPase complex of untreated cells and those grown in ouabain for 48 h. The overall mean rates of association in all experiments, averaged over untreated and ouabain-treated cells was $6.2 \pm 1.0 \times 10^7$ h^{-1} M^{-1} ($n = 6$) in 0 mM potassium and $4.5 \pm 0.7 \times 10^6$ h^{-1} M^{-1} ($n = 7$) in 5 mM potassium, a mean potassium effect of 14 times from 0 to 5 mM.

Dissociation rates

Ouabain bound to the pumps of HeLa cells is mainly internalised by the cells and then processed through a lysosomal compartment (Cook *et al.*, 1982). The main determinant of ouabain dissociation from the cells is thus by post plasma membrane events, so that ouabain cannot be used to measure a dissociation rate from the sodium pumps on the plasma membrane. Digoxin is handled by the cell via a similar route but at a much faster rate, for the lysosomal step does not impede the loss of digoxin from the cell (Griffiths *et al.*, 1983). In previous work we showed that the plasma membrane of HeLa cells loses ouabain and digoxin at the same rate (about 9% h^{-1}; Algharably *et al.*, 1986; a result also confirmed in the present experiments), and that the rate of loss of digoxin from whole cells is the same as from the membrane; whereas ouabain is lost much more slowly from whole cells. The loss of digoxin from whole cells is therefore a measure of ($k_{-1} + k_{int}$).

In Figure 7 the loss of digoxin from untreated cells is compared with that from cells treated for 48 h with 10 nM ouabain; the rates of loss were 9.6 ± 0.9 and 12.2 ± 0.9% h^{-1} respectively. We also checked if the degree of the digoxin block, used to measure the efflux, altered the rate of efflux, and so did a number of experiments varying the proportion of pumps blocked by digoxin. The results were: untreated cells 9.3 ± 0.8 ($n = 7$), chronic cells 11.8 ± 0.6 ($n = 4$), both with all pumps blocked by digoxin, and untreated cells with trace digoxin block (5–10%) 9.6 ± 2.2% h^{-1} ($n = 5$). Although there is a suggestion that the cells adapt to chronic block, the difference is not statistically significant.

Apparent K'_D in short term experiments

The ratio of the (mean) dissociation and (individual) association rate constants measured in these short term experiments gives an apparent binding constant, K'_D, for ouabain in normal growth medium ($[K]_0$ 5 mM) of 24 ± 4 nM (s.e.mean; $n = 7$), a value some 9 times higher than the 2.6 nM found in equilibrium conditions: the value in 0 K^+ medium is 1.92 ± 0.23 nM ($n = 6$).

To try to obtain a direct measure of the binding constant we measured binding curves for [^{3}H]-ouabain with incubation periods of 0.25 to 2 h. Figure 8 shows a typical experiment in which cells were exposed to a range of ouabain concentrations for 0.5 h in K^+-free medium. Half saturation of the pumps occurs at 34 nM. Increasing the time to 4 h only reduced the half block concentration to 18 nM, but the cells

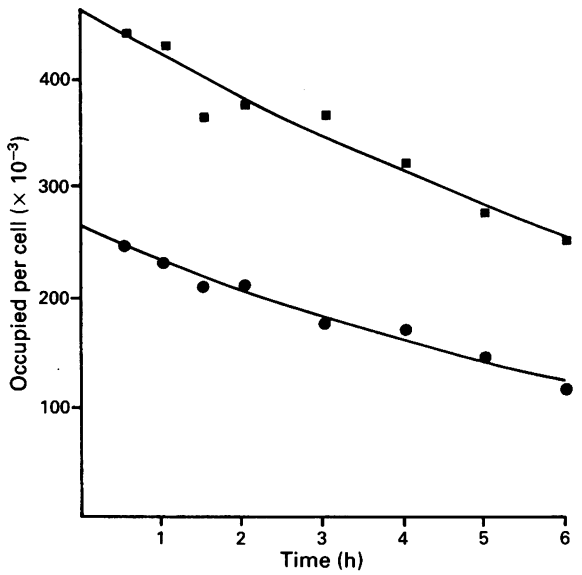

Figure 7 Rate of loss of digoxin from untreated and ouabain-treated cells. Cells were grown for 3 days in normal growth medium and then either changed into a similar or 10 nM ouabain medium for a further 2 days. After rinsing the cells were then put into 200 nM [^{3}H]-digoxin in potassium-free Krebs for 15 min, then washed at 37°C for 15 min to remove the non-specifically bound digoxin and then returned to the efflux medium. At the times indicated, aliquot plates were removed for counting of cell numbers and activity. Control cells (■) had an initial digoxin binding of 465 ± 13 × 10^3 sites per cell, and a rate of loss of digoxin of 9.6 ± 0.9% h^{-1}, and ouabain-treated cells (●) values of 265 ± 6 × 10^3 sites per cell and a rate of loss of 12.2 ± 0.9% h^{-1}. Each point is the mean of 3 observations, and the lines were fitted to an exponential dissociation equation by Enzfitter.

were then not in a good condition. In a similar experiment in 5 mM potassium, half block occurred at 100 nM.

The results so far show that there is a large discrepancy between the K'_D calculated from short term measurements and that found under equilibrium conditions in 5 mM potassium growth medium. We then extended these measurements over a range of $[K]_o$ values down to the lowest the cells would survive in, around 0.3 mM and so made a direct comparison in

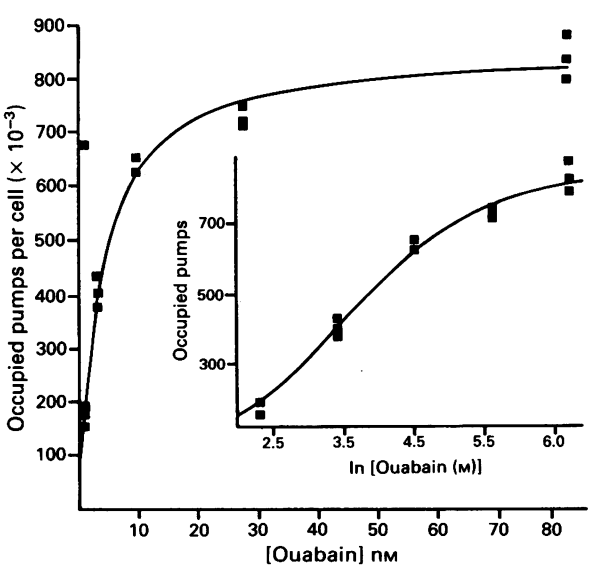

Figure 8 [^{3}H]-ouabain binding to HeLa cells after 0.5 h incubation. The main figure shows the number of molecules bound for each ouabain concentration on a linear scale, and the inset the usual log dose-response curve. The V_{max} is 858 ± 17 × 10^3 sites per cell and the K_m 34 ± 3 nM. Each point is a single value, and the Michaelis-Menten lines fitted by Enzfitter. The s.e.mean was less than 5% of the mean.

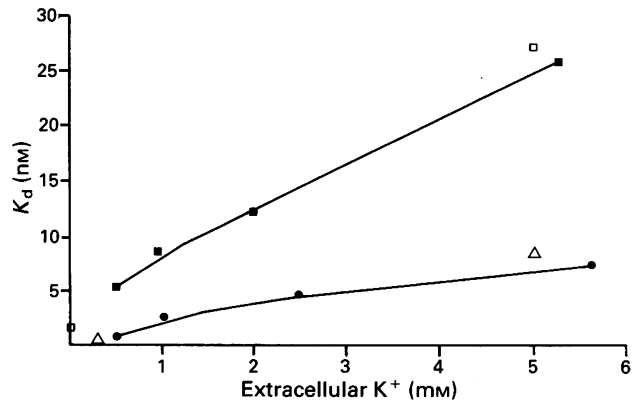

Figure 9 Effect of potassium on K'_D determined after chronic or acute exposure to ouabain in HeLa cells. The ordinate scale shows the apparent K'_D and the abscissa scale the potassium concentration in the growth or experimental medium. The data for chronic exposure to ouabain are taken from Figure 4, the acute results were obtained in the same experiment by measuring the initial rate of ouabain binding at potassium concentrations of 0.5, 1, 2.5 and 5 mM; the K'_D was then calculated using the mean value of dissociation rate of 9% h^{-1}. The lines are drawn by eye; (●) chronic exposure to ouabain; (■) acute exposure to ouabain. Also shown on the figure are single values from other chronic experiments (△), and other acute experiments (□).

the same experiment of the effect of potassium on chronic and acute binding. Figure 9 shows this comparison between the apparent K'_D found in 24 h chronic growth and that calculated from the initial rates of binding from 200 nM ouabain in the same potassium concentrations. It is clear that chronic growth binding decreases the apparent K'_D by a similar factor (around 4) at all potassium concentrations measured. As this experiment was only carried on for 24 h a rather larger discrepancy would be expected after 48 h.

Effect of pump occupancy on potassium influx

A possible explanation for the present results of a discrepancy in sensitivity between long and short term growths in ouabain is that HeLa cells have two isoforms for the sodium pump of differing sensitivities. This seems unlikely because cDNA probes only show one major band (α1) on Northern blots (see Discussion). Other evidence on this point can be obtained by examining the effect of an increasing block of sodium pumps on the active potassium influx, for two different sensititivies should show up as a discontinuous curve. Figure 10 shows the effect of the % occupancy of the sodium pumps by these cardiac glycosides on the ouabain-sensitive potassium influx, both in the presence and absence of bumetanide. There is no obvious discontinuity in the potassium influx with increasing block of the pump, a result consistent with the presence of only one isoform. If co-transport is present low concentrations of glycosides appear to 'stimulate' the ouabain-sensitive potassium influx (really an effect on co-transport; see Brown & Lamb, 1978; Bakker-Grunwald *et al.*, 1982). This perhaps explains the periodic claim that low concentrations of cardiac glycosides have their therapeutic action by pump stimulation.

In small cells such as HeLa, with a high surface to volume ratio, the most sensitive indicator of pump activity are changes in cell sodium and potassium. Figure 11 shows results from two experiments on different batches of cells done at different times. Both show a similar pattern; the potassium concentration falls by almost a fifth at the lowest ouabain concentration used (0.3 nM) and then continues to fall as the ouabain concentration increases; the sodium concentration shows an initial variable change and then a progressive rise. In other, less complete experiments, we found that 10 nM ouabain significantly increased the sodium concentration whereas 2 nM ouabain had a variable effect.

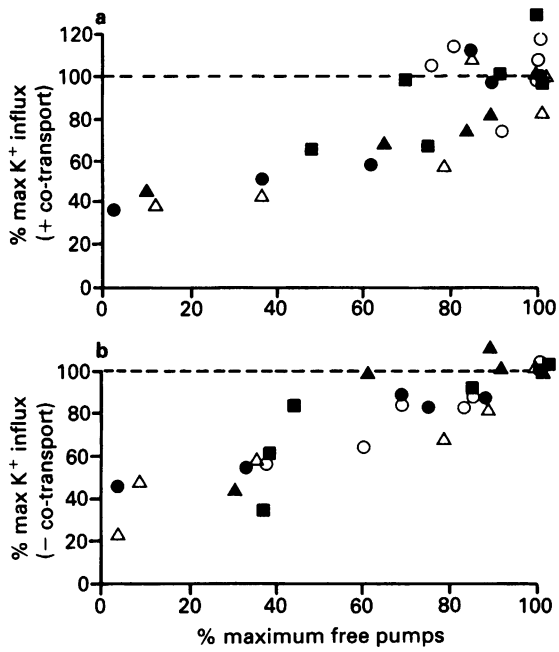

Figure 10 Effect of pump occupancy by various glycosides on the ouabain-sensitive potassium influx. HeLa cells were grown normally for 3 days and then for a further 2 days in various concentrations of ouabain (●); dihydro-ouabain (○); digoxin (▲); digitoxin (△); or acetylstrophanthidine (■). The plates were rinsed three times; half were then used to measure unblocked pumps and the other half to measure the potassium influx. Potassium influx (estimated with trace amounts of ^{86}Rb in 5 mM potassium salt solution) was measured in cells with no further drugs added or with 1 mM ouabain or with 1 mM ouabain and 10 μM bumetanide; calculation then gave ouabain-sensitive + co-transport influx (a) or ouabain-sensitive influx (b). Each point is the mean of 3 measurements made separately but on the same batch of cells. The s.e.mean of the values around 100% was 10%, composed of an error due to the influx and binding measurements. As the variance was constant this was also the error of the lowest values shown.

Association and dissociation experiments using membranes absorbed onto beads

Membranes prepared as described under Methods were loaded for various times up to equilibrium in 200 nM [^{3}H]-ouabain. At equilibrium the total binding was in the range of 22 to 32 pmol mg^{-1} protein, which corresponds to around 500,000 sodium pumps per cell; a normal value for intact cells. The ouabain dissociated from the membranes with a $t_{1/2}$ of around 3 h (a rate constant of $0.22 \pm 0.01\,h^{-1}$), a rate 2 to 3 times faster than from intact cells. In a single experiment in potassium-free conditions ouabain bound to the membranes with a $t_{1/2}$ of around 2 min (a rate constant of $0.37 \pm 0.10\,min^{-1}$). The calculated equilibrium binding constant from these values is 2.02 nM, a value similar to the 1.92 nM found in whole cells also in potassium-free conditions. These experiments suggest that the exchanges of ouabain between the medium and membranes on beads are somewhat faster than those with whole cells, but the K_D is of the same order. They do not explain the discrepancy between the short and long term effects of ouabain.

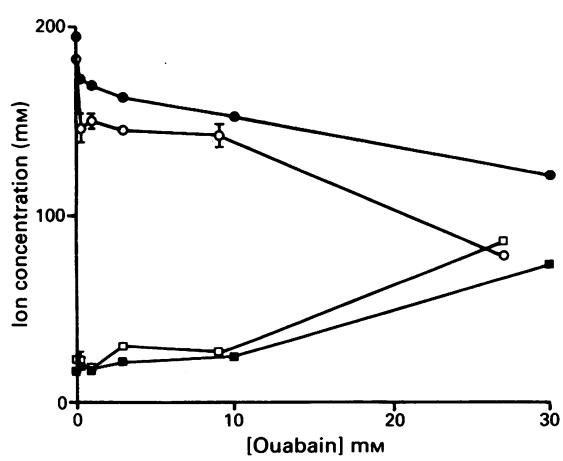

Figure 11 The effect of chronic ouabain treatment on cell sodium and potassium. Cells were grown normally for 3 days and then changed to medium containing the ouabain concentrations shown. Two days later the sodium and potassium contents were measured. In parallel plates cell numbers and water were measured. Two separate experiments on different batches of cells are shown. Each point was the mean of 4 replicate measurements; the variance was constant at 7.8 for sodium, and 22 for potassium; s.e.means are shown. The cell chloride in medium was 52 mM and in Krebs 74 mM; (●, ○), potassium; (■, □), sodium.

Discussion

The main point shown here is that equilibrium binding constants found by growth of cells in ouabain are much lower (by a factor of at least 4) than those expected from the ratios of the association and dissociation rate constants measured independently in short term experiments, either in intact cells or isolated membranes. This is different from the extensive study of the interaction of ouabain with Na^+, K^+-ATPase in turkey red cells (Furukawa *et al.*, 1980), which showed that the equilibrium dissociation constant (K_D) for the interaction predicted from the ratio of the dissociation and association rate constant agreed closely with independently measured values of K_D measured directly under equilibrium binding conditions. There are a number of differences between HeLa and turkey red cells, some of which might account for the observed difference; we have investigated a number of these differences.

(1) Long term growth in cardiac glycosides could have led to an alteration in the state of the cells, for as previously noted such growth greatly reduces cell division (Lamb & McCall, 1972) probably due to a reduction in protein synthesis. We find, however, that there is only a substantial effect on cell growth (probably via changes in cellular ions affecting cell division) at high levels of glycosides, so this cannot explain the effects at low glycoside concentrations. We think that this does affect the goodness of fit of the rectangular hyperbola at such high blocks; this may correspond to the toxic effects seen clinically.

Another effect of a rise in cell sodium following partial block of the pumps might be to increase the rate or magnitude of glycoside binding by increasing the fraction of time the pump was in the E2 state, as well as perhaps recruiting 'spare pumps'. This is unlikely, firstly because the rate of binding in fresh and ouabain-treated cells in these experiments was the same, and secondly we find that loading HeLa cells with sodium, by use of potassium-free medium, does not alter the sensitivity to ouabain (unpublished experiments).

(2) In short-term experiments, glycoside binding to the sodium pump in human, and some other cells is very sensitive to the potassium concentration in the medium, a finding confirmed in this study. However, it is not known if there is a quantitatively similar effect in longer term experiments such as those done here. If the potassium effect were much smaller, perhaps because ouabain binding could occur during the occasional backwards working of the pump which is known to occur normally, then this might explain our results. Our experiments show that although the potassium effect is perhaps somewhat smaller in long term experiments, the difference is less than a factor of two and so is unlikely to be the major explanation. This conclusion agrees with the clinical finding that low serum potassium potentiates glycoside effects.

(3) Internalisation of glycoside-pump complexes occurs in HeLa cells but not in red cells, and this could affect the process: (a) If each internalised pump is replaced by a new one then internalisation should decrease the affinity to cardiac gly-

cosides, rather than increase it, for it provides another way of replacing blocked pumps (Klausner *et al.*, 1985), although the effect should be quite small in HeLa cells with their slow rate of internalisation. (b) If some of the internalised pumps are not replaced by new ones then the total density of pumps (blocked + free) on the cell surface would be reduced, and this could provide an explanation for our observation. Pollack *et al.* (1981), however, showed that chronic growth marginally increases the density (by less than 20%), rather than decreases it, and we could find no change in the total number of membrane pumps on chronic growth (Owler, 1986). Our present results also show that the disappearance of blocked pumps equals the reappearance of fresh ones, implying a steady state of pump density. (c) Another possibility is that as internalisation probably involves various compartments, glycoside growth might alter rates between these so as to produce a false equilibrium constant. Mathematically we can find no way by which this can be made to alter the equilibrium values of blocked pumps, and so had to abandon this explanation.

For these various reasons we do not think that internalisation is a factor in the apparent increase in sensitivity to cardiac glycosides observed in prolonged growth.

(4) Another possible explanation for the present results would be for HeLa cells to have two isoforms for the sodium pump of differing sensitivities, and for the proportion of these isoforms expressed in the plasma membrane to differ between the acute and chronic treatment regimes. We investigated the following points in considering this explanation: (a) Northern blots of HeLa cell mRNA with a general a-cDNA probe (pHank) only show one major band (Cutler *et al.* 1988), indicating only one a-subunit, probably equivalent to $\alpha 1$ (Sweadner, 1989); (b) the association and dissociation rate constants of fresh cells and those treated chronically with ouabain are the same; (c) an increasing block of the sodium pumps with cardiac glycosides causes a continuous decrease in the active potassium influx, with no sign of the kind of discontinuity expected from two or more isoforms.

(5) The rectangular hyperbola is the appropriate representation of a reaction between one ligand and one receptor when equilibrium is reached. Before equilibrium is reached the curve is not of this form and values of rate constants obtained by fitting such a curve would be inaccurate. The magnitude of such errors, and the internal evidence for them, are explored in Appendix 1. They cannot explain our findings.

The temperature sensitivity of ouabain binding to HeLa cells measured at 2, 13 and 37°C gave an activation energy of 27.6 kcal mol^{-1}, a figure very similar to that obtained by Furukawa *et al.* (1980) for the binding of ouabain to turkey red blood cells, so that this aspect of our measurements agreed.

We think that the most likely explanation for the apparent difference in sensitivity of short and long term sensitivity to cardiac glycosides involves the following combination of events in cells grown chronically in cardiac glycosides: a slightly reduced sensitivity of cardiac glycoside binding to potassium, slower removal of glycosides from the membranes due to raised cell sodium (Pollack *et al.*, 1981) and better access of cardiac glycosides to the sodium pump. This latter factor probably is largely responsible for underestimating the association rate constant in short term experiments and so underestimating the sensitivity.

A possible drawback to this latter explanation has been pointed out by Klausner *et al.* (1985) in a slightly different context. This is, that such diffusional slowing should also affect the rate of dissociation and so not have an effect on the equilibrium dissociation constant. We think that this does not apply to these cells, for we are measuring the sum of dissociation and internalisation and think that internalisation dominates the reaction. Indeed it may be that the diffusional delay impedes dissociation so much that the membrane is largely cleared by internalisation.

However, the cause of this delay has not been established in this work. The possibilities are either that all the pumps are partially hidden from the bathing medium, perhaps by the normal coating of molecules found on cells, or that a proportion of the pumps are not accessible to the bathing medium and so can only be reached rather slowly. We examine such models mathematically in Appendix 2 and show that if pumps cycle into and out of such an inaccessible region this would explain our results. Possible regions are the coated pits which exist on the cell surface of most cells.

Although the explanation for these results is not clear, it is obvious that the isoform present in these human cultured cells is very sensitive to cardiac glycosides when measured in normal growth medium containing 5 mM potassium under equilibrium conditions. As the apparent sensitivity of the HeLa Na^+, K^+-ATPase in short term experiments is similar to that of other types of human cells (heart cell membranes; Erdmann, 1981: leucocytes; Boon *et al.*, 1984); it may well be that the true sensitivity of these other cells is also some 4 to 10 times the apparent value. This sensitivity is such that if this isoform is present in human heart the normal plasma concentrations of cardiac glycosides of a few nM should give a substantial block of the sodium pumps. This finding also does have the consequence that it is not a straightforward matter to obtain a correct measure of the pharmacological properties of the various isoforms of the sodium pump in short term experiments for comparison with the other measures of these isoforms.

These results were obtained on cells in culture, so the question arises as to whether similar results would be obtained in animals *in vivo*. In dogs treated chronically with digoxin to test if the sodium pumps adapt to the continued presence of therapeutic doses, an incidental finding of Ku *et al.* (1977), not discussed in the paper, was that the sensitivity to chronic digoxin was some 2 to 15 times greater than to acutely applied digoxin. This suggests that cultured cells are a good model for the *in vivo* situation.

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Appendix 1

Chronic observations made before equilibrium is reached

The time dependent solution of the simple reaction equation, $S + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SR$, gives the proportion of pumps that are blocked after time t in concentration s of ouabain as

$$b(t) = \frac{s}{(k_{-1}/k_1 + s)}[1 - e^{-(k_1 s + k_{-1})t}]$$

$$= B_{equil}[1 - e^{-(k_1 s + k_{-1})t}]$$

As a function of s, this differs from the equilibrium equation because of the distorting factor

$$[1 - \exp^{-(k_1 s + k_{-1})t}].$$

If the experimenter believes the reaction to be in equilibrium after time t and fits a rectangular hyperbola $Vs/(K_D + s)$ to data obtained at that time from cells exposed to a range of concentrations s, the estimates V and K_D will be biased as estimates of the true $V = N$ and $K_D = k_{-1}/k_1$. Clearly the extent of the bias depends primarily on the value of $k_1 t$, the dominant term in the exponent when k_{-1} is small, as in these experiments.

The results of generating data by this equation and fitting a rectangular hyperbola are shown below, for a typical acute experiment, with five levels of s: 0, 1, 3, 9, 27 nM, the true $K_D = 2.7$ nM and the true $V = 1000$.

The shorter the duration of the experiment, the more will the K_D from a fitted rectangular hyperbola overestimate the true K_D. For the fitted value to be double the true value, $k_1 t$ has to be as low as $0.25\,nM^{-1}$ (e.g. $k_1 = 0.001\,nM^{-1}\,min^{-1}$ after 4 h). The fact that the rectangular hyperbola is the wrong form of curve for the data will be difficult to infer from the residuals in the presence of typical experimental variation.

If equilibrium has not been reached within 24 h then the true K_D will be less than the estimate obtained from fitting a rectangular hyperbola to the 24 h data. However, with the true $k_1 = 0.001\,nM^{-1}\,min^{-1}$, at 24 h $k_1 t = 1.44\,nM^{-1}$ and the effect of stopping early on K_D or V can be seen from Table 1 to be trivial.

Appendix 2

Models with inaccessibility

The observation that the equilibrium K_D in chronic experiments is less than the ratio k_{-1}/k_1 of rate constants in acute experiments can be generated by the following mechanism.

Suppose that there is a continual movement of all pumps from an accessible state A to an inaccessible state I at rate θ_1, the reverse movement occurring at rate θ_2. Ouabain can be associated with, or dissociated from, a pump only while it is accessible, an inaccessible pump being locked in the state, free F or blocked B, it was in when it became inaccessible. One, but not the only, possibility is that a pump becomes inaccessible when it enters a coated pit. Although such pits are usually considered as the location of internalisation, no internalisation need be invoked to generate the required property. We describe three models differing in the behaviour of blocked pumps. They have particularly simple solutions if it is assumed that equilibrium has been reached between A_F and I_F before the cell is placed in the glycoside medium. First assume that transition rates between A and I are the same for free or blocked pumps.

$$I_F \underset{\theta_2}{\overset{\theta_1}{\rightleftharpoons}} A_F \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A_B \underset{\theta_2}{\overset{\theta_1}{\rightleftharpoons}} I_B$$

The proportion of sites in the accessible state remains constant at $\theta_2/(\theta_1 + \theta_2)$. Solution of the resulting differential equations gives the proportion of blocked pumps at time t as

$$\frac{k_1 s}{(k_1 s + k_{-1})}[1 - ce^{-\alpha_1 t} - (1 - c)e^{-\alpha_2 t}]$$

where α_1, α_2 are the real, positive, unequal roots of the quadratic $x^2 - (\theta_1 + \theta_2 + k_1 s + k_{-1})x + \theta_2 (k_1 s + k_{-1}) = 0$ and $c = [\theta_2 (k_1 s + k_{-1}) - \alpha_2 (\theta_1 + \theta_2)]/[(\alpha_1 - \alpha_2)(\theta_1 + \theta_2)]$.

Table 1

$k_1 t$	K_D	V	*Residual s.s.*	*Residuals about fitted hyperbola*				
				0	1	3	9	27 nM
1.9	2.70	1000.2	0.03	0	−0.1	0.1	0	0
1.5	2.71	1000.3	0.56	0	−0.6	0.4	0.2	−0.2
1.1	2.74	1002.4	9.1	0	−2.3	1.5	0.8	−0.9
0.7	2.92	1011.8	122	0	−8.1	3.9	5.0	−4.1
0.3	4.38	1071.7	1160	0	−18.0	−4.8	25.0	−13.4
0.1	13.34	1292.5	220	0	−6.5	−8.6	9.7	−2.8

This has the same equilibrium relation (t large) as the simple exponential model

$$\frac{k_1 s}{(k_1 s + k_{-1})}[1 - e^{-(k_1 s + k_{-1})t}]$$

Moreover an acute experiment will be virtually unable to distinguish between the two models if c is close to zero or to 1. For example, with $(k_1 s + k_{-1}) = 0.2\,\text{min}^{-1}$, $\theta_1 = 0.1875$ and $\theta_2 = 0.1125$ so that 5/8 of pumps are inaccessible at any instant then $c = 0.9375$, $\alpha_1 = 0.05$, $\alpha_2 = 0.45$ and the mixture of exponentials is almost indistinguishable, on the basis of an acute experiment lasting up to 1 h, from a single exponential with rate constant of 0.058, a value that is roughly one-quarter of that (0.2) in the model with inaccessible states. The values in the example were selected to correspond to values in Klausner *et al.* (1985).

The second model postulates that blocked pumps cannot become inaccessible. The proportion of blocked pumps at time t is again a mixture of two exponentials

$$\frac{k_1 s}{[k_1 s + k_{-1}(1 + \theta_1/\theta_2)]}[1 - ce^{-\alpha_1 t} - (1 - c)e^{-\alpha_2 t}]$$

where α_1, α_2 are the real, positive, unequal roots of the quadratic

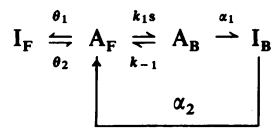
$$x_2 - (\theta_1 + \theta_2 + k_1 s + k_{-1})x + [\theta_2(k_1 s + k_{-1}) + \theta_1 k_{-1}]$$

and

$$c = [(k_1 s - \alpha_2) + k_{-1}(1 + \theta_1/\theta_2)]/(\alpha_1 - \alpha_2).$$

Although, when the dissociation rate k_{-1} is small, the asymptote and the exponents differ little from the earlier model, the mixing constant c is different. With the values used above $c = 0.42$. Such an equal mixture of the two exponentials is more readily distinguishable from a single exponential. Moreover, the best-fitting exponential has an estimated rate constant 0.16, not greatly different from the 0.2 used to generate the data. Thus this model does not generate patterns which match the experimental results described in the main paper.

A third model of this type postulates that inaccessible blocked pumps revert to accessibility only by losing their bound ligand:



One possible mechanism for this is that the inaccessible sites are internalised pumps, models for which are discussed in detail by Klausner *et al.* (1985). In this case radioligand binding will measure A_B only, not $(A_B + I_B)$ as assumed in the other models. In general the rates of internalisation may be different for unblocked and for blocked pumps, denoted θ_1 and α_1 respectively, and the transitions from I_F and I_B to A_F are likely to be replacement by new pumps, occurring at a constant rate β. However, in such a model the number of accessible pumps will vary with the degree of blocking: at equilibrium

$$A_F + A_B = \frac{\beta}{\theta_1}\left[1 - \frac{k_1 s(\alpha_1 - \theta_1)}{(\alpha_1 k_1 s + \alpha_1\theta_1 + \theta_1 k_{-1})}\right]$$

The time-dependent behaviour of the proportion of blocked pumps is again a mixture of two exponentials which can have properties of either of the previous models according to the numerical values of the parameters.

The first and third mechanisms thus provide possible explanations of the difference between acute and chronic experiments.

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Opioid activity of dermenkephalin analogues in the guinea-pig myenteric plexus and the hamster vas deferens

†Sandrine Sagan, *A.D. Corbett, †M. Amiche, †A. Delfour, †P. Nicolas & [1]H.W. Kosterlitz

Unit for Research on Addictive Drugs and *Division of Physiology, School of Biomedical Sciences, Marischal College, University of Aberdeen, AB9 1AS and †Laboratoire de Bioactivation des Peptides, Institut Jacques Monod, Université Paris 7, 2 Place Jussieu, 75251 Paris Cedex 05, France

1 To elucidate the structural features required for selective and potent action of dermenkephalin at the δ-opioid receptor, a series of analogues of dermenkephalin and dermorphin were tested for their effectiveness in depressing electrically-evoked contractions of the vas deferens of the hamster (δ-opioid receptors) and the guinea-pig myenteric plexus-longitudinal muscle preparation (μ- and κ-opioid receptors).

2 Dermenkephalin was more selective and more potent at δ-receptors than the δ-ligand [D-Pen^2, D-Pen^5]-enkephalin. The responses to dermenkephalin in the hamster vas deferens were increased by addition of peptidase inhibitors; the maximum effect was obtained with 3 μM thiorphan.

3 [L-Met^2]-dermenkephalin had 0.2% and [L-Ala^2]-dermorphin 0.01% of the agonist activity of the corresponding endogenous peptides which have D-amino acids in position 2. The pharmacological activity of these analogues was unaffected by inhibition of peptidases. This emphasizes the role that the D-configuration plays in determining the bioactive folding of these highly active peptides.

4 Dermenkephalin-(1-6)-NH_2 was more potent at δ-receptors than at μ-receptors whereas, dermenkephalin-(1-4)-NH_2 is a selective μ-agonist, having no activity at δ-receptors.

5 Substitution of the C-terminal tripeptide of dermorphin with the C-terminal tripeptide of dermenkephalin abolished the μ-receptor preference of dermorphin. The resulting hybrid peptide, Tyr-D-Ala-Phe-Gly-Leu-Met-Asp-NH_2 was as potent as dermenkephalin at δ-receptors. A shift towards a preference for δ-receptors was obtained when the C-terminal tetrapeptide of dermorphin was replaced by the C-terminal tetrapeptide of dermenkephalin.

6 Substitution of Asp by Asn in position 7 of dermenkephalin caused an increase in μ-receptor potency and a decrease in δ-receptor potency, resulting in a 20 fold decrease in μ-receptor selectivity. Dermenkephalin-(1-6)-NH_2 and [Asn^7]-dermenkephalin have almost identical δ-receptor agonist potencies and ratios of IC_{50} in the myenteric plexus to IC_{50} in the hamster vas deferens.

7 The results obtained emphasise the importance of a negative charge at the C-terminus of dermenkephalin for selectivity at the δ-opioid receptor. Furthermore, the hydrophobic residues Leu^5 and Met^6 may be critical in ensuring tight binding to the receptor which results in high agonist potency.

Keywords: Opioid peptides; peptide analogues; μ-receptor; δ-receptor; hamster vas deferens; guinea-pig ileum; myenteric plexus

Introduction

Most of the endogenous opioid peptides arise from the processing of one of three distinct precursors, pro-opiomelanocortin (Nakanishi *et al.*, 1979), pro-enkephalin (Noda *et al.*, 1982) or pro-dynorphin (Kakidani *et al.*, 1982). Each of these peptides interacts with more than one type of opioid receptor (Kosterlitz, 1985) which makes it difficult to explore the physiological role of the μ-, δ- and κ-opioid receptors. A fourth group of opioid peptides has been found to be present in the skin of frogs belonging to the subfamily *Phyllomedusinae* (Montecucchi *et al.*, 1981; Erspamer & Melchiorri, 1983; Erspamer *et al.*, 1989). These heptapeptides, dermorphin (Montecucchi *et al.*, 1981), dermenkephalin (Mor *et al.*, 1989; Amiche *et al.*, 1989) [also known as deltorphin (Kreil *et al.*, 1989) or dermorphin gene-associated peptide (Lazarus *et al.*, 1989)], deltorphin I and deltorphin II (Erspamer *et al.*, 1989; Richter *et al.*, 1990), are devoid of structural homology with mammalian opioids and contain a common N-terminal sequence Tyr-D-aa-Phe, in which 'D-aa' is either D-Ala or D-Met. Thus, they are unique among peptides synthesized by animal cells in having a D-amino acid in their sequence, even though the D-residue is encoded by the usual L-amino acid codons in the cDNA (Richter *et al.*, 1987).

These amphibian peptides have singular pharmacological patterns since they are selective for one of the opioid receptor types. Dermorphin, Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2, has high affinity and selectivity for the μ-opioid receptor (Broccardo *et al.*, 1981; Yamashiro *et al.*, 1983; Rossi *et al.*, 1986; Amiche *et al.*, 1990). In contrast, dermenkephalin, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, and the deltorphins I and II, Tyr-D-Ala-Phe-aa-Val-Val-Gly-NH_2 where 'aa' is either Asp or Glu, have high affinity and selectivity for the δ-opioid receptor (Amiche *et al.*, 1989; Erspamer *et al.*, 1989; Kreil *et al.*, 1989; Lazarus *et al.*, 1989); their affinities and selectivities are the equal of, or are superior to, those of the synthetic δ-ligands, [D-Pen^2, D-Pen^5]-enkephalin (Mosberg *et al.*, 1983), [D-Ser^2, (OtBu), Leu^5]-enkephalyl-Thr^6 and [D-Ser^2, (OtBu), Leu^5]-enkephalyl-Thr^6 (Delay-Goyet *et al.*, 1988).

In binding assays, it has been demonstrated that the C-terminal tripeptide of dermenkephalin is critical in specifying the δ-selectivity of the molecule. Moreover, the preferred opioid binding of dermorphin is changed from the μ-site to the δ-site when the C-terminal tripeptide of dermorphin is replaced by the C-terminal tripeptide of dermenkephalin (Sagan *et al.*, 1989a,b). However, binding assays provide information only on the interaction of a compound with the recognition site of a receptor but they do not determine whether a peptide acts as agonist or antagonist. In the present investigation, we have determined with bioassays in isolated tissues, the opioid activities of a series of dermenkephalin and dermorphin analogues. The main assay preparations used were the myenteric plexus-longitudinal muscle of the guinea-pig small intestine which has μ- and κ-opioid receptors but no δ-receptors (Lord *et al.*, 1977) and the hamster vas deferens

[1] Author for correspondence at: Unit for Research on Addictive Drugs, School of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen AB9 1AS.

which contains δ-receptors but not μ- or κ-receptors (McKnight *et al.*, 1985); the mouse vas deferens which contains μ-, δ- and κ-receptors (Hughes *et al.*, 1975; Schultz & Wüster, 1981) also was used. Since the binding studies have shown that dermorphin and dermenkephalin have negligible κ-affinity (Amiche *et al.*, 1989), comparison of the results obtained in the myenteric plexus with those in the hamster vas deferens should demonstrate the amino acids required for selective and potent agonist action at the μ- and δ-opioid receptors. The selective κ-opioid antagonist nor-binaltorphimine was used to confirm that none of the analogues had κ-opioid activity (Portoghese *et al.*, 1987).

Methods

Bioassays in isolated tissues

The myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine and the vasa deferentia of the hamster and the mouse were set up for field stimulation in 3 ml siliconised organ baths as described previously (McKnight *et al.*, 1983; 1985). Dunkin-Hartley guinea-pigs (Tuck), Golden Syrian hamsters (MB strain; Intersimian) and TO mice (Tuck) were used. When the myenteric plexus preparation was used, hexamethonium bromide (70 μM; May & Baker), mepyramine maleate (0.13 μM; May & Baker) and choline chloride (20 mM; BDH) were added to the Krebs solution (Kosterlitz & Watt, 1968).

Complete dose-response curves were constructed for each of the peptides; potencies were expressed as IC_{50} values (nM). Between each dose of the peptide under investigation, a standard agonist was used to monitor any changes in tissue sensitivity during the experimentation. The δ-opioid agonist [D-Pen^2, D-Pen^5]-enkephalin (Mosberg *et al.*, 1983) was the standard for the vasa deferentia and the μ-agonist [D-Ala^2, MePhe^4,Gly-ol^5]-enkephalin (Kosterlitz & Paterson, 1981) for the myenteric plexus. The possibility of dermenkephalin or dermorphin having any antagonist activity was estimated by the 'single dose' method of Kosterlitz & Watt (1968).

Since it is well known that many opioid peptides are rapidly degraded by tissue peptidases, the potencies of dermorphin, dermenkephalin and their analogues were determined in the hamster vasa deferentia before and after the addition of a cocktail of peptidase inhibitors: bestatin (10 μM), amastatin (10 μM), thiorphan (10 μM) and captopril (10 μM) (McKnight *et al.*, 1983; 1985). In a few experiments the effects of different concentrations of individual peptidase inhibitors were also examined. The tissues were exposed to the peptidase inhibitors for at least 20 min before reassessing activity of the opioid compounds; full dose-response curves were constructed before and after addition of antipeptidases.

In preliminary experiments, it was found that in the myenteric plexus and in the mouse vas deferens the potencies of dermenkephalin and dermorphin were unchanged by inhibition of tissue peptidases. Consequently, full dose-response curves for the analogues were not constructed in either tissue after inhibition of peptidases; at the end of each assay a single concentration of analogue was added to the tissue after the addition of peptidase inhibitors to the bathing Krebs solution.

Peptides and drugs

Dermenkephalin, dermorphin and related peptides (Table 1) were prepared by stepwise solid phase synthesis as described (Amiche *et al.*, 1988). After treatment with liquid HF and ether extraction, the peptides were purified by a combination of Sephadex gel filtration, ion exchange chromatography and preparative high performance liquid chromatography (h.p.l.c.). Homogeneity of the synthetic peptides was assessed by analytical h.p.l.c., thin layer chromatography, amino acid analysis and fast atomic bombardment mass spectrometry (Nicolas *et al.*, 1986). Stock solutions of the peptides were prepared in distilled water and stored at −25°C. Drugs were added to the bath from siliconised glass microsyringes in volumes not exceeding 50 μl.

The opioid agonists and antagonists used were [D-Pen^2,D-Pen^5]-enkephalin and [D-Ala^2,MePhe^4,Gly-ol^5]-enkephalin (Peninsula), naltrindole and nor-binaltorphimine (Research Biochemicals) and naloxone HCl (Endo Laboratories). The inhibitors of peptidases were: amastatin and bestatin (Sigma), captopril (Squibb) and thiorphan (Peninsula). Dilutions were made in Krebs solution before use.

Results

Opioid activity of dermenkephalin and dermorphin in bioassays

Dermenkephalin had very low agonist activity in the guinea-pig myenteric plexus preparation (IC_{50} = 5000 nM) but was much more active in the hamster vas deferens (Table 2). The inhibitory effects of dermenkephalin in the hamster vas deferens were blocked by either the selective δ-opioid antagonist naltrindole (Portoghese *et al.*, 1988; Figure 1) or by 100 nM naloxone; 10 nM naloxone did not antagonize the action of dermenkephalin (data not shown).

Dermorphin was very potent in the myenteric plexus preparation but it was inactive in the hamster vas deferens (Table 2). The inhibitory effects in the myenteric plexus were antagonized by a low concentration of naloxone (10 nM), indicative of an action at μ-receptors but they were not antagonized by the selective κ-opioid antagonist nor-binaltorphimine (10 nM).

Dermorphin and dermenkephalin were equiactive in the mouse vas deferens with IC_{50} values of ~2 nM (Table 2). Neither of the peptides had antagonist activity in any of the bioassay tissues.

There is no evidence from the bioassays that either of the compounds has affinity for κ-receptors. Therefore, the ratio of the IC_{50} value in the guinea-pig myenteric plexus to the IC_{50} value in the hamster vas deferens is a measure of the degree of selectivity of the peptide for the δ-receptor or for the μ-receptor. If the ratio is high, e.g. 385 for dermenkephalin, the

Table 1 Amino acid sequence of amphibian skin opioid peptides and dermenkephalin analogues used in this investigation

Peptide	*Primary structure*
Dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
Dermenkephalin	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2
[L-Ala^2]-dermorphin	Tyr-L-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
[L-Met^2]-dermenkephalin	Tyr-L-Met-Phe-His-Leu-Met-Asp-NH_2
Dermenkephalin-(1-6)-NH_2	Tyr-D-Met-Phe-His-Leu-Met-NH_2
Dermenkephalin-(1-4)-NH_2	Tyr-D-Met-Phe-His-NH_2
[D-Met^6]-dermenkephalin	Tyr-D-Met-Phe-His-Leu-D-Met-Asp-NH_2
[Asn^7]-dermenkephalin	Tyr-D-Met-Phe-His-Leu-Met-Asn-NH_2
[Leu^5,Met^6,Asp^7]-dermorphin	Tyr-D-Ala-Phe-Gly-Leu-Met-Asp-NH_2
[His^4,Leu^5,Met^6,Asp^7]-dermorphin	Tyr-D-Ala-Phe-His-Leu-Met-Asp-NH_2

Table 2 The inhibitory effects (IC_{50}, nM) of dermorphin, dermenkephalin and related analogues on electrically-evoked contractions of the myenteric plexus-longitudinal muscle of the guinea-pig and the vasa deferentia of the hamster and mouse

Peptide	*Hamster vas deferens* Without peptidase inhibitors	With peptidase inhibitors	*Guinea-pig myenteric plexus*	*Ratio IC_{50} in myenteric plexus: IC_{50} in hamster vas deferens*	*Mouse vas deferens*
Dermorphin	>10,000	>10,000	1.4 ± 0.4	<0.00014	2.4 ± 0.2
Dermenkephalin	380 ± 70	13 ± 1.5	5000 ± 350	385	1.35 ± 0.6
[L-Ala^2]-dermorphin	>10,000	>10,000	9200 ± 800	<1	N.D.[b]
[L-Met^2]-dermenkephalin	>10,000	7000 ± 1000	>10,000	>1.5	450 ± 67
Dermenkephalin-(1-6)-NH_2	585 ± 87	40 ± 5	430 ± 70	11	2.9 ± 0.3
Dermenkephalin-(1-4)-NH_2	>10,000	>10,000	205 ± 31	<0.02	1360 ± 400
[D-Met^6]-dermenkephalin	180 ± 40	180 ± 40	2000 ± 150	11	3.7 ± 0.5
[Asn^7]-dermenkephalin	370 ± 63	37 ± 3.5	700 ± 122	19	N.D.[b]
[Leu^5,Met^6,Asp^7]-dermorphin	3015 ± 430	16 ± 2.5	36 ± 7	2	1.9 ± 0.3
[His^4,Leu^5,Met^6,Asp^7]-dermorphin	410 ± 75	15 ± 3	260 ± 42	17	1.3 ± 0.2
[D-Pen^2,D-Pen^5]-enkephalin	41 ± 10	41 ± 10	3000 ± 300	73	1.9 ± 0.6
[D-Ala^2,MePhe^4,Gly-ol^5]-enkephalin	>10,000	>10,000	10.5 ± 1.7	<0.001	N.D.[b]

The values are the means ± s.e.mean of 3–4 observations. [a] The Krebs solution contained the following peptidase inhibitors (all 10 μM): bestatin, amastatin, captopril and thiorphan. No peptidase inhibitors were necessary for either the myenteric plexus preparation or the mouse vas deferens. [b] Not determined.

degree of δ-selectivity is high; if the ratio is very low, e.g <0.00014 for dermorphin then the peptide has a high degree of μ-selectivity.

The ratio of IC_{50} in the myenteric plexus to IC_{50} in the hamster vas deferens was higher for dermenkephalin than for [D-Pen^2,D-Pen^5]-enkephalin (Table 2). In addition, dermorphin was more potent at μ-receptors than [D-Ala^2,MePhe^4,Gly-ol^5]-enkephalin.

Effects of peptidase inhibitors on the agonist potency of dermenkephalin and dermorphin

The combination of peptidase inhibitors, bestatin, amastatin, captopril and thiorphan, increased the potency of dermenkephalin in the hamster vas deferens about 30 fold (Table 2). Therefore in the hamster vas deferens, the activities of all peptides were determined before and after the addition of antipeptidases (Table 2). In a few experiments different combinations and concentrations of peptidase inhibitors were examined in the hamster vas deferens. Thiorphan by itself was responsible for protection of dermenkephalin, the maximum effect being achieved by 3 μM (Figure 2).

In contrast, in the myenteric plexus and the mouse vas deferens the potencies of dermenkephalin, dermorphin and their analogues were unchanged by inhibition of peptidases.

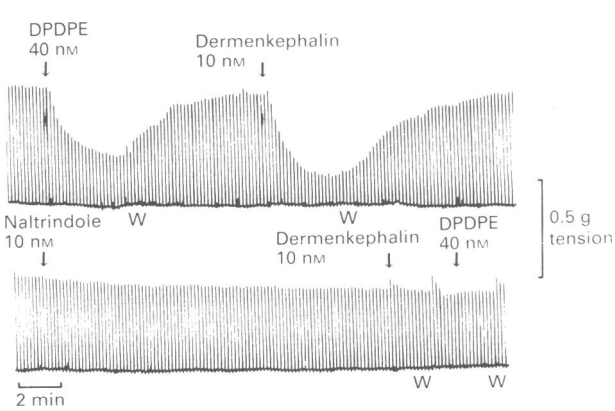

Figure 1 The effects of dermenkephalin, [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) and the δ-antagonist, naltrindole, on the electrically-evoked contractions of the hamster vas deferens. The Krebs solution contained the following peptidase inhibitors: amastatin, bestatin, captopril and thiorphan, all at a concentration of 10 μM. Drugs were added at the arrows and washed out at W.

Agonist activity of some dermenkephalin analogues in bioassays

In the myenteric plexus preparation, the selective κ-opioid antagonist nor-binaltorphine (10 nM) had no effect on the agonist activity of the dermenkephalin analogues added to the organ baths in concentrations about their IC_{50} value. Thus, since these analogues are devoid of κ-agonist activity, comparison of the data from the myenteric plexus with those from the hamster vas deferens permits the direct correlation of μ-potency with δ-potency.

In the myenteric plexus, the hexapeptide dermenkephalin-(1-6)-NH_2 was 11 times more potent than dermenkephalin whereas, in the δ-selective hamster vas deferens, dermenkephalin was 3 times more potent than dermenkephalin-(1-6)-NH_2 (Table 2). Hence, the ratio of δ-activity to μ-activity was 385 for dermenkephalin but only 11 for dermenkephalin-(1-6)-NH_2 (Table 2). Further shortening of the peptide to dermenkephalin-(1-4)-NH_2 resulted in a complete loss of δ-activity whereas the μ-potency remained similar to that of dermenkephalin-(1-6)-NH_2 (Table 2). Thus, for dermenkephalin-(1-4)-NH_2 the ratio of δ- to μ-activity is less than 0.02.

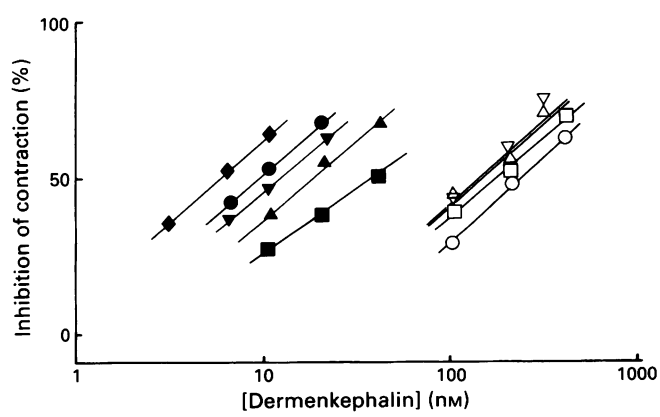

Figure 2 The effects of peptidase inhibitors on the agonist activity of dermenkephalin in the hamster vas deferens; (○) without inhibitors; (□) 10 μM bestatin; (△) 10 μM bestatin and 10 μM amastatin; (▽) 10 μM bestatin, 10 μM amastatin and 10 μM captopril; (●) 10 μM bestatin, 10 μM amastatin, 10 μM captopril and 10 μM thiorphan; (■) 0.3 μM thiorphan; (▲) 1 μM thiorphan; (▼) 3 μM thiorphan; (◆) 10 μM thiorphan. Each point is the mean of 3–4 observations.

These results suggest that the structural features necessary for δ-receptor selectivity of dermenkephalin are located within the C-terminal tripeptide. In this regard, neutralizing the C-terminal negative charge of dermenkephalin by replacing Asp by Asn in position 7 caused a decrease in δ-receptor potency and an increase in μ-receptor potency resulting in a 20 fold decrease in δ-selectivity (Table 2). Interestingly, both dermenkephalin-(1-6)-NH_2 and [Asn^7]-dermenkephalin have almost identical activity ratios and δ-receptor potencies (Table 2).

The importance of the C-terminal region of dermenkephalin for opioid receptor selectivity and pharmacological potency is demonstrated further by comparing the activities of dermenkephalin and dermorphin to those of the hybrid molecules [Leu^5,Met^6,Asp^7]-dermorphin and [His^4,Leu^5,Met^6,Asp^7]-dermorphin, i.e. dermorphin-(1-4)-dermenkephalin-(5-7) and dermorphin-(1-3)-dermenkephalin-(4-7) (Table 2). Substitution of the C-terminal tripeptide of dermorphin with the C-terminal tripeptide of dermenkephalin abolished the μ-receptor preference of dermorphin, the hybrid peptide being equiactive in the hamster vas deferens and the myenteric plexus preparation (Table 2). The IC_{50} for [Leu^5,Met^6,Asp^7]-dermorphin in the hamster vas deferens was 16 nM, the same as that for dermenkephalin. Replacement of the C-terminal tetrapeptide of dermorphin with the C-terminal tetrapeptide of dermenkephalin caused no change in δ-activity, when compared to the tripeptide hybrid but the μ-activity was less with an IC_{50} value of 260 nM. Thus, [His^4,Leu^5,Met^6,Asp^7]-dermorphin has a preference for the δ-opioid receptor (Table 2).

The role of the D-amino acids

The relationship between opioid receptor selectivity and the stereochemistry of the second amino acid of dermenkephalin and dermorphin was assessed by replacing the naturally-occurring D-amino acids by the L-amino acid. [L-Met^2]-dermenkephalin was inactive in the hamster vas deferens and in the guinea-pig myenteric plexus preparation (Table 2). Similar results were obtained with [L-Ala^2]-dermorphin.

Whether the D-configuration is of importance for determining folding of thse molecules or confers resistance to proteolysis, has been considered by measuring agonist activity in the presence of protease inhibitors. In the hamster, the δ-activity of dermenkephalin was increased 30 fold by addition of peptidase inhibitors which had barely any effect on [L-Met^2]-dermenkephalin, as the IC_{50} value changed from >10,000 nM to 7000 nM (Table 2). The potencies of dermorphin and [L-Ala^2]-dermorphin in the myenteric plexus were unchanged by peptidase inhibition.

The influence of the stereochemistry of the sixth amino acid of dermenkephalin was investigated by changing L-Met^6 to D-Met^6. In the hamster vas deferens [D-Met^6]-dermenkephalin had only 0.07% the activity of dermenkephalin (Table 2). There was a small increase in μ-receptor potency in the myenteric plexus from 5000 nM to 2000 nM.

Discussion

The results of this investigation confirm the high potency and selectivity of dermenkephalin for δ-opioid receptors and of dermorphin for μ-receptors. Of the bioassay tissues used, the hamster vas deferens contains only δ-receptors but the myenteric plexus has μ- and κ-receptors. Since dermorphin, dermenkephalin and their analogues have no activity at κ-receptors (Amiche *et al.*, 1989; this study), it is permissible to compare IC_{50} values in the hamster vas deferens with IC_{50} values in the myenteric plexus preparation as a direct correlation of δ-potency with μ-potency. Comparison of the results obtained in the hamster vas deferens with those in the mouse vas deferens indicates that the vas deferens of the mouse, which contains μ-, δ- and κ-receptors (Schultz & Wüster, 1981), is not a reliable tissue in which to assess agonist potency and the receptor selectivity of opioids (Table 2).

We have used the isolated tissue bioassays to determine the opioid activity of some analogues of dermorphin and dermenkephalin and thus elucidate the structural features required for the selective and potent action of dermenkephalin at δ-receptors. It has been proposed by Schwyzer (Schwyzer, 1986) that opioid peptides have a common N-terminal sequence which is the 'message' segment triggering the receptor responses but have different C-terminal 'address' segments responsible for their receptor type preference. It is of interest that dermorphin and dermenkephalin have similar N-terminal sequences, Tyr-D-aa-Phe, but they have distinct C-terminal regions. Furthermore, n.m.r. studies have shown that the N-terminal regions of dermorphin and dermenkephalin have similar conformational features (Temussi *et al.*, 1989). Therefore, the side-chains of the amino acids in the C-terminal region, and their sequence, should be the only factors directing receptor selectivity of amphibian skin opioid peptides.

The present results confirm the data from binding assays in homogenates of rat brain (Sagan *et al.*, 1989a,b) that agonist potency and discrimination between μ- and δ-opioid receptors depends on the C-terminal sequence of dermenkephalin. Since dermenkephalin-(1-4)-NH_2 retains less than 0.4% of the δ-receptor potency of dermenkephalin-(1-6)-NH_2, the presence of Leu^5 and/or Met^6 is important for the opioid activity of dermenkephalin. The fact that dermenkephalin-(1-4)-NH_2 and dermenkephalin-(1-6)-NH_2 have comparable potencies at the μ-receptor, suggests that the side-chains of Leu^5 and/or Met^6 are critical for the full expression of the agonist activity of dermenkephalin. The finding that changing the stereochemistry of the sixth amino acid by replacing L-Met^6 by D-Met^6 causes a loss of δ-potency, implies that both the constitution and the proper orientation of the C-terminal tripeptide of dermenkephalin are major contributors to receptor selectivity.

Interestingly, dermenkephalin-(1-6)-NH_2 and [Asn^7]-dermenkephalin which are equipotent in the hamster vas deferens, also have similar ratios of μ-activity to δ-activity, 11 and 19. This indicates that the negatively charged side-chain of the endogenous Asp^7 makes a significant contribution to the δ-addressing ability of the C-terminal region. This is in accordance with Schwyzer's model of membrane requirement for opioid selection in which the δ-receptor would be less accessible to positively charged than to neutral opioid agonists. However, since deletion or modification of Asp^7 mainly affects the μ-potency (which is increased) rather than the δ-potency of dermenkephalin, the contribution of Asp^7 to δ-selectivity is due to its depressant effect on affinity for the μ-receptor. The importance of the C-terminal sequence of dermenkephalin in determining receptor selection and agonist potency is supported by our finding that it is possible to invert the selectivity of dermorphin, from μ-activity > δ-activity to δ-activity > μ-activity, by exchanging its C-terminal part with that of dermenkephalin. A decisive shift towards δ-receptor selectivity was obtained by replacing the C-terminal tetrapeptide of dermorphin with the C-terminal tetrapeptide of dermenkephalin. The resulting hybrid peptide, [His^4,Leu^5,Met^6,Asp^7]-dermorphin, exhibited good selectivity and high potency at δ-receptors. This observation shows that His^4 is significantly involved in directing dermenkephalin towards the δ-receptor. Therefore, the role of the membrane in determining opioid receptor selectivity may involve only the 'address' of the C-terminal region of dermenkephalin.

The configuration of the Met-residue in position 2 makes a major contribution to pharmacological potency since [L-Met^2]-dermenkephalin is inactive in the hamster vas deferens as well as in the myenteric plexus preparation. The D-configuration is of importance in determining active folding of these molecules rather than conferring resistance to peptidases as the potency of [L-Met^2]-dermenkephalin was unaffected by protease inhibitors.

In conclusion, these findings are consistent with a moderate but definite role for the side-chains of D-Met[2] and His[4] in directing selectivity of dermenkephalin. They demonstrate that a negative charge at the C-terminus of dermenkephalin is important for δ-opioid receptor selectivity. Furthermore, the data strongly suggest that Leu[5] and/or Met[6] are involved in ensuring high agonist potency at the δ-site rather than in controlling receptor selectivity. Thus, dermenkephalin, dermorphin and their analogues may provide a starting point for the design of selective agonists which may be used for the exploration of the ill-defined physiological roles of μ- and δ-opioid receptors.

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Comparison of the electrophysiological effects of Org 7797, disopyramide, mexiletine and propafenone in anaesthetized dogs with myocardial infarcts

J.K. Campbell, R.J. Marshall & [1]E. Winslow

Organon Laboratories Limited, Newhouse, Lanarkshire, ML1 5SH

1 The electrophysiological effects of intravenously administered Org 7797 were compared with those of disopyramide (class Ia), mexiletine (Ib) and propafenone (Ic) in anaesthetized dogs with 5–6 day-old left ventricular myocardial infarcts.

2 Org 7797 (0.5 mg kg^{-1}) slowed conduction at all levels of the myocardium as shown by increases in St-A, AH, HV and QRS intervals, very modestly prolonged atrial and ventricular refractory periods and slightly shortened ventricular repolarization. Sinus node recovery time was increased whilst the RR interval was unchanged. A higher dose (2 mg kg^{-1}) prolonged RR and rendered 5 out of 8 dogs unable to follow an atrial pacing stimulus of mean cycle length 322 ms.

3 Electrophysiological changes induced by propafenone (2 mg kg^{-1}) were qualitatively similar to those of Org 7797 (0.5 mg kg^{-1}).

4 Electrophysiological changes induced by mexiletine (2 mg kg^{-1}) were small or insignificant. The most noticeable effect was a modest increase in the St-A interval and a slight shortening of ventricular repolarization. A higher dose (8 mg kg^{-1}) additionally slowed conduction in the His-Purkinje system and in the ventricular myocardium.

5 Disopyramide (2 and 5 mg kg^{-1}) prolonged all cardiac intervals including JTc, QTc and QT during pacing and prolonged cardiac refractory periods.

6 It was concluded that the electrophysiological profile of Org 7797 is more like that of the Ic agent propafenone than that of the class Ia and Ib drugs, disopyramide and mexiletine.

Keywords: Electrophysiology; anaesthetized dog; myocardial infarction; Org 7797; propafenone; mexiletine; disopyramide

Introduction

Most clinically available antiarrhythmic agents block the fast inward sodium current in cardiac tissue (class I agents). However, individual class I agents display a variety of electrophysiological profiles and have been broadly subclassified into Ia, Ib and Ic agents on the basis of both their electrophysiological actions *in vivo* (Harrison, 1985) and on the speed at which they bind to and dissociate from cardiac sodium channels *in vitro* (Campbell, 1983). Org 7797 ((16α,17β)-17-methylamino-oestra-1,3,5(10)-triene-3,16-diol(z)-2-butenedioate), is a new steroidal antiarrhythmic agent (Winslow *et al.*, 1989a; Janse *et al.*, 1990; Kirchhof *et al.*, 1991) currently undergoing clinical evaluation and which, in terms of its kinetics of sodium channel block in isolated porcine cardiac tissue has been subclassified as a Ic agent (Winslow *et al.*, 1989b). The aim of the present study was to evaluate and compare the electrophysiological actions of Org 7797 *in vivo* with those of disopyramide, mexiletine and propafenone, antiarrhythmic agents belonging to classes Ia, Ib and Ic respectively. Drug doses were chosen to include accepted therapeutic doses in man or in animal models of ischaemic-induced arrhythmias. Since electrophysiological results from clinical studies imply that the effects of sodium channel blocking agents on infra-nodal conduction may be enhanced in patients with existing conduction defects or coronary heart disease (Ross *et al.*, 1978; Marrot *et al.*, 1976), dogs with 5–6 day old myocardial infarcts were used for this study. Part of this work has already been published in abstract form (Winslow *et al.*, 1989a).

Methods

Beagle dogs of either sex (10.0 to 14.4 kg) were anaesthetized with sodium thiopentone (25 mg kg^{-1}, i.v.). Anaesthesia was subsequently maintained with halothane (1–2%) in N_2O/O_2 (1:1). Using aseptic techniques, the left carotid artery was isolated and a guide catheter inserted and positioned at the left coronary ostium under fluoroscopic control. A Fogarty 2F embolectomy catheter was passed through the guide catheter into the left anterior descending coronary artery. The catheter balloon was inflated by injecting contrast medium to occlude the artery close to its origin. If ECG leads I, II or III showed no evidence of ischaemia 2 to 3 min after occlusion, the balloon was deflated and repositioned in the circumflex coronary artery. After 2 h of artery occlusion, the balloon was deflated, the guide and embolectomy catheters removed, the neck wound closed and the animal allowed to recover from anaesthesia. Xylocaine spray and acramide antibiotic powder were applied around the neck wound and ampicillin given intramuscularly. Heparin (1000 units i.v.) was administered prior to surgery to minimize the risk of thrombus formation around the catheters and lignocaine (8 mg kg^{-1}, i.m.) was given 5 min prior to reperfusion in an attempt to reduce the risk of fatal arrhythmias.

Five or six days after myocardial infarction, animals were re-anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.). 6F quadripolar and bipolar intracardiac electrodes were positioned via the left jugular vein in the right atrium and right ventricle respectively. A bipolar electrode was positioned at the aortic root via the left femoral artery for His bundle recording. Electrophysiological measurements were made during normal sinus rhythm (NSR) and during pacing at a basic cycle length of 333 ms where possible, or, if not, at 20 ms less than the sinus cycle length. Atrial and ventricular effective refractory periods (ERP) were determined during atrial pacing or during simultaneous atrial and ventricular pacing respectively by interpolating an extra stimulus after every 12th basic stimulus.

The AH interval was measured from the first deflection of the atrial electrogram to the start of the largest His deflection. The HV interval was measured from the start of the largest His deflection to the start of the ventricular electrogram. The

[1] Author for correspondence.

Table 1 Baseline values (ms) for electrophysiological parameters before drug administration

Normal sinus rhythm	n	*HV*	*QRS*	*AH*	*PR*	*RR*
Org 7797	8	27 ± 3	45 ± 2	81 ± 4	90 ± 3	424 ± 19
Propafenone	9	27 ± 1	44 ± 1	71 ± 4	85 ± 3	382 ± 20
Mexiletine	7	25 ± 2	43 ± 3	78 ± 4	88 ± 4	387 ± 54
Disopyramide	6	28 ± 1	50 ± 5	71 ± 2	82 ± 3	333 ± 21

Pacing		*HV*	*QRS*	*QT*	*VERP*	*AH*	*St-A*	*AERP*	*SNRT*	*RR*
Org 7797	8	29 ± 3	45 ± 3	198 ± 8	154 ± 2	81 ± 4	24 ± 3	141 ± 7	473 ± 61	322 ± 7
Propafenone	9	26 ± 2	43 ± 1	201 ± 12	152 ± 7	67 ± 4	23 ± 2	130 ± 7	416 ± 24	319 ± 14
Mexiletine	7	27 ± 1	44 ± 6	201 ± 17	162 ± 9	70 ± 5	20 ± 3	148 ± 14	436 ± 38	321 ± 6
Disopyramide	6	28 ± 2	48 ± 3	195 ± 7	142 ± 2	65 ± 2	22 ± 2	120 ± 4	357 ± 9	306 ± 6

Values are the mean ± s.e.mean of n observations. VERP and AERP are the ventricular and atrial refractory periods respectively.

St-A interval was measured from the start of the stimulus artefact to the earliest atrial deflection. Sinus node recovery time (SNRT) was measured from the start of the last atrial complex during pacing to the beginning of the first atrial complex when normal sinus rhythm was resumed. The RR, QRS and QT intervals were measured from the lead II ECG. Derived intervals were:

$$QTc = QT \div \sqrt{RR}$$

$$JTc = (QT - QRS) \div \sqrt{RR}$$

Drugs were given over a 1 min period via the left femoral vein in a volume of 10–20 ml distilled water and electrophysiological measurements made before and 2, 5 and 10 min after administration. Refractory periods were determined 5 min after administration. Further doses of the same test drug were given when control values had been reattained. Each dog received only one drug.

At the end of each experiment, the heart was removed and perfused retrogradely via the aorta with triphenyltetrazolium for estimation of infarct size. Scores of 1, 2 or 3 were given for an endocardial infarct area of <10%, 10–25% and >25% of the total left ventricular endocardial surface respectively.

Statistical analysis of the results

Significant differences between pre- and post-drug values were determined by a paired t test. Results are presented as the mean % change to ease comparison of different drug-induced effects. Student's unpaired t test was used to detect significant differences in infarct size.

Drugs used

Drugs used were disopyramide phosphate (Searle), mexiletine hydrochloride (Boehringer Ingleheim), propafenone hydrochloride (Helopharm) and Org 7797 maleate (Organon). Doses quoted refer to the free base.

Results

Mean baseline values for each drug tested were within the normal range for pentobarbitone anaesthetized animals and are given in Table 1.

Electrophysiological effects during normal sinus rhythm

With the exception of the effect of propafenone on JTc, maximum changes in response to all 4 drugs were seen within 5 min of administration and are summarized in Table 2.

Org 7797 (0.5 and 2 mg kg^{-1}) dose-dependently lengthened the PR, AH, QRS and HV intervals whilst only the higher dose prolonged the RR interval. QTc was unchanged by the low dose and prolonged by the high dose but JTc was significantly shortened by both doses.

Propafenone (0.5 and 2 mg kg^{-1}) also dose-dependently prolonged PR, AH and HV but was about four times less potent then Org 7797. QRS was unchanged by the lower dose and only modestly (and insignificantly) prolonged by the higher dose. RR and QTc remained unchanged whilst a very small (2%) but significant shortening of JTc was seen 10 min after administration of the higher dose.

Mexiletine (0.5 and 2 mg kg^{-1}) induced only very small or insignificant effects. PR and QRS were minimally prolonged by 2 mg kg^{-1} whilst QTc and JTc were, if anything, slightly shortened. A higher dose (8 mg kg^{-1}) induced prolongation of the QRS and HV intervals and slightly lengthened the PR interval; RR and AH remained unchanged, whilst QTc and JTc were shortened.

Disopyramide (2 and 5 mg kg^{-1}) dose-dependently prolonged all intervals.

Figure 1 compares the effects of 0.5 mg kg^{-1} Org 7797 on intracardiac conduction and ventricular repolarisation over the 10 min observation period with those of 2 mg kg^{-1} propafenone, mexiletine and disopyramide. The effects of all 4 drugs on conduction intervals declined with time as did shortening of JTc in response to Org 7797 and mexiletine. However, disopyramide-induced JTc lengthening was sustained.

Table 2 Mean maximum percentage changes in electrophysiological parameters during normal sinus rhythm

	Org 7797		*Propafenone*		*Mexiletine*			*Disopyramide*	
	0.5	2.0	0.5	2.0	0.5	2.0	8.0	2.0	5.0 mg kg^{-1}
RR	+2 ± 2	+22 ± 2**	0 ± 3	+6 ± 4	+5 ± 2	−3 ± 2	+4 ± 2	+14 ± 2**	+43 ± 4**
PR	+15 ± 2**	+50 ± 3**	+4 ± 1**	+14 ± 3**	0 ± 1	+3 ± 1*	+7 ± 2*	+15 ± 3**	+37 ± 9**
AH	+15 ± 3**	+45 ± 7**	+2 ± 1*	+13 ± 3**	+3 ± 2	+1 ± 1	+2 ± 2	+11 ± 2**	+24 ± 3**
HV	+25 ± 2**	+119 ± 33**	+6 ± 1**	+18 ± 4**	+4 ± 3	+3 ± 2	+20 ± 3**	+15 ± 3**	+27 ± 4**
QRS	+30 ± 6**	+107 ± 17**	0 ± 2	+11 ± 5	+1 ± 5	+5 ± 1**	+21 ± 3**	+11 ± 3**	+36 ± 3**
QT_c	+1 ± 1	+13 ± 2**	−1 ± 1	−1 ± 1	−6 ± 2	−4 ± 2	−7 ± 3*	+5 ± 1**	+6 ± 1**
JT_c	−6 ± 1**	−5 ± 1**	−1 ± 1	−2 ± 1*	−5 ± 1*	−6 ± 3	−13 ± 4*	+6 ± 2*	+11 ± 3*

Results are the mean ± s.e.mean of 6–9 observations.
* $P < 0.05$ and ** $P < 0.01$ denote significant changes.

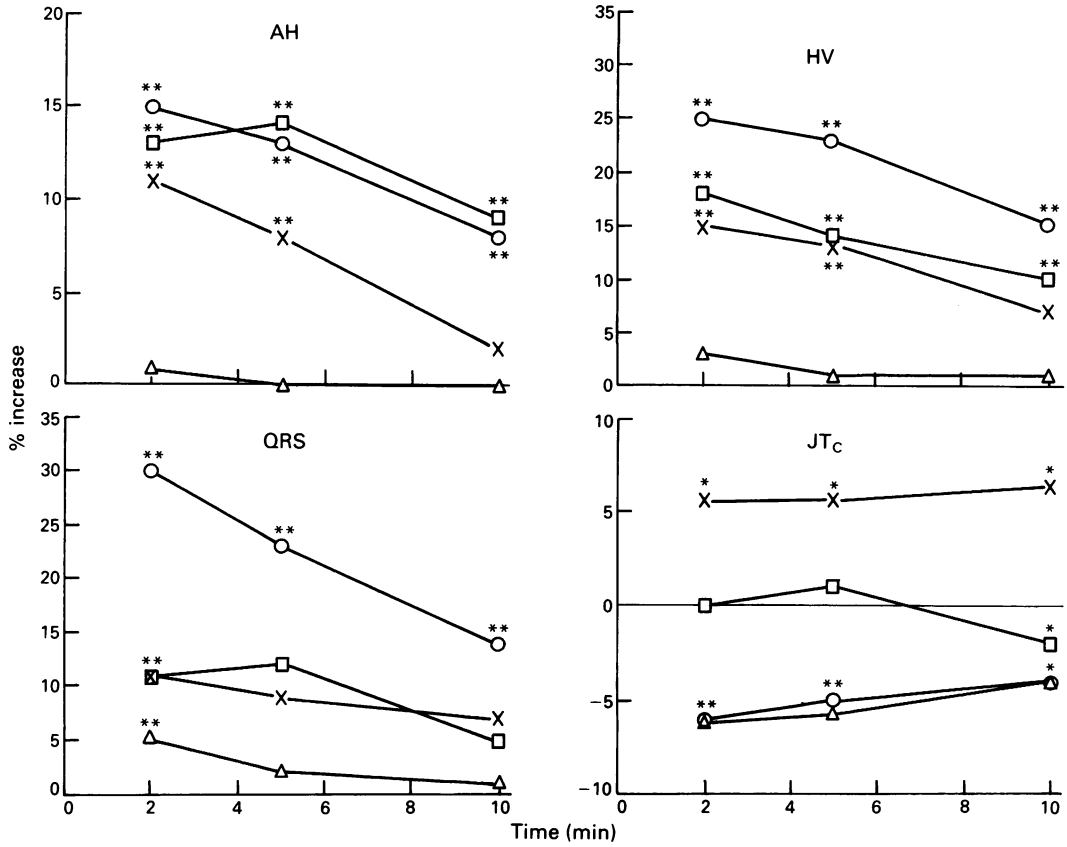

Figure 1 Comparative effects of 0.5 mg kg^{-1} Org 7797 (○) with those of 2 mg kg^{-1} propafenone (□), mexiletine (△) and disopyramide (×) on intracardiac conduction and ventricular repolarization during normal sinus rhythm. * $P < 0.05$ and ** $P < 0.01$ denote significant changes. Each result is the mean of from 6 to 9 observations.

Ventricular electrophysiological effects during pacing

Two to three minutes after administration, 7 of the 8 dogs given the high dose of Org 7797 (2 mg kg^{-1}) were unable to follow the atrial pacing stimulus and by 5 min only 3 dogs had recovered this ability. Two of the dogs given the lower dose (0.5 mg kg^{-1}) failed to follow the driving stimulus 2 min after dosing in comparison with 2/9 and 1/7 dogs given 2 mg kg^{-1} propafenone and 8 mg kg^{-1} mexiletine respectively. However, these drug effects had worn off by 5 min. The high dose of disopyramide (5 mg kg^{-1}) also rendered 3/6 dogs unresponsive to atrial pacing 5 min after administration. Also 2 dogs developed transient episodes of ventricular tachycardia.

Figure 2 compares mean maximum changes in HV, QRS and QT induced by 0.5 mg kg^{-1} Org 7797, with those of propafenone, mexiletine and disopyramide. Both Org 7797 and propafenone increased HV and QRS whilst QT was not significantly changed. Again the effect of Org 7797 on QRS appeared more pronounced than that seen in response to propafenone.

Mexiletine induced a dose-dependent increase in HV and QRS but only the higher dose (8 mg kg^{-1}) induced changes comparable to those of Org 7797 and propafenone. QT was, if anything, slightly and dose-dependently shortened.

Disopyramide dose-dependently increased HV, QRS and QT Figure 3 compares effects on the ventricular effective refractory period (ERP). Org 7797 induced only a small (4%) but significant increase in ERP whilst propafenone and disopyramide at 2 mg kg^{-1} induced more marked increases (9%). The high dose of disopyramide prolonged ERP by 21% whilst mexiletine (2 and 8 mg kg^{-1}) if anything, shortened the refractory period.

Supraventricular electrophysiological effects during pacing

Mean maximum changes in AH, St-A intervals and atrial effective refractory periods are summarized in Figure 4. Org 7797 (0.5 mg kg^{-1}) induced a marked increase in St-A (81%) and modestly prolonged the AH interval (16%) but caused only a small increase in the effective refractory period (8%). Propafenone (2 mg kg^{-1}) increased AH to a similar extent (22%) as Org 7797 but induced more marked prolongation of the refractory period (26%). Mexiletine 2 mg kg^{-1} was without effect on AH or ERP but modestly prolonged St-A (by 19%). At the higher dose (8 mg kg^{-1}), St-A and ERP were markedly increased (by 78 and 28% respectively) whilst only a modest and non-significant increase in AH was observed (15%).

Disopyramide (2 and 5 mg kg^{-1}) also lengthened AH, St-A and ERP but unlike the other 3 drugs its effects on AH and the refractory period were more pronounced than its effects on St-A.

Effects on sinus node recovery time are summarized in Figure 5. Org 7797 and 2 mg kg^{-1} propafenone or disopyramide induced modest increases in SNRT although the result obtained with propafenone did not reach statistical significance. Mexiletine even at a high dose was without effect.

Infarct size

All dogs used in the study were found to have an infarct. Mean infarct size was similar in all groups (scores of 2.3 ± 0.3, 2.3 ± 0.3, 2.5 ± 0.2 and 2.7 ± 0.2 in the Org 7797, propafenone, mexiletine and disopyramide groups respectively).

Discussion

The results of the present study suggest that the *in vivo* electrophysiological profile of Org 7797 is more like that of the Ic

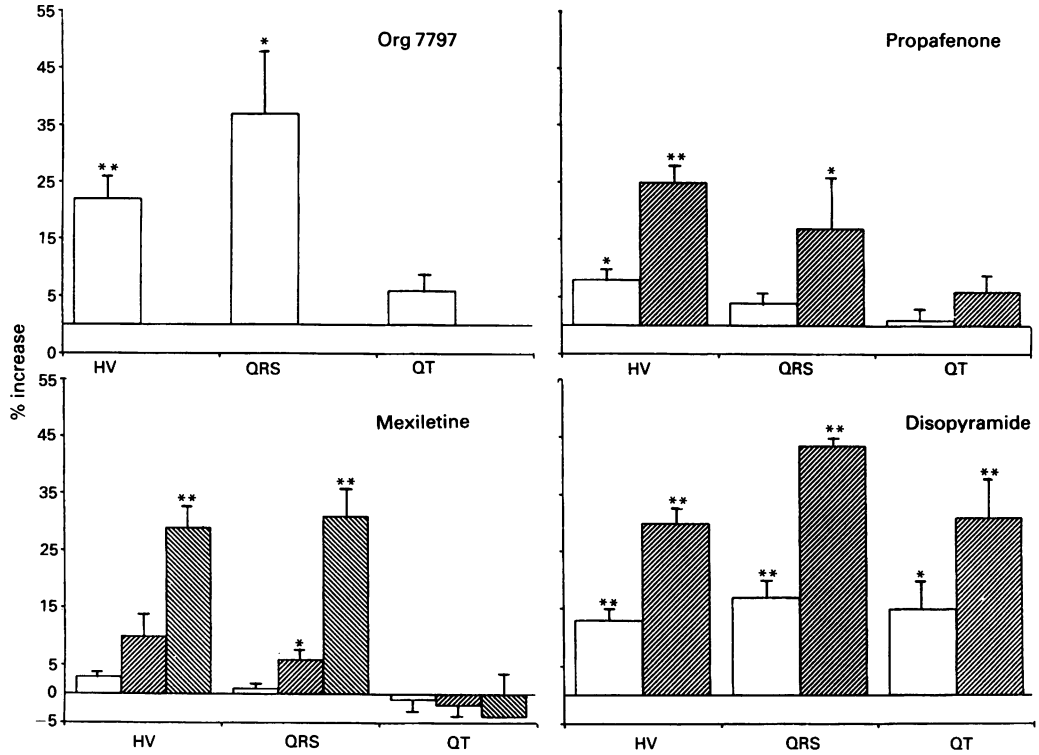

Figure 2 Maximum drug-induced effects on HV, QRS and QT intervals during pacing. Doses (mg kg^{-1}) used are denoted by □ (0.5 Org 7797); □ and ▨ (0.5 and 2.0 propafenone); ▨, ▧ and □ (0.5, 2.0 and 8.0 mexiletine); ▨ and □ (2.0 and 5.0 disopyramide). Each result is the mean of from 6 to 9 observations; vertical bars show s.e.mean. * $P < 0.05$ and ** $P < 0.01$ denote significant changes.

drug propafenone than of either mexiletine (Ib) or disopyramide (Ia). Org 7797 was some 4 times more potent than propafenone. Org 7797 (2 mg kg^{-1}) rendered 5 out of 8 dogs unable to follow an atrial pacing stimulus of mean cycle length 322 ms and induced substantial bradycardia. At a lower dose, shown previously to prevent ischaemia-induced ventricular fibrillation in dogs (Winslow *et al.*, 1989a), Org 7797 (0.5 mg kg^{-1}), like propafenone (2 mg kg^{-1}), slowed conduction at all levels of the myocardium. This was shown by prolongation of St-A (81%), AH (16%), HV (22%) and QRS (37%) intervals reflecting slowing of conduction in the atria, AV node, His-Purkinje system and ventricular myocardium respectively. Only small increases in the ventricular and more noticeably the atrial effective refractory period (of 4 and 8% respectively) were seen in response to Org 7797 whereas, relative to effects on ventricular and intraatrial conduction, refractory periods were increased to a greater extent by propafenone (9 and 26% respectively) than by Org 7797. The

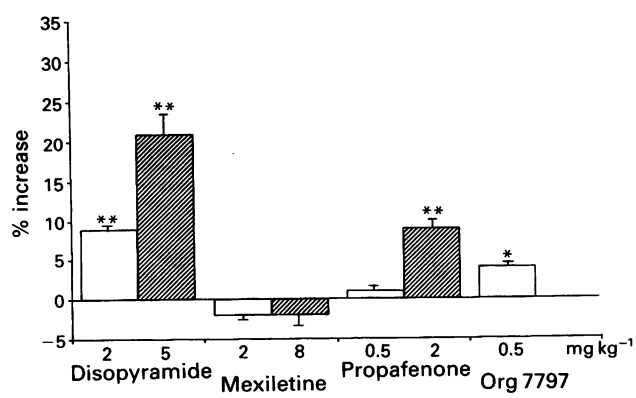

Figure 3 Changes in ventricular effective refractory periods 5 min after drug administration. Each result is the mean of from 6 to 9 observations; vertical bars show s.e.mean. * $P < 0.05$ and ** $P < 0.01$ denote significant changes.

electrophysiological effects of both drugs were seen in the absence of significant changes in QTc or paced QT and are qualitatively similar to clinical findings with propafenone (Waleffe *et al.*, 1981; Doherty *et al.*, 1984; Hammill *et al.*, 1987). Neither drug altered sinus cycle length but sinus node recovery time (SNRT) was significantly increased by Org 7797 and appeared to increase following propafenone. A similar dose of propafenone in man has been variably reported to have either no effect or to slightly increase SNRT (Shen *et al.*, 1984; Breithardt *et al.*, 1984). In the high range of therapeutic doses, propafenone has ancillary β-adrenoceptor blocking activity (Brown *et al.*, 1986) an action expected to oppose sodium channel-mediated shortening of cardiac action potentials. This may explain the very small shortening effect of propafenone on JTc observed in our study, in which the combination of myocardial infarction and pentobarbitone anaesthesia enhances sympathetic tone (Alter III *et al.*, 1979). On the other hand, β-adrenoceptor blockade might be expected to contribute to prolongation of cardiac refractory periods.

The results obtained with propafenone differ from those obtained by Karaguezian *et al.* (1984) in non-infarcted pentobarbitone-anaesthetized dogs where 4 mg kg^{-1} propafenone, whilst slowing atrial conduction (by 50%) and prolonging AV nodal refractoriness, failed to prolong either the HV interval or the ventricular effective refractory period. However, clinical studies have demonstrated that propafenone-induced slowing of conduction in the His-Purkinje system is enhanced in patients with underlying conduction abnormalities (Seipel & Breithardt, 1984; Connolly *et al.*, 1983a,b). Our use of dogs with left ventricular infarcts may therefore explain the prominent effects we found on HV conduction with a relatively low dose of the drug.

In the present study electrophysiological responses to an antiarrhythmic dose of mexiletine (2 mg kg^{-1}) were small compared to those of propafenone (2 mg kg^{-1}) and Org 7797 (0.5 mg kg^{-1}).

The most noticeable effect of this dose of mexiletine was a modest increase (19%) in St-A reflecting slowing of intraatrial

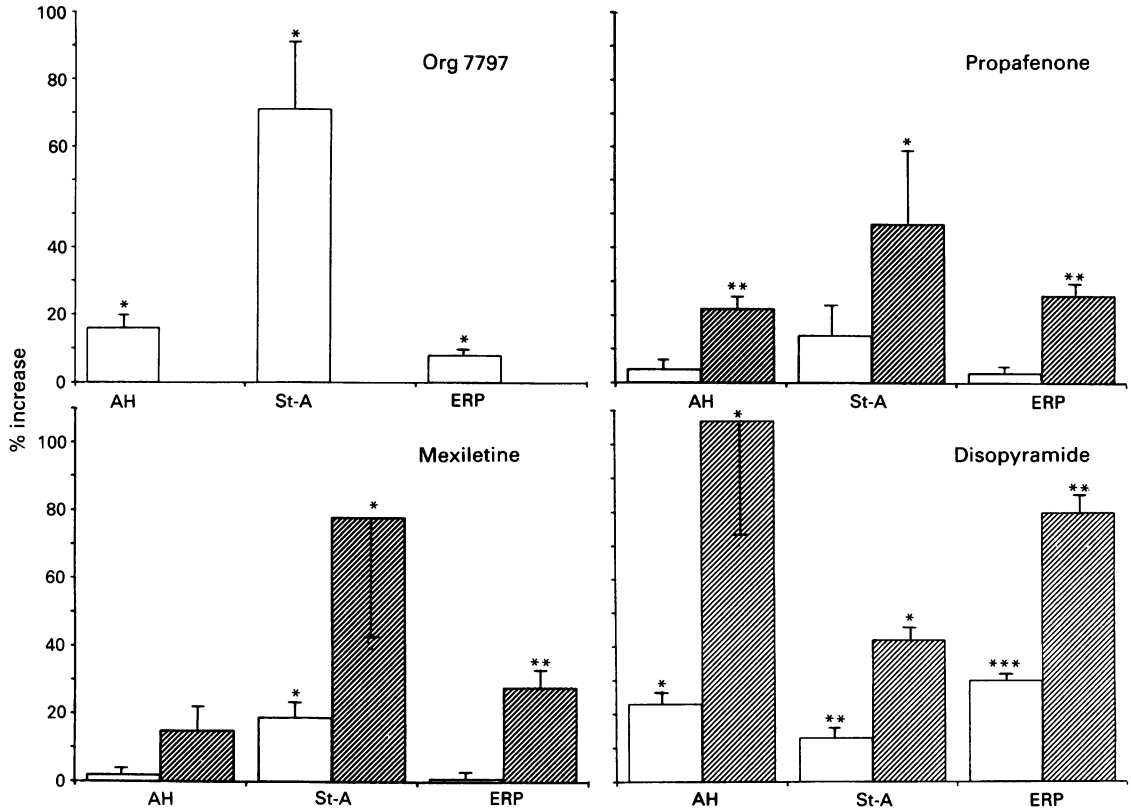

Figure 4 Maximum drug-induced effects on AH, St-A and the atrial effective refractory period (ERP) during pacing. Doses ($mg\,kg^{-1}$) used are denoted by □ (0.5 Org 7797); □ and ▨ (0.5 and 2.0 propafenone); □ and ▨ (2.0 and 5.0 mexiletine); □ and ▨ and (2.0 and 5.0 disopyramide). Each result is the mean of from 6 to 9 observations; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ denote significant changes.

conduction together with a modest but non-significant increase in HV (10%) and a small but significant increase in QRS (5–6%). JTc was, if anything, shortened but no other electrophysiological changes were seen. Intravenous (2–3 $mg\,kg^{-1}$) canine studies (Yoon & Han, 1982; Costard-Jackle & Franz, 1989), intravenous clinical studies (Seipel & Breithardt, 1978; Jewitt, 1978) and clinical oral studies (reviewed by Kreuger *et al.*, 1987; Nestico *et al.*, 1988) have reported little or no effect of mexiletine on intracardiac conduction, refractory periods or repolarization although shortening of the latter has sometimes been observed. Experimental studies using 2 $mg\,kg^{-1}$ do however suggest that mexiletine may slow ventricular conduction (Bajaj *et al.*, 1987) and prolong the ventricular refractory period (Costard-Jackle & Franz, 1989) but only at cycle lengths substantially shorter than those used in our study whilst at long cycle lengths, prominent shortening of ventricular repolarization is seen. Our results are in general agreement with those obtained by others but deviate in that we found a definite slowing of conduction in both atria and ventricular tissue at physiological pacing rates. Again, this may be due to the presence of infarction.

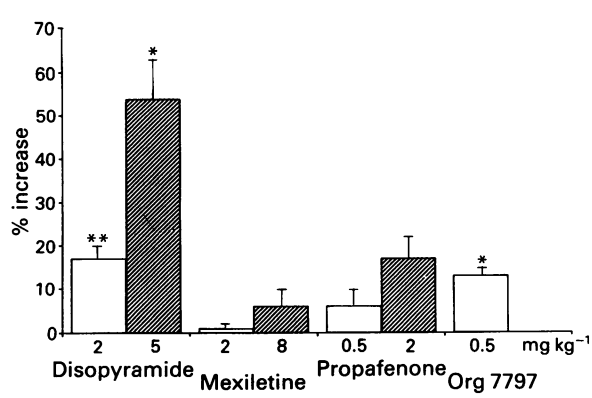

Figure 5 Maximum drug-induced changes in sinus node recovery time. Each result is the mean of from 6 to 9 observations; vertical bars show s.e.mean. * $P < 0.05$ and ** $P < 0.01$ denote significant changes.

A dose of mexiletine (8 $mg\,kg^{-1}$) close to that which produces convulsions in conscious dogs (10 $mg\,kg^{-1}$, Hashimoto *et al.*, 1984) produced some effects similar to those of Org 7797 and propafenone (slowing of conduction in ventricular, His-Purkinje and especially atrial tissue and a marked increase in the atrial effective refractory period) whilst repolarization during normal sinus rhythm was shortened. Responses to this dose of mexiletine differed from those to Org 7797 and propafenone in that neither the ventricular refractory period, SNRT nor AV nodal conduction were significantly altered. These results suggest that even at a high dose, the profile of mexiletine differs from that of Org 7797.

In contrast to Org 7797, disopyramide (2 $mg\,kg^{-1}$) in antiarrhythmic doses (Marshall & Parratt, 1979; Hashimoto *et al.*, 1982) increased JTc (by 6%) and QT during pacing (by 16%) indicating a clear prolongation of ventricular repolarization. The ventricular refractory period was lengthened to a greater extent (9%) than that seen in response to Org 7797 whilst effects on His-Purkinje and ventricular conduction were less pronounced (HV and QRS during pacing were lengthened by 13 and 17% respectively). In comparison to Org 7797, disopyramide markedly prolonged the atrial refractory period (by 30%) whilst its effect in slowing intraatrial conduction was much less prominent (St-A increased by only 13%). This dose of disopyramide also prolonged the RR interval. However, increases in SNRT and the AV interval in response to both drugs were of similar magnitude. Three of the six dogs given the higher dose of disopyramide (5 $mg\,kg^{-1}$) failed to follow the atrial pacing stimulus and two others responded to ventricular pacing with transient episodes of ventricular tachycardia suggesting that this dose is close to toxic levels.

The effects of disopyramide on ventricular repolarization, atrial conduction and refractory periods are similar to those reported from both canine (Guimond *et al.*, 1982; Giacomini *et al.*, 1985; Kidwell *et al.*, 1989) and clinical (Dreifus *et al.*, 1976; Marrott *et al.*, 1976) studies using i.v. doses of 1.5–4.0 mg kg^{-1}. However, results from non-infarcted animal studies including those of Lee *et al.* (1985) have shown either no change or only very small increases in HV and QRS in response to 4 mg kg^{-1} disopyramide or doses giving mid-therapeutic plasma levels (3–6 mg ml^{-1}), results in agreement with clinical studies using 1.5–1.7 mg kg^{-1} (Dreifus, 1975; Dreifus *et al.*, 1976). However Ross *et al.* (1978) and Marrott *et al.* (1976) both report an increase in the HV interval at doses of 1.5–2 mg kg^{-1} in patients with intraventricular conduction defects or suspected coronary heart disease. Again, these findings and the results of our studies seem to suggest that left ventricular infarction increases sensitivity to sodium channel blocking agents.

In clinical studies RR and SNRT are either unchanged or shortened by disopyramide, effects which are probably a consequence of the well-documented anticholinergic actions of this agent (Mirro *et al.*, 1980). However in experimental studies using pentobarbitone-anaesthetized dogs in which vagal activity is already depressed, disopyramide, as in our study, prolongs both RR and SNRT (Kidwell *et al.*, 1989).

In conclusion, the results of these studies suggest that in agreement with *in vitro* studies (Winslow *et al.*, 1989b), Org 7797 can be classified as a Ic agent. According to Harrison's clinical classification (1985), Ic agents widen QRS and slow conduction at low doses, have little effect on repolarization and induce only small increases in refractory periods. This is the profile observed with Org 7797 in these studies. Propafenone exerted similar effects but relative to its effects on conduction, refractory periods were more prolonged than with Org 7797. Class Ia agents prolong both repolarization and refractory periods at low doses whilst Ib agents have a limited effect on QRS and conduction and shorten repolarization, effects essentially observed with disopyramide and mexiletine, respectively, in this study. Our results also suggest that left ventricular infarction may enhance slowing of conduction induced by class Ia, Ib or Ic antiarrhythmic agents.

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Different pharmacological profiles of big-endothelin-3 and big-endothelin-1 *in vivo* and *in vitro*

[1]Pedro D'Orléans-Juste, Sabine Télémaque & Audrey Claing

Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada

1 Human big-endothelin-1 (big-ET-1) and endothelin-1 (ET-1) are equipotent as pressor agents and produce a significant change in mean arterial blood pressure (MAP) in anaesthetized guinea-pigs (2 nmol kg^{-1}: peak Δ MAP: 23 ± 6 mmHg and 26 ± 5 mmHg, respectively).

2 Unlike big-ET-1, big-endothelin-3 (big-ET-3) (10 and 20 nmol kg^{-1}) induces no pressor responses whereas endothelin-3 (ET-3) at 2 nmol kg^{-1} induces a significant increase of blood pressure in anaesthetized guinea-pigs (peak Δ MAP: 27 ± 5 mmHg) with a shorter duration than ET-1 and big-ET-1.

3 Big-ET-1 at concentrations 40 times higher than those required for ET-1 (2.5 nM) releases prostacyclin (PGI_2) (maximal release: 2.7 ± 0.8 ng ml^{-1}; 2.9 ± 0.9 ng ml^{-1}, respectively) and thromboxane B_2 (TxB_2) (maximal release: 6.7 ± 1.3 ng ml^{-1}; 6.8 ± 1.1 ng ml^{-1}, respectively) from guinea-pig perfused lungs. ET-3 (2.5 nM) is also a potent releaser of PGI_2 and TxB_2 from the guinea-pig lungs (maximal release: PGI_2: 2.4 ± 1.0 ng ml^{-1}; TxB_2: 3.8 ± 0.6 ng ml^{-1}). Conversely, big-ET-3 (100 nM) does not increase basal release of eicosanoids.

4 Phosphoramidon (50 μM), a metalloprotease inhibitor, markedly reduced the eicosanoid releasing properties of big-ET-1 ($n = 4$, $P < 0.01$) in guinea-pig perfused lungs without affecting the release stimulated by ET-1.

5 Our results suggest that big-ET-1 is converted to ET-1 via a phosphoramidon-sensitive endothelin converting enzyme (ECE) to release eicosanoids. The ECE is present in the guinea-pig pulmonary vasculature. Furthermore, our results suggest that the ECE activity is specific for big-ET-1 and may not convert big-ET-3 to its active metabolite, ET-3.

Keywords: Big–endothelin-1; big-endothelin-3; endothelin-converting enzyme; eicosanoids; phosphoramidon

Introduction

Recent studies have shown that the intermediate precursor of endothelin-1 (ET-1), porcine big-ET-1 (1-39), may be readily converted to its active metabolite ET-1 *in vivo* (Kashiwabara *et al.*, 1989). Although Yanagisawa *et al.* (1988a, b) had initially reported that porcine big-ET-1 was processed by a chymotrypsin-like enzyme, recent reports have shown that the human precursor of ET-1, big-ET-1 (1-38), was converted to ET-1 via a phosphoramidon-sensitive endothelin converting enzyme (ECE) *in vivo* (Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990).

Unless specified otherwise, the human intermediate precursor of ET-1 is referred to as big-ET-1 throughout the present study.

Similar to ET-1 (De Nucci *et al.*, 1988), big-ET-1 induces the release of prostanoids *in vivo* and *in vitro* (D'Orléans-Juste *et al.*, 1990a, b; 1991). We have also recently shown that phosphoramidon abolished the prostanoid release induced by big-ET-1 without affecting the release induced by ET-1 in rat perfused lungs, suggesting that a phosphoramidon-sensitive ECE may be present in the rat pulmonary vasculature (D'Orléans-Juste *et al.*, 1991).

Endothelin-3 (ET-3) is an isomeric homologue of ET-1 (Yanagisawa *et al.*, 1988b). Although ET-3 is not released by endothelial cells (Matsumoto *et al.*, 1989), it has been shown to be present in significant levels in human plasma (Yamaji *et al.*, 1990) and specific ET-3 receptors have been identified in cultured bovine endothelial cells (Emori *et al.*, 1990). The genomic fragment encoding ET-3 was originally isolated from the nucleotide sequence of porcine endothelin mRNA (Inoue *et al.*, 1989) and the transcription of the ET-3 gene isolated from a hypothalamic cDNA library has been reported (Bloch *et al.*, 1989). These authors found that ET-3 was cleaved from a 41 amino acid intermediate (big-ET-3), and suggested that the enzyme which processes big-ET-3 to ET-3 may be similar to the one responsible for the conversion of big-ET-1 to ET-1. Big-ET-3 and big-ET-1 are structurally different as the former encloses the structure of ET-3 but not ET-1 in its N-terminal portion and also differs from big-ET-1 in its C-terminal portion (Bloch *et al.*, 1989).

Although the cDNA cloning and chromosome assignment of the gene encoding the precursor of ET-3 (big-ET-3) have been identified (Bloch *et al.*, 1989), the pharmacological profile of big-ET-3 has not yet been documented. In anaesthetized rats, ET-3 has a similar pharmacological profile to ET-1 but is less potent as a pressor agent (Yanagisawa *et al.*, 1988a, b; Wallace *et al.*, 1989; Inoue *et al.*, 1989).

The aim of the present study was to compare the pressor effects induced by big-ET-3 and big-ET-1 *in vivo* and their eicosanoid releasing properties in perfused guinea-pig lungs. Secondly, the presence of a functional phosphoramidon-sensitive ECE was indirectly monitored in the guinea-pig pulmonary vasculature.

Our results suggest that the phosphoramidon-sensitive ECE is specific for big-ET-1 and cannot process big-ET-3 to ET-3 *in vivo* and *in vitro*.

Some of these results were presented at a meeting of the British Pharmacological Society (D'Orléans-Juste *et al.*, 1990a).

Methods

In vivo *experiments*

Male guinea-pigs (Dunkin-Hartley, 350 g) were anaesthetized with urethane (0.75 mg kg^{-1}, i.p.). A catheter was passed into the right carotid artery to monitor mean arterial blood pressure (MAP) which was detected with a Statham pressure transducer and recorded on a Grass physiograph. Big-ET-1, ET-1, ET-3 (2 nmol kg^{-1}), big-ET-3 (10–20 nmol kg^{-1}) or indomethacin (5 mg kg^{-1}) were administered as boluses via a cannula in the left jugular vein.

[1] Author for correspondence.

Measurement of eicosanoids from isolated perfused lungs

Male guinea-pigs (Dunkin-Hartley, 300–400 g) were anaesthetized with sodium pentobarbitone (Sagatal; 135 mg kg^{-1}, i.p.) and their spinal cord sectioned. Following thoracotomy, the pulmonary artery was cannulated for perfusion of the pulmonary circulation. The lungs were then suspended in a heated chamber (37°C) and perfused (5 ml min^{-1}) with an oxygenated (95% O_2: 5% CO_2) Krebs solution (Bakhle *et al.*, 1969). The lungs were left to stabilize for 40 min before big-ET-1 (100 nM) as well as ET-1 and ET-3 (2.5 nM) were infused. In some experiments, phosphoramidon (50 μM, 60 min) or indomethacin (5 μM, 60 min) were infused through the lungs prior to the administration of the peptides. The effluent from the lungs was collected (1 min samples) before, during and after infusion of the various peptides. The samples were stored (−20°C) and the stable, hydrolytic metabolites of prostacyclin (PGI_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TxB_2), were later determined by radioimmunoassay (Salmon, 1978).

Synthetic big-ET-1, big-ET-3, ET-1 and ET-3 were purchased from Peptide Institute (Osaka, Japan), phosphoramidon was obtained from Peninsula Laboratories (Belmont, U.S.A.), sodium pentobarbitone (Sagatal) from May and Baker (Dagenham, U.K.) and urethane (ethyl carbamate) from MCB Manufacturing Chemists Inc. (Cincinnati, U.S.A.). Indomethacin, heparin, 6-keto-$PGF_{1\alpha}$, TxB_2 and the TxB_2 antiserum were purchased from Sigma Chemicals (St. Louis, U.S.A.). The 6-keto-$PGF_{1\alpha}$ antiserum was kindly provided by Dr J.A. Salmon (Wellcome, U.K.). The TxB_2 antiserum has a 100% cross-reactivity with TxB_2, less than 2% with PGD_2 and $PGF_{2\alpha}$ and less than 0.1% cross-reactivity with 6-keto-$PGF_{1\alpha}$, PGE_1 and PGE_2 (Granström *et al.*, 1976). The 6-keto-$PGF_{1\alpha}$ antiserum has a 100% cross-reactivity with 6-keto-$PGF_{1\alpha}$, less than 10% with PGE_1, PGE_2, PGD_2 and $PGF_{2\alpha}$ and less than 0.1% with TxB_2 (Salmon, 1978). The eicosanoid antisera used in the present study did not cross react with ET-1, ET-3, big-ET-1 or big-ET-3. The tracers, [^{3}H]-6-keto-$PGF_{1\alpha}$ and [^{3}H]-TxB_2, were purchased from Amersham (Oakville, Canada). All agents were dissolved either in saline or distilled water except for indomethacin which was dissolved in Trizma base (0.2 M, pH: 7.4).

All values in the text and figures are expressed as means ± s.e.mean of n observations. When repeated measurements were taken, the results were analysed by one way analysis of variance followed by a least significance difference procedure to determine the nature of the response (SPS, Chicago, Ill., U.S.A.). In all other experiments, statistical comparisons between groups were made by an unpaired Student's t test. A P value of <0.05 was considered statistically significant.

Results

Lack of pressor effects of big-ET-3 when compared to ET-1, ET-3 and big-ET-1

The mean arterial blood pressure (MAP) in all guinea-pigs tested was 50 ± 4 mmHg (n = 18), prior to administration of the peptides.

ET-1 (2 nmol kg^{-1}), increased MAP in the guinea-pig by 26 ± 5 mmHg (n = 5, $P < 0.05$) (Figure 1a). Big-ET-1 (2 nmol kg^{-1}) also induced a marked increase in MAP (23 ± 6 mmHg, n = 4, $P < 0.05$). The MAP remained significantly increased for more than 5 min following administration of both peptides (Figure 1a).

Similarly, ET-3 (2 nmol kg^{-1}) induced a marked hypertensive effect (Δ MAP: 27 ± 5 mmHg, n = 5, $P < 0.05$) which however was short lasting (Figure 1b). In contrast, big-ET-3 at 10 nmol kg^{-1} (Figure 1b) and 20 nmol kg^{-1} (Δ MAP: 1.0 ± 0.5 mmHg, n = 2) was found to be inactive.

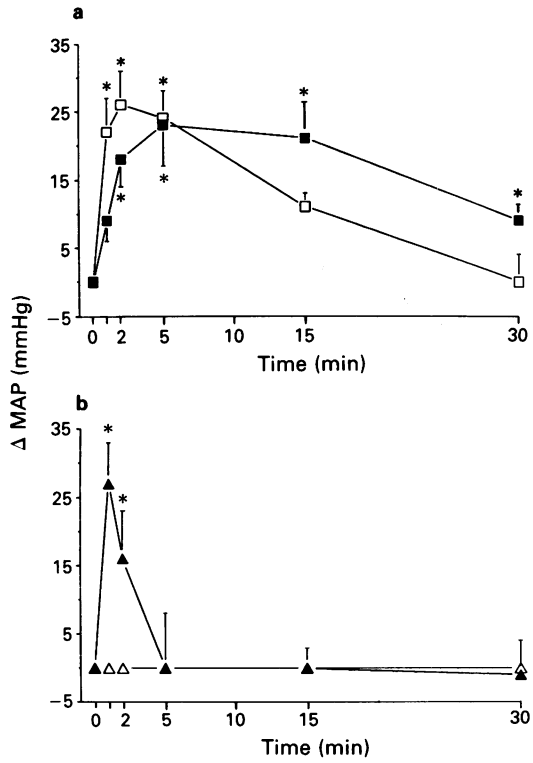

Figure 1 Changes in mean arterial pressure (Δ MAP) induced by big-endothelin-1 (big-ET-1: ■, 2 nmol kg^{-1}) or endothelin-1 (ET-1: □, 2 nmol kg^{-1}) (a), and endothelin-3 (ET-3: ▲, 2 nmol kg^{-1}) or big-endothelin-3 (big-ET-3: △, 10 nmol kg^{-1}) (b), in the anaesthetized guinea-pig. Each point represents the mean of at least four experiments; vertical bars show s.e.mean. *$P < 0.05$.

The pressor responses to big-ET-1, ET-1 and ET-3 (2 nmol kg^{-1}) were unaffected in guinea-pigs pretreated with indomethacin (5 mg kg^{-1}) given 20 min prior to the administration of peptides (peak Δ MAP; ET-1: 25 ± 3 mmHg; ET-3: 27 ± 2 mmHg; big-ET-1: 27 ± 7 mmHg; n = 6).

Effect of phosphoramidon on prostacyclin and thromboxane B_2 release induced by big-ET-1 and ET-1

The infusion of ET-1 (2.5 nM) through the guinea-pig perfused lungs significantly increased the release of PGI_2 (from: 0.4 ± 0.1 to 2.9 ± 0.9 ng ml^{-1}, n = 6, $P < 0.05$) and TxB_2 (from 1.3 ± 0.5 to 6.8 ± 1.1 ng ml^{-1}, n = 6, $P < 0.05$). The maximal release was reached 3 to 4 min following the start of the infusion (Figure 2). Similarily, big-ET-1 (100 nM) significantly increased the basal release of eicosanoids from the perfused lungs 4 to 6 min after the beginning of the infusion (PGI_2: from 0.6 ± 0.3 to 2.7 ± 0.8 ng ml^{-1}, n = 6, $P < 0.05$; TxB_2: from 1.2 ± 0.5 to 6.7 ± 1.3 ng ml^{-1}, n = 6, $P < 0.05$) (Figure 3).

Pretreatment of the lungs with phosphoramidon (50 μM) for 60 min prior to the infusion of peptides did not affect the PGI_2 and TxB_2 releases induced by ET-1 (Figure 2) but markedly reduced the release of eicosanoids induced by big-ET-1 (Figure 3). Indeed, PGI_2 and TxB_2 levels measured from phosphoramidon-treated lungs did not significantly increase when compared to basal levels, following a 3 min infusion of big-ET-1.

Release of eicosanoids from guinea-pig perfused lungs by ET-3 but not big ET-3

Infusion of ET-3 (2.5 nM) induced a significant release of PGI_2 and TxB_2 (Figure 4, n = 5, $P < 0.05$). Like ET-1, the

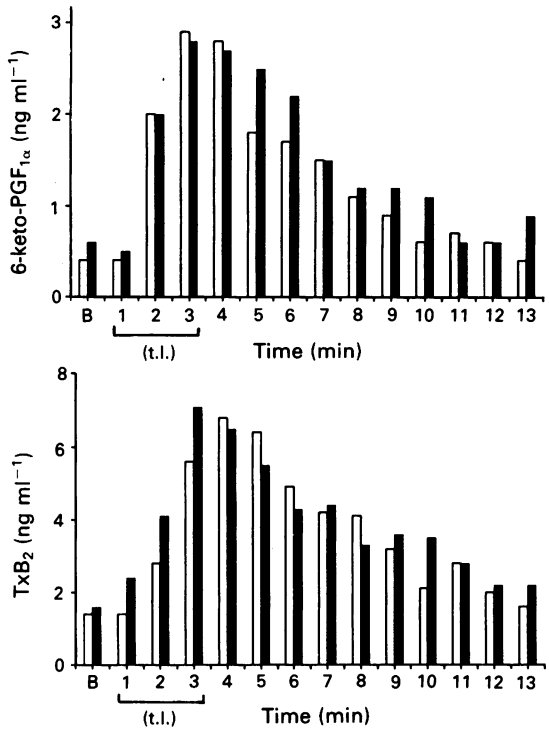

Figure 2 Time course of prostacyclin (measured as 6-keto-PGF$_{1\alpha}$) and thromboxane A (measured as TxB$_2$) release following infusion of endothelin-1 (ET-1: 2.5 nM) in the guinea-pig perfused lung, in the absence (open columns) or in the presence (closed columns) of phosphoramidon (50 μM). Eicosanoids were monitored for 10 min following the interruption of a 3 min infusion of ET-1 in the lung circulation (t.i.). B: basal levels of eicosanoids measured prior to the administration of peptides. Each result was the mean of at least four determinations; s.e.mean (10 to 15%) was omitted for the sake of clarity.

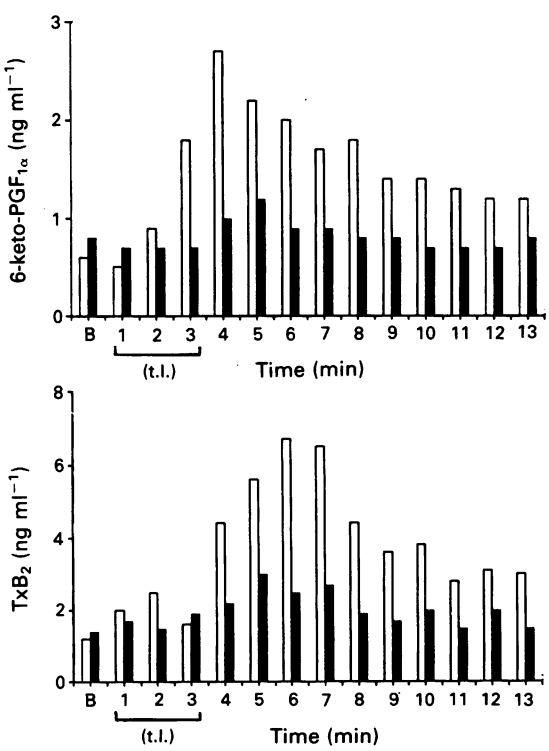

Figure 3 Time course of prostacyclin (measured as 6-keto-PGF$_{1\alpha}$) and thromboxane A$_2$ (measured as TxB$_2$) release by big-endothelin-1 (big-ET-1: 100 nM) in the guinea-pig perfused lung, in the absence (open columns) or in the presence (closed columns) of phosphoramidon (50 μM). Eicosanoids were monitored for 10 min following the interruption of a 3 min infusion of big-ET-1 in the lung circulation (t.i.). B: basal levels of eicosanoids measured prior to the administration of peptides. Each result was the mean of at least four determinations; s.e.mean (10 to 15%) were omitted for the sake of clarity.

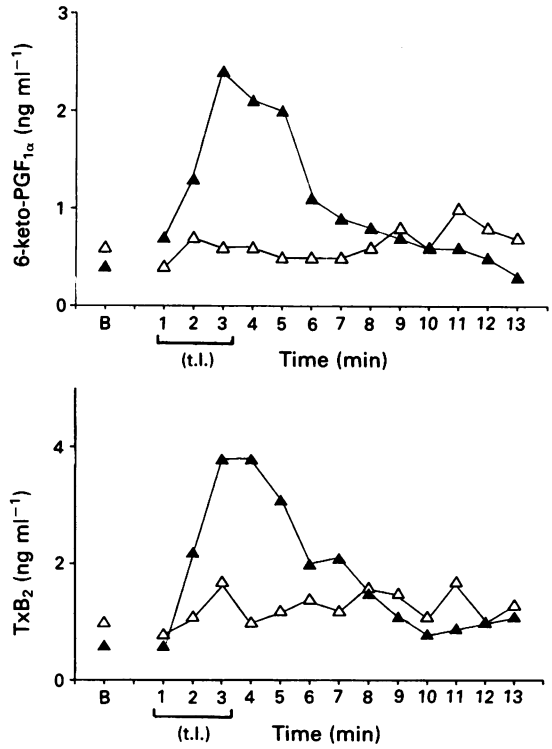

Figure 4 Release of prostacyclin (measured as 6-keto-PGF$_{1\alpha}$) and thromboxane A$_2$ (measured as TxB$_2$) induced by endothelin-3 (ET-3: ▲, 2.5 nM) but not big-endothelin-3 (big-ET-3: △, 100 nM) from guinea-pig perfused lung. Eicosanoids were monitored for 10 min following the interruption of a 3 min infusion of either peptide in the lung circulation (t.i.). B: basal levels of eicosanoids measured prior to the administration of peptides. Each result was the mean of at least four determinations; s.e.mean (5 to 15%) were omitted for the sake of clarity.

maximum release induced by ET-3 was reached 3 min after beginning the infusion (Figure 4). On the other hand, big-ET-3 (100 nM) infused for 3 min did not induce any significant release of eicosanoids from the perfused lungs (Figure 4).

Indomethacin (5 μM) infused through the lungs abolished the release of eicosanoids induced by all peptides (maximal releases induced by ET-1, ET-3 and big-ET-1 in presence of indomethacin; PGI_2: $<0.3 \pm 0.1$ ng ml^{-1}, $P < 0.05$, $n = 3$ for each peptide; TxB_2: $<0.9 \pm 0.2$ ng ml^{-1}, $P < 0.05$, $n = 3$ for each peptide).

Discussion

The endothelin converting enzyme (ECE), which has been reported to be responsible for the conversion of big-ET-1 to ET-1 *in vivo* and *in vitro* (Matsumura *et al.*, 1990; D'Orléans-Juste *et al.*, 1991), has yet to be biochemically characterized and the major target organ possessing ECE activity has still to be identified. The main purpose of the present study was to assess the presence of the ECE in the guinea-pig pulmonary vessels and to verify whether the enzyme may biotransform the precursor of ET-3, big-ET-3, *in vivo* and *in vitro*, by monitoring two well characterized pharmacological properties of endothelins, namely pressor responses and induction of eicosanoid release (Yanagisawa *et al.*, 1988a; de Nucci *et al.*, 1988).

ET-1 and ET-3 are both pressor agents in the anaesthetized guinea-pig (Whittle *et al.*, 1989; Pons *et al.*, 1991). The hypertensive effect of endothelins is the result of a complex chain of events. The pressor responses to ET-1 in the anaesthetized guinea-pig have been reported to be partly mediated by eicosanoids (most probably thromboxane, Whittle *et al.*, 1989).

These authors have demonstrated that indomethacin significantly reduced the responses produced by low doses of ET-1 but had much less effect on those evoked by higher doses of the compound. They thus suggested that the hypertensive properties of higher doses of ET-1 (1 nmol kg^{-1}) were mostly due to a direct vasoconstrictor effect of ET-1 on resistance vessels.

In contrast, Pons *et al.* (1991) have recently reported that indomethacin did not modify the pressor responses of mechanically ventilated guinea-pigs to ET-1 and ET-3. Furthermore, these authors have shown that, although ET-3 was equipotent to ET-1, the former induced a pressor response of much shorter duration. In the present study, we confirmed, in spontaneously breathing guinea-pigs, the results obtained by Pons *et al.* (1991). The short duration of the pressor effect of ET-3 was initially reported by Yanagisawa *et al.* (1988b) in anaesthetized rats. Interestingly, ET-3 has been found to be less potent than ET-1 as a vasoconstrictor in porcine isolated coronary artery (Inoue *et al.*, 1989) and rat aortic strips (Yanagisawa *et al.*, 1988b).

Because equimolar doses of ET-1 and ET-3, induced a similar increase in MAP in anaesthetized guinea-pigs, the latter model was used in the present study initially to evaluate the pressor responses to big-ET-1 and 3, the two intermediate precursors of ET-1 and ET-3 (Yanagisawa *et al.*, 1988a; Bloch *et al.*, 1989). It was shown that big-ET-1 and ET-1, applied intravenously, were equipotent as pressor agents in the guinea-pig (Fukuroda *et al.*, 1990), suggesting that big-ET-1 may be converted almost totally in that animal.

Interestingly, big-ET-3 at concentrations up to 10 times higher than big-ET-1, ET-1 and ET-3, did not affect MAP in the anaesthetized guinea-pig.

Big-ET-1 and ET-1 when administered systemically not only induce pressor effects but also increase airways resistance (Whittle *et al.*, 1989; Fukuroda *et al.*, 1990). Contrary to its effect on blood pressure, the increase of airways resistance induced by ET-1 is totally abolished by indomethacin (Whittle *et al.*, 1989). It thus appears that endothelins exert an indirect contractile effect on the airways smooth muscle, mediated by eicosanoids, and one can suggest a similar phenomenon for the bronchoconstrictor effect of big-ET-1 in the anaesthetized guinea-pig.

To validate this hypothesis, the eicosanoid releasing profile of big-ET-1, ET-1 and ET-3 was established in the guinea-pig perfused lung. Our results show that big-ET-1, like ET-1 and ET-3, albeit at concentrations 40 times higher than those of the latter peptides, increased the basal release of PGI_2 and TxA_2 from the guinea-pig pulmonary vasculature. Conversely, big-ET-3 did not stimulate the release of eicosanoids.

Matsumura *et al.* (1990) and Fukuroda *et al.* (1990) have recently shown in the anaesthetized rat and guinea-pig that an inhibitor of metalloproteases, phosphoramidon, abolished the pressor responses to big-ET-1 but not that of ET-1. Fukuroda *et al.* (1990) have also shown that phosphoramidon abolished the increase in pulmonary resistance induced by big-ET-1 in the guinea-pig. It thus appears that inhibition of ECE is accompanied by a significant reduction of the two main pharmacological effects of big-ET-1, namely increase of blood pressure and airways resistance.

As the increase of pulmonary resistance induced by ET-1 is totally mediated by eicosanoids in the guinea-pig (Whittle *et al.*, 1989), we can assume that the effects of big-ET-1 on the airways dynamics are produced by the release of eicosanoids stimulated by ET-1 which has been generated from the conversion of big-ET-1 via the ECE. In the present study, by the use of phosphoramidon, we were able to abolish the big-ET-1-induced eicosanoid release from the guinea-pig lungs, without affecting the ET-1-induced release of PGI_2 and TxB_2. These results are in accordance with previous observations we reported on the inhibition, with phosphoramidon, of the big-ET-1-induced prostanoid release from rat perfused lungs (D'Orléans-Juste *et al.*, 1991). From these observations, it is clear that big-ET-1 is activated by a phosphoramidon-sensitive enzyme, which is present in the lung vasculature.

We suggest that big-ET-3 is inactive as a pressor agent and as an eicosanoid releaser due to a lack of biotransformation by the ECE which effectively converts big-ET-1 to ET-1. The lack of activity of the ECE to transform big-ET-3 to ET-3 *in vivo* supports the observations made by Okada *et al.* (1990) who reported a weak conversion of big-ET-3 to ET-3 by the ECE when compared to big-ET-1 in cultured bovine endothelial cells.

Because of its sensitivity to phosphoramidon, the ECE has been classified as a neutral metalloprotease (Matsumura *et al.*, 1990). However phosphoramidon, which was initially identified as an inhibitor of thermolysin (Suda *et al.*, 1973), is not totally specific for the ECE as it also inhibits the neutral endopeptidase 24.11 and thus prevents the degradation of many peptides (Mumford *et al.*, 1981; Rouissi *et al.*, 1990). Nonetheless, phosphoramidon has been shown to be a potent inhibitor of partially purified ECE from rabbit lung membranes (McMahon *et al.*, 1991). Interestingly, these authors have shown that phosphoramidon was more potent than 1–10 phenanthroline and far more potent than thiorphan in inhibiting the conversion of big-ET. As we have not measured enzyme activity directly in the present study, the isolation and further purification of the ECE in addition to the development of more specific inhibitors will be required to elucidate further the specific nature of that enzyme.

The fact that the ECE does not biotransform big-ET-3 suggests that big-ET-1 may be a specific substrate for the converting enzyme. Interestingly, the ECE is also active against porcine big-ET-1 (1–39) (McMahon *et al.*, 1991). Both human and procine big-ET-1 show a remarkable homology of structure except for the replacement of a valine (Val^{28}) by an isoleucine (Ile^{28}) in the porcine structure and different C-terminal sequences (Pro^{36}-Arg^{37}-Ser^{38} in the human peptide; Pro^{36}-Ser^{37}-Arg^{38}-Ser^{39} in the porcine peptide).

Although porcine big-ET-1 and ET-1 are equipotent as pressor peptides in anaesthetized rats (McMahon *et al.*, 1991), the addition of an extra amino acid (L-arginine) at the C-terminal end of the big-ET (1–39) structure renders the peptide completely inactive as a pressor peptide and as a vasoconstrictor of isolated vascular tissues unless the analogue, namely big-ET (1–40), is preincubated with chymotrypsin (McMahon *et al.*, 1989). Hence, the C-terminal portion of big-ET (1–39) appears to be critical for the activity of the ECE.

These results indicate that the ECE is quite selective for its substrate and its selectivity may be determined by both the N-terminal (ET-1 (1–21)) and the C-terminal sequences of both porcine and human big-ET-1. Since big-ET-1 and 3 differ in their N- and C-terminal sequences, the above interpretation is proposed to explain why big-ET-1 is converted in the guinea-pig lung and big-ET-3 is not.

In conclusion, the results presented in our study indicate that the potent pressor effects of endothelins are largely direct. ET-1, ET-3 and big-ET-1, when administered intravenously, are potent bronchoconstrictors, these effects being predominantly modulated by the release of eicosanoids. Big-ET-1 but not big-ET-3 is possibly converted in the pulmonary vasculature of the guinea-pig and produces similar biological effects to ET-1 and ET-3. The enzyme which converts big-ET-1 in the pulmonary vasculature is sensitive to phosphoramidon and shows some selectivity for both the N- and the C-terminal sequences of human and porcine big-ET-1. The selectivity of the ECE may explain why big-ET-3 is not converted *in vivo* and *in vitro*.

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L-Glutamate diethyl ester and deaminated analogues as excitatory amino acid antagonists in rat cerebral cortex

[1]J.P. Turner & B.S. Meldrum

Department of Neurology, Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF

1 The effects of L-glutamate diethyl ester (GDEE) HCl, glutarate diethyl ester (GlrDEE) and glutarate dimethyl ester (GlrDME) on depolarizing responses to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate (Kain), N-methyl-D-aspartate (NMDA) and quisqualate (Quis), and spontaneous paroxsymal discharges (SPDs) were examined. Experiments were performed on slices of rat cingulate cortex using the *in vitro* grease gap recording technique in nominally Mg^{2+}-free Krebs medium.

2 GDEE HCl (3–14 mM) caused a concentration-dependent depolarization of the d.c. baseline potential. L-Glutamate (0.1–0.5 mM), HCl (15 mM) and sucrose (30 mM) also depolarized the baseline. GlrDEE (3–12 mM) and GlrDME (4–26 mM) had no consistent effect on baseline potential.

3 GDEE HCl (10 mM) had no effect on depolarizing responses to AMPA, Kain and NMDA, but caused potentiation of those to Quis with a dose-ratio of 0.53 (0.44–0.63) ($n = 4$). In two other experiments, where the depolarization of the baseline induced by GDEE HCl was large, a depression of Quis response amplitude was observed.

4 GlrDEE (10 mM) antagonized depolarizing responses to Kain, and to a lesser extent NMDA, with dose-ratios of 2.14 (1.92–2.38) and 1.61 (1.39–1.87), respectively. This concentration of GlrDEE had no effect on AMPA responses, but potentiated Quis responses, with a dose-ratio of 0.64 (0.58–0.71).

5 GlrDME (10 mM) antagonized depolarizing responses to Kain and to Quis, with dose-ratios of 1.66 (1.48–1.85) and 1.22 (1.15–1.29), respectively, and had no effect on responses to NMDA.

6 The SPDs were inhibited by GDEE HCl (IC_{50} 6.7 ± 0.37 mM), GlrDEE (IC_{50} 5.6 ± 0.38 mM) and GlrDME (IC_{50} 10.4 ± 0.73 mM).

7 In conclusion, there is little evidence that GDEE HCl is an antagonist of the postsynaptic excitatory amino acid receptors in the rat neocortex, and its effects may result from its contamination with L-glutamate and increased osmolarity of the bathing medium at high concentrations. The deaminated analogues of GDEE are very weak Kain antagonists.

Keywords: Amino acids; cerebral cortex; glutamate; synaptic receptors

Introduction

The electrophysiological study of excitatory amino acid (EAA) action has led to the proposal of three classes of EAA postsynaptic receptors, based on the action of specific or relatively selective antagonists (Watkins & Evans, 1981). These receptors have been designated α-amino-3-hydroxy-5-methylisoxazole-4-propionate/quisqualate (AMPA/Quis)-, kainate (Kain)- and N-methyl-D-aspartate (NMDA)-preferring receptors on the basis of their agonist specificity. The differentiation between NMDA and non-NMDA receptors is made more definitive by the specific blockade of NMDA receptors by antagonists. In addition, the quinoxaline-2,3-diones are potent and relatively selective antagonists of non-NMDA receptors (Honoré *et al.*, 1988). The distinction between the non-NMDA receptors AMPA/Quis and Kain is, however, less clearly defined and is based upon the relative selectivity of antagonists such as γ-D-glutamylglycine and γ-D-glutamyl-aminomethylsulphonate (Davies & Watkins 1981; 1985). The original distinction was made with the observation of a selective blockade of microiontophoretically applied Quis and AMPA with L-glutamate diethyl ester (GDEE) in the spinal cord (McLennan & Lodge, 1979; Krogsgaard-Larsen *et al.*, 1980). This has resulted in the proposal of specific antagonist properties for GDEE against AMPA/Quis compared with Kain and NMDA (McLennan, 1983). However, GDEE has also been shown to suppress spontaneous activity and acetylcholine excitation in the spinal cord (MacDonald *et al.*, 1977; McLennan & Lodge, 1979), and to hyperpolarize spinal neurones (Zieglgänsberger & Puil, 1973) indicating a more non-specific inhibitory action. In addition, a subsequent *in vitro* study of GDEE in the frog spinal cord showed no antagonist activity at the three postsynaptic EAA receptors at concentrations up to 5 mM, and some apparently non-specific effects, with the inhibition of synaptic activity (Evans *et al.*, 1979). In the hippocampus, though, a more selective action of GDEE against EAAs over ACh has been observed (Segal, 1976; Spencer *et al.*, 1983).

The low potency of GDEE when bath-applied has limited its further use as a pharmacological tool in electrophysiological experiments. However, this has not prevented the use of GDEE in both biochemical and behavioural experiments as an antagonist of EAA-mediated action. GDEE has been shown to inhibit the release of [^{3}H]-D-aspartate from, and the mobilization of intracellular Ca^{2+} in cultured cerebellar granule cells (Drejer *et al.*, 1986; Bouchelouche *et al.*, 1989). Behavioural studies have shown that GDEE and some deaminated analogues protect against seizures induced by DL-homocysteine thiolactone or Quis, but not seizures induced by pentylenetetrazole (Freed, 1986; Freed & Braun, 1988).

The aim of the present study was to evaluate GDEE HCl, and two deaminated analogues glutarate diethyl ester (GlrDEE) and glutarate dimethyl ester (GlrDME), against depolarizing responses to the EAA receptor agonists AMPA, Kain, NMDA and Quis, and the spontaneous paroxysmal discharges (SPDs) induced by the removal of Mg^{2+} from the bathing medium, in slices of the rat cingulate cortex *in vitro*.

Methods

All experiments were performed on the rat cingulate cortex preparation (Harrison & Simmonds, 1985). Male Wistar rats (200–300 g) were killed by decapitation and the whole brain removed. Once submerged in chilled Krebs medium containing 1 mM Mg^{2+}, serial transverse sections of forebrain (500 μm)

[1] Author for correspondence at present address: Dept. of Visual Science, Institute of Ophthalmology, Judd St., London WC1H 9QS.

were made with a Vibroslice (Campden Instruments). Sections at the mid-line and lateral to the cingulate cortex resulted in wedge-shaped pieces of tissue containing cingulate cortex and underlying white matter. The slices were then incubated in Krebs medium and gassed for 1.5 h before being placed in two-compartment baths. The majority of the cortical tissue was in one compartment, with the remaining cortex and underlying white matter projecting through a greased slot in a two-piece barrier dividing the two compartments. Each compartment was perfused independently with Krebs medium at 22 ± 1°C. The Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2 $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.0 and glucose 11, and was gassed continuously with 95% O_2 and 5% CO_2. However, the Krebs in the cortical compartment was modified so that it contained no Mg^{2+}. This allowed the appearance of SPD-like activity, probably through the removal of the voltage-dependent block of NMDA receptors by Mg^{2+} (Nowak *et al.*, 1984) and the increased efflux of L-glutamate from presynaptic terminals (Smith *et al.*, 1989). In those experiments where drug effects on responses to bath applied EAA agonists were examined, 0.1–0.2 μM tetrodotoxin (TTX) was present in the Krebs medium in the cortical compartment to suppress this SPD-like activity.

Drug solutions diluted in the Krebs medium were passed through the cortical compartment only. The potential difference between the two compartments was recorded continuously with a pen recorder, and negativity induced in the cortical compartment was interpreted as a depolarization of the cortical tissue. The recordings were made by use of amplifiers with low pass frequency filters, so that high frequency noise was eliminated. Although, this interfered with the frequency response of the recording system, limiting any potential conclusions about changes in amplitude and duration of high frequency events such as the SPDs, their frequency could be measured. Once the frequency of the SPD-like activity had stabilized, the control frequency was measured over a 20 min period, and then the prospective agents perfused at a range of increasing concentrations in the Krebs medium, for 60 min each. The frequency over the last 20 min period was then measured. At the end of the drug applications, the slices were returned to perfusion with control Krebs medium for at least 60 min to monitor recovery, and again the frequency was measured over a 20 min period. Any drug-induced changes were expressed as a percentage inhibition of control frequency. The results were fitted by non-linear regression analysis based on the logistic equation;

$$y = 100 \times (C^p/C^p + K^p)$$

where y = % inhibition, C = concentration, K = IC_{50} and p = slope constant and for each experiment an IC_{50} value was calculated. All statistical analyses were performed using Student's *t* test.

In experiments examining the influence of GDEE HCl, GlrDEE and GlrDME on the depolarizing responses to EAAs, AMPA (RS-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid, Tocris), Kain (kainic acid, Sigma), NMDA (N-methyl-D-aspartic acid, Tocris) and Quis (quisqualic acid, Sigma) were routinely used as agonists. Concentrations on the approximately linear portion of the dose-response (DR) curves for these agonists were selected (AMPA 2.5–5 μM, Kain 5–20 μM, NMDA 10–40 μM and Quis 5–20 μM) and perfused for 2 min. Responses were measured at peak amplitude. The prospective agents were perfused for 30 min prior to and then during the re-determination of agonist responses bridging the control responses. From the parallel shift of the DR lines between these responses, dose-ratios for the EAA agonists were calculated from equipotent concentrations of the agonists in the absence and presence of GDEE and deaminated analogues. All the agonists were prepared as 10 mM solutions in Krebs medium, except tetrodotoxin (TTX, Sigma), which was prepared as a 1 mM solution in 0.01 M acetic acid, and diluted in the Krebs medium immediately before use. GDEE HCl (L-glutamate diethyl ester HCl salt >99% purity by thin layer chromatography (t.l.c.), Sigma), GlrDEE (glutarate diethyl ester >99% purity by gas chromatography (g.c.), Sigma), GlrDME (glutarate dimethyl ester ≈98% purity by g.c., Sigma), L-glutamate Na, glutaric acid and sucrose (all Sigma) were dissolved directly into the Krebs medium. All base chemicals (Analar) were obtained from BDH.

Results

Baseline effects

GDEE HCl depolarized the cortical tissue in the absence and presence of TTX. At concentrations between 3 and 14 mM GDEE HCl, in the absence of TTX, there was an initial rapid depolarizing phase, which reached a plateau within 10–15 min (Figure 1a). This effect was concentration-dependent (Figure 2). There were additional ramp-like depolarizing shifts with 14 mM GDEE HCl, ranging between 0–0.15 mV h^{-1}, following the initial rapid depolarization. In the presence of TTX, 10 mM GDEE HCl produced a mean depolarization of 0.16 ± 0.022, consistent with the depolarizations seen in the absence of TTX (Figure 2). In addition, where the tissue had been exposed to AMPA or Quis, 10 mM GDEE HCl also produced ramp-like depolarizing shifts with rates ranging between 0.1–0.18 mV h^{-1} and 0.09–0.48 mV h^{-1} for AMPA and Quis, respectively. The rates of the ramp-like depolarizing shifts for NMDA- and Kain-exposed slices never exceeded 0.05 mV h^{-1} in the presence of 10 mM GDEE HCl and TTX. In contrast, both GlrDEE (3–12 mM) and GlrDME (4–26 mM) had no concentration-dependent effects on baseline potential in the absence of TTX ($P > 0.5$ and $P > 0.05$, respectively, correlation analysis). In the case of GlrDEE, there were no significant changes ($P > 0.05$) in baseline potential at any individual concentrations. However, in the presence of TTX, 10 mM GlrDEE caused a small but significant hyperpolarization of baseline (0.08 ± 0.030 mV, $n = 20$). With GlrDME, in the absence of TTX, the effect of individual concentrations on baseline potential varied, with significant mean hyperpolarizing responses at some concentrations (4, 5.5, 13 and 16 mM, range of means 0.07–0.14 mV, $P < 0.05$) and not others (7.5, 8, 10, 14 and 26 mM). In the presence of TTX, 10 mM GlrDME produced a hyperpolarization of 0.14 ± 0.031 mV ($n = 17$). Although 2 and 6 mM HCl (in the absence of TTX) had no significant effect on the baseline d.c. potential ($P > 0.2$), the application of 15 mM HCl (Figure 1d) resulted in a small depolarization, which rapidly attained a plateau of 0.16 ± 0.03 mV ($n = 4$, $P < 0.05$). L-Glutamate (0.1 and 0.2 mM) produced small transient depolarization of the baseline when first applied, which rapidly returned towards the control level in the absence of TTX (Figure 3a). However, with 0.5 mM L-glutamate there was a sustained depolarization of 0.10 ± 0.014 mV ($n = 5$, $P < 0.005$) following the initial transient depolarization (Figure 3a). No effect on the baseline was observed with 5 mM glutaric acid (data not shown) and 10 mM sucrose (both $P > 0.2$), but 30 mM sucrose (Figure 3b) resulted in a small depolarization 0.07 ± 0.014 mV ($n = 6$, $P < 0.01$) in the absence of TTX.

Excitatory amino acid-induced depolarizations in the presence of tetrodotoxin

GDEE HCl (10 mM) had no significant effect on responses to AMPA, Kain and NMDA (Figure 4). Against Quis, however, there was a potentiation of the depolarizing responses producing leftward parallel shifts of the DR line with a mean of 0.28 log units ($n = 4$). In two other experiments where there was a relatively rapid rate of baseline depolarization by GDEE HCl, responses to Quis became progressively more depressed in amplitude, as the baseline depolarized (Figure 5). This effect was reversed after returning to control Krebs medium. No such effect was observed with AMPA responses.

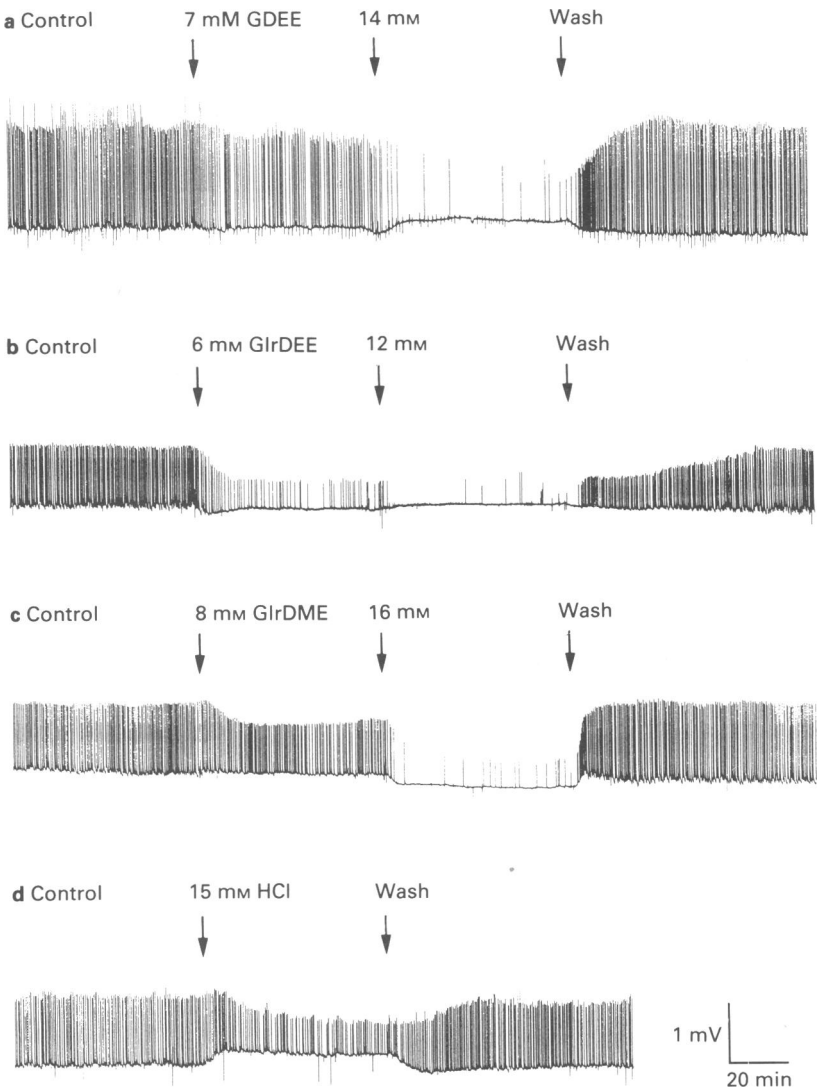

Figure 1 Continuous chart recordings (d.c.) of the spontaneous paroxysmal discharge (SPD)-like activity in the absence of Mg^{2+}, with the effect of increasing concentrations of (a) L-glutamate diethyl ester (GDEE), (b) glutarate diethyl ester (GlrDEE), (c) glutarate dimethyl ester (GlrDME) and (d) HCl on the baseline potential and the frequency of SPDs, and the reversal upon return to control Krebs medium. Cortical negativity is upwards.

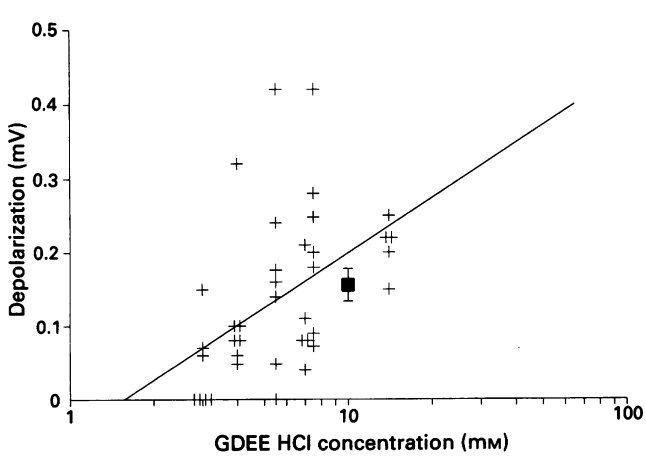

Figure 2 The concentration-effect data for the initial depolarization of baseline potential by L-glutamate diethyl ester (GDEE) HCl in the absence of tetrodotoxin (TTX). The points represent values from individual experiments ($n = 38$). Using linear regression analysis, a line was fitted with a slope of 0.25, and a correlation coefficient of 0.47 ($P < 0.003$). The filled square and vertical line represent the mean ± s.e.mean of the initial depolarization produced by 10 mM GDEE HCl in the presence of TTX ($n = 22$).

GlrDEE (10 mM) had a different spectrum of activity to GDEE, antagonizing responses to Kain and NMDA (Figures 6 and 4), with parallel rightward shifts of the DR lines. For three of the five experiments against Kain responses in Figure 4 recovery was monitored, with a complete reversal of the antagonist action observed in two, but not in the third. However, like GDEE, GlrDEE had no effect against responses to AMPA and potentiated those to Quis, producing parallel leftward shifts of the DR line with a mean of 0.20 log units.

GlrDME (10 mM) antagonized responses to Kain and to a much lesser extent to Quis, but had no effect on responses to NMDA (Figure 4). For two of the five experiments against responses to Kain recovery was monitored, with a complete reversal of the antagonism by GlrDME in both. GlrDME was not tested against AMPA.

Spontaneous paroxysmal discharge-like activity

GDEE HCl, GlrDEE and GlrDME all reduced the frequency of SPD-like activity in a concentration-dependent manner (Figures 1 and 7). The most potent was GlrDEE with an IC_{50} of 5.6 ± 0.38 mM ($n = 12$). The threshold concentration for this effect was about 4 mM with complete inhibition at 12 mM. Complete recovery of control frequency was observed within

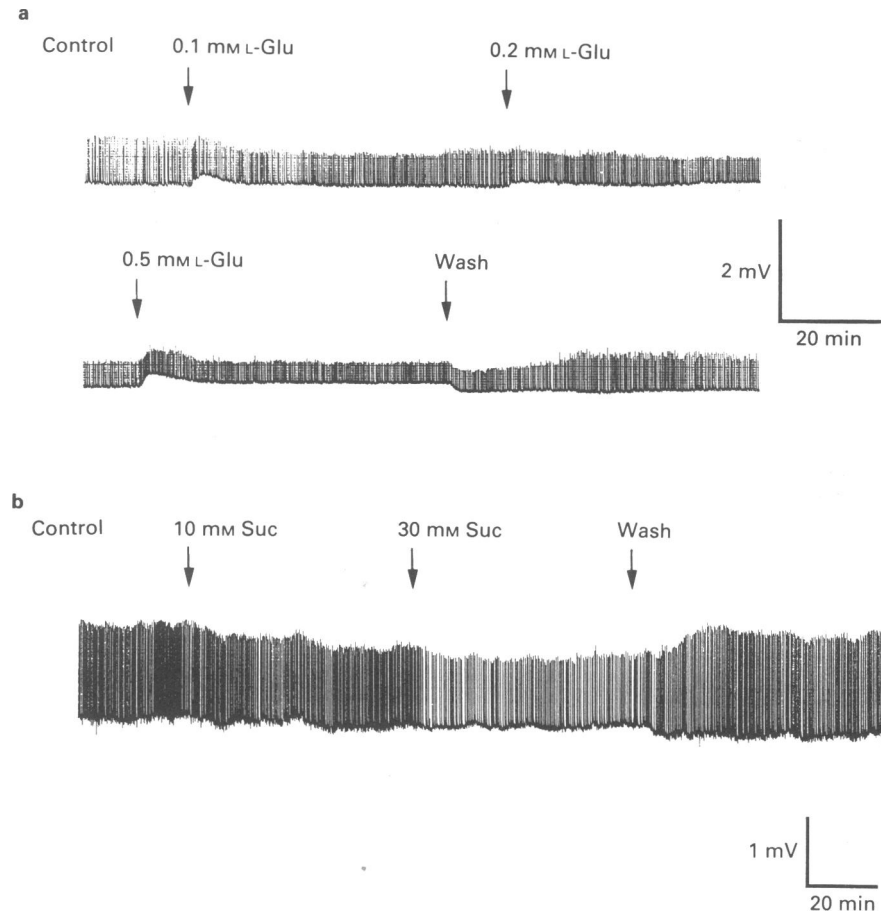

Figure 3 Continuous chart recordings (d.c.) of the spontaneous paroxysmal discharge (SPD)-like activity in the absence of added Mg^{2+}, with the effect of increasing concentrations of (a) L-glutamate (L-Glu), and (b) sucrose (Suc) on baseline potential and the frequency of SPDs, and the reversal upon return to control Krebs medium. Cortical negativity is upwards.

40 min of returning slices to control Krebs medium. GDEE HCl was slightly less potent with an IC_{50} of 6.7 ± 0.37 mM ($n = 13$). A virtually complete inhibition was observed at about 14 mM. Recovery was again achieved after about 40 min on return to control medium. GlrDME was the least potent as an inhibitor of the SPDs, with an IC_{50} of 10.4 ± 0.73 mM ($n = 23$). The threshold concentration for the effect was about 4.5 mM, with complete inhibition occurring at 20 mM. Again complete recovery was observed within about 40 min of return to control Krebs medium.

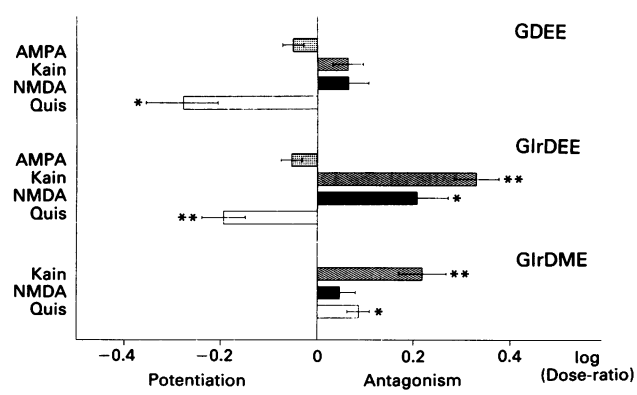

Figure 4 The effect of L-glutamate diethyl ester (GDEE), glutarate diethyl ester (GlrDEE) and glutarate dimethyl ester (GlrDME) (all 10 mM) on the dose-response lines for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate (Kain), N-methyl-D-aspartate (NMDA), and quisqualate (Quis) in the presence of tetrodotoxin. Lateral leftward shifts or potentiation and rightward shifts or antagonism were measured as log dose-ratios. The horizontal lines represent the s.e.mean and the bars the means of $n = 5$ experiments, except those for AMPA and Quis in GDEE, $n = 4$. * $P < 0.05$, ** $P < 0.01$.

In view of the high concentrations required, it is possible that the buffering capacity of the Krebs medium is compromised, so the effect of these compounds on the pH of the Krebs medium was investigated. Before the drug solutions were perfused, the pH of continuously gassed control Krebs medium was monitored. Drug was then added to the Krebs medium and the pH was continuously monitored until a new pH was obtained, stable for 10 min, while being continuously gassed. Monitoring continued in the perfusion reservoir prior to and during perfusion. The lag time between perfusion reservoir and the tissue bath was ≈30 s, and the pH increase between reservoir and bath was found to be the same with either control Krebs medium or that acidified with 15 mM HCl (0.16–0.21 and 0.16–0.20 respectively, $n = 7$). Neither GlrDEE nor GlrDME had any effects on pH, up to concentrations of 30 mM (increase in pH < 0.05 pH units). However, the addition of GDEE HCl to the Krebs medium resulted in acidification, such that at 10 mM and 30 mM concentrations, there were decreases in pH of 0.12 and 0.34 pH units, respectively.

In a few experiments the effect of decreasing pH on the SPDs was examined by the addition of HCl solution to the Krebs medium. The effect of HCl on the pH of the Krebs medium was monitored in the same way as that with GDEE HCl, GlrDEE and GlrDME. The acidified Krebs medium was then perfused for 60 min, as with the drug applications. Again any change in the frequency of the SPDs was measured as a percentage inhibition of control frequency. The addition of HCl to give final concentrations of 2 mM and 6 mM produced decreases in pH of <0.05 and 0.10 pH units, respectively, but

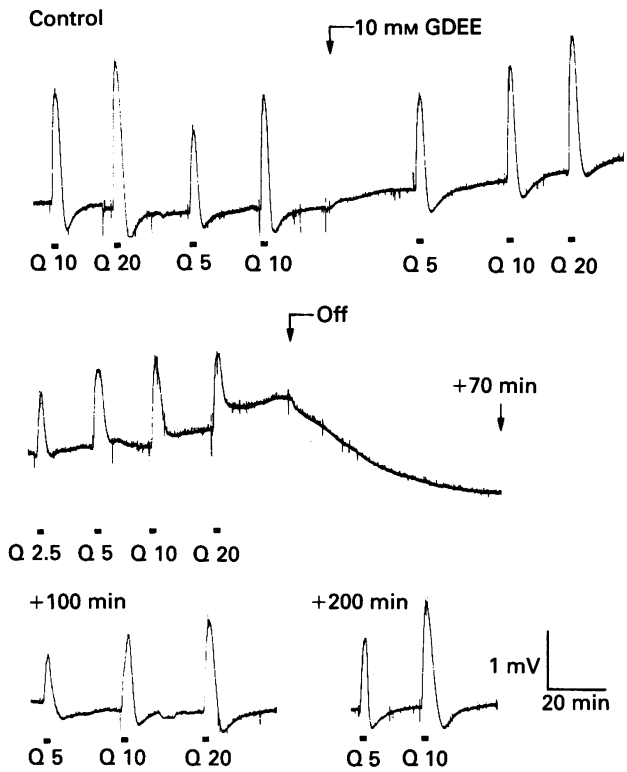

Figure 5 A continuous recording of d.c. potential in the presence of tetrodotoxin (TTX) showing the depolarizing responses to quisqualate (Quis, Q), in the absence and presence of 10 mM L-glutamate diethyl ester (GDEE). It illustrates the large ramp-like depolarization of baseline (0.48 mV h^{-1}) coincident with occlusion of Quis responses seen in two experiments. The reversal of this depression is illustrated on the lowest trace at +100 min and +200 min after re-application of control Krebs medium.

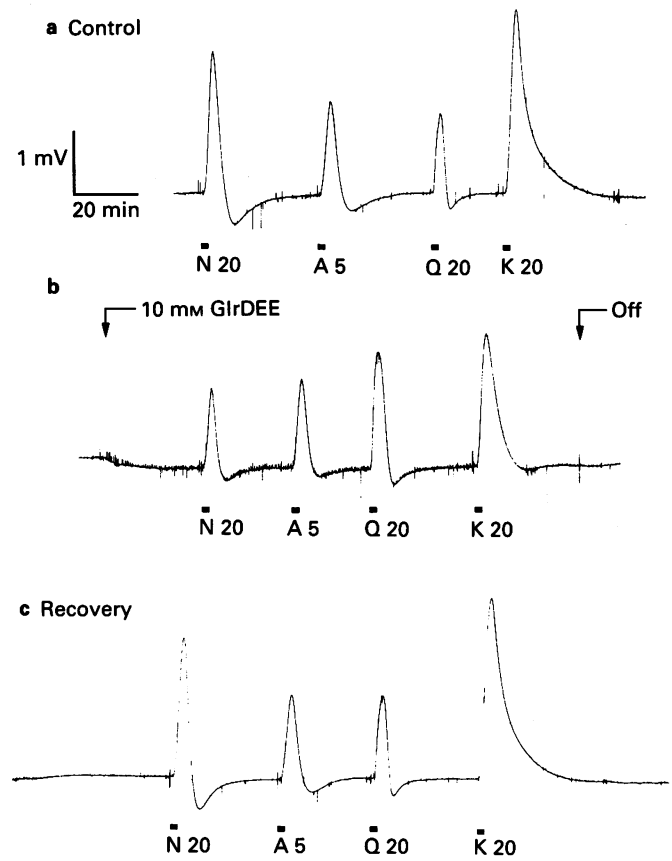

Figure 6 A continuous recording of d.c. potential showing the depolarizing responses to 20 μM N-methyl-D-aspartate (NMDA, N), 5 μM α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA, A) 20 μM quisqualate (Quis Q) and 20 μM kainate (Kain, K), from the cortical wedge preparation in the absence (a) and the presence (b) of 10 mM glutarate diethyl ester (GlrDEE). Responses to NMDA and Kain were depressed, that to Quis was augmented, and AMPA little affected. The recovery (c) of NMDA, Kain and Quis response amplitude occurred after a period of wash with control medium.

had no significant effect ($P > 0.1$) on the frequency of SPDs (both $n = 6$). However, a final concentration of 15 mM HCl, producing a decrease in pH of 0.40 pH units, significantly ($P < 0.05$) reduced SPD frequency by 38.9 ± 8.5% ($n = 4$).

In view of the fact that these compounds possibly contain upto 1–2% impurities such as L-glutamate or glutaric acid, the effects of these compounds were examined. L-Glutamate (0.1–0.5 mM), although producing transient increases in frequency of SPDs at the onset of application, had no significant effect ($P > 0.05$) on the SPD frequency when measured 60 min after application ($n = 4$). However, there was a tendency towards a decrease in frequency, and a reduction in SPD amplitude, the reduction in SPD amplitude not being reversed 60 min after wash out. The application of 5 mM glutaric acid had no significant effect ($P > 0.05$) on the frequency of SPDs ($n = 6$). L-Glutamate (0.1–3 mM) and 0.1 and 0.3 mM glutaric acid had no effect on pH, whilst 1 and 5 mM glutaric acid reduced pH by 0.07 and 0.20 pH units, respectively. Increasing the osmolarity of the Krebs medium with concentrations of 10 and 30 mM sucrose resulted in no effect at 10 mM, but a significant depression of SPD frequency at 30 mM by 32.0 ± 2.72% ($n = 5$, $P < 0.001$).

Discussion

L-Glutamate diethyl ester

The results from the present study indicate that GDEE HCl has no antagonist activity on postsynaptic EAA ionophore-receptors, consistent with the observations in the spinal cord (Evans *et al.*, 1979), and *Xenopus* oocyte expression studies (Hirono *et al.*, 1988). Since responses to AMPA are unaffected by GDEE HCl, the potentiation of responses to Quis is also not due to a direct action on the AMPA/Quis receptor. There

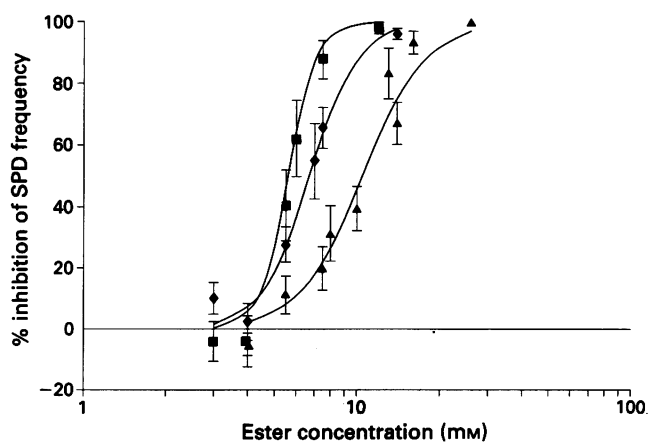

Figure 7 Dose-effect curves for L-glutamate diethyl ester (◆) and deaminated analogues glutarate diethyl ester (■) and glutarate dimethyl ester (▲) showing the inhibition of spontaneous paroxysmal discharge (SPD) frequency observed in 0 Mg^{2+} Krebs medium. The vertical lines represent the s.e.mean and the points the means of 4–10 values for the inhibition of SPD frequency expressed as a percentage of control.

is some evidence that GDEE displaces one of the components of Cl^--dependent [^{3}H]-L-glutamate binding (Werling *et al.*, 1983), which probably represents Cl^--dependent L-glutamate uptake, in view of its comparable pharmacological profile to [^{3}H]-AP4 binding (Butcher *et al.*, 1983) and its sensitivity to freeze-thawing. Since Quis is much more potent than AMPA in displacing this binding and Quis is a known substrate of Cl^--dependent uptake (Zaczek *et al.*, 1987), a block of this uptake would explain these results. Another consequence of uptake blockade is that it could lead to an enhanced tonic activation of EAA receptors by endogenous L-glutamate, and hence the depolarization of baseline observed with GDEE HCl. However, the contamination of GDEE HCl by up to 1% L-glutamate, which probably explains its weak agonist activity in *Xenopus* oocyte expression studies (Hirono *et al.*, 1988), means that the concentrations of GDEE HCl used in this study would contain 0.03–0.14 mM L-glutamate. The application of 0.1–0.5 mM L-glutamate did not greatly affect baseline potential, so the depolarizing action of the L-glutamate *per se*, does not explain the depolarizing action of GDEE HCl. Recent evidence indicates that the main component of the depolarizing action of bath applied L-glutamate in brain slices is due to the electrogenic nature of Na^+-dependent L-glutamate uptake (Frenguelli *et al.*, 1991). Therefore, it is possible that the depolarizing action of GDEE HCl is the result of the blockade of Cl^--dependent L-glutamate uptake combined with the enhanced electrogenic Na^+-dependent uptake of endogenous and contaminating L-glutamate. The reason for the greater depolarizing action of GDEE HCl on slices exposed to AMPA and Quis remains unclear, unless the activation of the AMPA/Quis receptor has a long term effect on L-glutamate uptake mechanisms. The reduction of Quis responses seen with very large depolarizing shifts may simply reflect occlusion by the rising baseline.

The depression of the frequency of the SPDs by GDEE HCl observed in 0 Mg^{2+} Krebs is consistent with the depression of synaptic activity seen in the spinal cord. Previous study has shown that the SPDs are mediated via the action of released EAAs on postsynaptic NMDA receptors, since the frequency of SPDs is very sensitive to NMDA receptor antagonists (Horne *et al.*, 1986), but not the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (Fletcher *et al.*, 1988). Therefore, the lack of any EAA antagonist activity for GDEE HCl, indicates that some other mechanism is involved. In addition, this lack of EAA antagonist activity means that the decrease in extracellular pH produced by GDEE HCl is insufficient to affect NMDA receptors, which have been shown to be very sensitive to changes in pH in the physiological range (Treynalis & Cull-Candy, 1990). Indeed, concentrations of HCl and glutaric acid which produced changes in pH of the Krebs medium that would be effective in producing some reduction in the level of NMDA receptors activation, caused little or modest change in SPD frequency. However, the increase in pH between perfusion reservoir and tissue bath is probably sufficient to account for this discrepancy. Certainly a previous study indicates that SPDs in the cortical wedge preparation are very sensitive to changes in extracellular pH produced by manipulation of the CO_2 concentration in the Krebs medium (Aram & Lodge, 1987). However, it is unlikely that a decrease in extracellular pH is the major contributory factor to the inhibition of the SPDs by GDEE HCl in this study.

It is possible that the osmolarity of the Krebs medium is affected by the high concentrations of GDEE HCl used in this present study. Attempts to increase osmolarity with sucrose resulted in inhibition of the SPDs, consistent with the fact that increased osmolarity has been shown to decrease neuronal excitability (Rosen & Andrew, 1990). However, this effect was a partial inhibition at a concentration in excess of concentrations of GDEE HCl producing near maximal inhibition. This would suggest that increased osmolarity and certainly increased molarity are only contributory non-specific factors in the inhibitory action of GDEE HCl on synaptic activity. Indeed, since GDEE suppresses the release of [^{3}H]-D-aspartate from cerebellar granule cells (Drejer *et al.*, 1986), GDEE HCl could block the presynaptic release of EAAs, inhibiting the appearance of SPD-like activity. In addition, non-specific effects such as membrane stabilization and blockade of other ion channels cannot be ruled out as mediators of the inhibitory action of GDEE.

Glutarate diethyl ester and glutarate dimethyl ester

In contrast to GDEE HCl, GlrDEE and GlrDME have some EAA antagonist activity, indicating that deamination of the L-glutamate diester structure confers a different activity on these compounds. In the case of GlrDEE it has both NMDA and Kain, but not AMPA receptor antagonist activity, indicating a separation of AMPA and Kain receptors. Much electrophysiological evidence indicates that the actions of Kain are mediated via the AMPA/Quis receptor expressed in the *Xenopus* oocyte (Verdoorn *et al.*, 1989) and isolated hippocampal neurones (Kiskin *et al.*, 1986), and this is supported by antagonist studies in the cortical wedge preparation (Fletcher *et al.*, 1988). However, another expression study in the *Xenopus* oocyte has revealed the presence of a Kain receptor not activated by AMPA (Umbach & Gundersen, 1989), indicating the possibility of postsynaptic ionophore-receptors for Kain distinct from the AMPA/Quis receptors. GlrDEE also potentiated responses to Quis, indicating there may also be an interaction of GlrDEE with Cl^--dependent L-glutamate uptake. Although the NMDA antagonist activity exhibited by GlrDEE would explain the inhibition of SPDs, it is more likely that a non-specific mechanism similar to that for GDEE is involved, with the NMDA antagonism contributing to the increased potency compared to GDEE HCl. GlrDME showed some antagonist activity against both Kain and Quis, and not NMDA, at a concentration that is not near maximal for the inhibition of the SPDs. Therefore, the action of GlrDME on SPDs cannot be ascribed to any one EAA receptor, and probably results from a non-specific mechanism of action as well.

As with GDEE HCl, the contamination of the deaminated analogues of GDEE must be considered. With these compounds the most probable contaminant is glutaric acid, which is likely to be as much as 1 and 2%, i.e. concentrations in this study of 0.03–0.12 and 0.08–0.52 mM, for GlrDEE and GlrDME, respectively. Since glutaric acid at these concentrations, and GlrDEE and GlrDME all had little effect on the pH of the Krebs medium, it is unlikely that acidification of the Krebs medium leading to NMDA receptor blockade is responsible for the antagonism of NMDA responses by GlrDEE or the inhibition of SPDs. As with GDEE HCl, the underlying mechanism may involve, in part at least, the decrease in neuronal excitability resulting from the increase in extracellular osmolarity (when high concentrations are used).

These results, together with a previous study (Evans *et al.*, 1979), provide little evidence for the action of GDEE as an antagonist of postsynaptic ionophore-receptors in the CNS, when bath applied. The fact that GDEE was effective as a selective AMPA/Quis antagonist in iontophoretic experiments remains unresolved. As a result, when GDEE is applied by a method other than iontophoresis, it is impossible to determine whether GDEE is acting as a selective AMPA/Quis antagonist or via some less specific mechanism. Therefore, in behavioural studies where seizures induced by EAA have been blocked by GDEE (Freed, 1986; Freed & Braun, 1988), the antagonism of seizures cannot necessarily be attributed to the antagonism of EAA postsynaptic ionphore-receptors, although in the case of the deaminated analogues of GDEE this may contribute. Finally, it is clear from this study that the lack of AMPA/Quis receptor antagonist action for GDEE in the rat neocortex prevents its use as a tool in receptor classification in these types of experiment.

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Effect of nedocromil sodium on allergen-, PAF-, histamine- and bradykinin-induced airways vasodilatation and pulmonary obstruction in the pig

[1]K. Alving, R. Matran & J.M. Lundberg

Department of Pharmacology, Karolinska Institutet, Box 60 400, S-104 01 Stockholm, Sweden

1 The influence of nedocromil sodium on the nasal and bronchial effects induced by allergen, platelet-activating factor (PAF), capsaicin, histamine and bradykinin aerosol challenge in ascaris-sensitized and pentobarbitone-anaesthetized pigs was studied. Blood flow changes in the bronchial and nasal circulation were measured with ultrasonic flow probes around the supplying arteries, and vascular resistance was calculated. Changes in pulmonary resistance (R_{pulm}), dynamic compliance (C_{dyn}), mean arterial pressure (MAP) and heart rate (HR) were also determined.

2 Allergen and PAF aerosol challenge in the lung produced similar effects consisting of both bronchial and nasal vasodilatation, bronchoconstriction (increase in R_{pulm} and decrease in C_{dyn}) and increases in MAP and HR. Local pretreatment with nedocromil sodium (80 mg, aerosol) reduced the peak and duration of both the bronchial vasodilatation and increase in R_{pulm}, while only the duration of the change in C_{dyn} was significantly decreased. Nedocromil sodium did not alter the increases in MAP and HR. The nasal vasodilatation evoked by PAF, but not allergen, challenge in the lung was reduced by nedocromil sodium.

3 Allergen challenge in the nose induced vasodilatation of long duration which was reduced by local nedocromil sodium pretreatment (50 $\mu g\,kg^{-1}$, intra-arterially).

4 The vasodilator response to histamine aerosol was attenuated in the nasal, but not the bronchial circulation by local nedocromil sodium pretreatment. Histamine-induced bronchoconstriction was not altered by nedocromil sodium.

5 Bradykinin aerosol-induced vasodilatation in the nasal and bronchial circulation was markedly and equally reduced by local nedocromil sodium and systemic capsaicin (50 $mg\,kg^{-1}$, s.c. 2 days before) pretreatment. The vasodilatation caused by i.v. bradykinin challenge was not reduced by capsaicin pretreatment. Capsaicin aerosol-induced vasodilatation in the nasal and bronchial circulation was not affected by local nedocromil sodium pretreatment.

6 In conclusion, nedocromil sodium blocks some local vascular and bronchial effects, but not increases in MAP and HR, induced by allergen and PAF aerosol in the pig. Bradykinin-induced vasodilatation in the airways, which seems to be largely dependent on capsaicin-sensitive sensory nerves, is markedly inhibited by nedocromil sodium pretreatment, whereas capsaicin-induced vasodilatation is not affected by nedocromil sodium. It may be suggested that nedocromil sodium acts by inhibiting some common process involved in the release of mediators from inflammatory cells (when stimulated by allergen and PAF) and sensory nerves (when stimulated by bradykinin and histamine, but not capsaicin).

Keywords: Nedocromil sodium; bronchial circulation; nasal circulation; sensory nerves; capsaicin; allergen; platelet-activating factor; histamine; bradykinin

Introduction

Nedocromil sodium, which has now been used as an anti-asthmatic agent for several years, is known to attenuate allergen- (e.g. Dahl & Pedersen, 1986) and excercise- (Roberts & Thomson, 1985) induced immediate bronchoconstriction as well as immediate symptoms of rhinitis upon allergen challenge (Corrado *et al.*, 1987) in man. Regarding the mechanism of action, nedocromil sodium may influence a variety of inflammatory cells, including mast cells (Wells *et al.*, 1986). In addition, it has been proposed that nedocromil sodium inhibits sensory nerve activation since it reduces, for example, SO_2- (Altounyan *et al.*, 1986) and bradykinin- (Barnes, 1986) induced reflex bronchoconstriction in man and citric acid-induced cough in dogs (Jackson, 1988). The activation of sensory nerves in the airways by histamine (Coleridge & Coleridge, 1977) and bradykinin (Kaufman *et al.*, 1980) is now well recognized and has also been shown to result in the release of sensory neuropeptides like substance P (Saria *et al.*, 1988). Capsaicin specifically stimulates sensory nerves (see Buck & Burks, 1986), but may do so by a mechanism different from that seen with bradykinin, since capsaicin-induced reflex bronchoconstriction in man is not affected by nedocromil sodium (Hansson *et al.*, 1988). Furthermore, whereas capsaicin-evoked excitation of sensory nerves is specifically blocked by ruthenium red (Maggi *et al.*, 1988), bradykinin-induced excitation is attenuated by protein kinase C inhibitors (Dray *et al.*, 1988).

We have previously shown that capsaicin, histamine and allergen produce clear-cut vasodilatation in the pig bronchial and nasal circulations upon local aerosol challenge and that these vasodilator responses are reduced after systemic pretreatment with capsaicin 2 days before (Alving *et al.*, 1990; Matran *et al.*, 1990). In this study, we examine the effects of nedocromil sodium on the pig airways vascular and bronchial responses to aerosol challenge with capsaicin, histamine, bradykinin, allergen and platelet-activating factor (PAF). We have also studied the effect of systemic capsaicin pretreatment on bradykinin-induced airways vasodilatation and compared it with nedocromil sodium.

Parts of these results have been published in a preliminary form (Lundberg *et al.*, 1990).

Methods

The experiments were approved by the Local Ethical Committee for Animal Research.

[1] Author for correspondence.

Sensitization

Twelve pigs were sensitized with s.c. injections of 1 mg *Ascaris suum* antigen in a suspension of $Al(OH)_3$ at the age of 6 weeks (10–12 kg body weight), followed by a booster injection 4 weeks later. Experiments were performed after a further 2-week period. Successful sensitization was verified by skin injections of ascaris antigen in saline.

Systemic capsaicin pretreatment

Eight unsensitized pigs (20–25 kg body weight) were fasted overnight and premedicated with ketamine hydrochloride (20 mg kg^{-1}, i.m.). Anaesthesia was induced by sodium pentobarbitone (12 mg kg^{-1}) introduced into an ear vein, and muscle relaxation was achieved by pancuronium bromide (0.2 mg kg^{-1}, i.v.) given intermittently during the pretreatment procedure. The pigs were mechanically ventilated by use of an Engström respirator through an oral endotracheal tube with a mixture of air and oxygen.

Anaesthesia was maintained by a continuous i.v. infusion of pentobarbitone (10 mg kg^{-1} h^{-1}) and Ringer solution (150 ml h^{-1}). The efficacy of anaesthesia was tested by pinching the ear and nipples when muscle relaxation was weak. Atropine (1 mg kg^{-1}, i.v.) was given before capsaicin injections to avoid excessive exocrine secretion in the airways. Capsaicin was dissolved to a concentration of 25 mg ml^{-1} in a vehicle consisting of 10% ethanol and 20% Tween 80 in saline. Capsaicin was then injected s.c. in increasing doses (6 injections in different regions) to a total cumulative dose of 50 mg kg^{-1} over a period of 2 h. Anaesthesia was maintained for another 2 h before the pentobarbitone infusion was withdrawn, and spontaneous breathing was restored after a further 30–60 min, after which the pigs were extubated. Four pigs received injections of the vehicle by a similar procedure, and served as controls together with 4 untreated animals (all unsensitized). Compared to vehicle-treated animals, capsaicin-treated pigs were more drowsy for the rest of the day but the two groups were drinking and eating normally the next day (resting day). The experiments were performed on the third day. The neural effects of systemic capsaicin pretreatment of pigs have been described in detail elsewhere (Alving *et al.*, 1991a).

Experimental procedure

Pigs were anaesthetized as above, intubated through a low tracheostomy and subsequently mechanically ventilated. Briefly (for details see Alving *et al.*, 1990), the femoral vein was cannulated for continuous infusion of pentobarbitone (10 mg kg^{-1} h^{-1}), pancuronium (0.6 mg kg^{-1} h^{-1}) and Ringer solution with glucose (200 ml h^{-1}). The femoral artery was cannulated for continuous recordings of mean arterial pressure (MAP) and heart rate (HR), and heparin (5000 iu) was given as a bolus injection. Arterial Po_2, Pco_2 and pH were regularly monitored and body temperature was maintained at 38–39°C. After a right-side thoracotomy the bronchial artery was dissected free, and an ultrasonic flow probe connected to a T202 S recorder (Transonic System Inc., NY, U.S.A.) was placed around the vessel for continuous recording of absolute blood flow. The right internal maxillary artery (mainly supplying the nasal mucosa) was similarly exposed for blood flow measurements. A small collateral branch of the internal maxillary artery was cannulated for infusion of nedocromil sodium. The transpulmonary pressure and pulmonary airflow were measured and the signals were processed on-line by a microcomputer to give R_{pulm} and C_{dyn}. All parameters were continuously recorded on a Grass polygraph. Nedocromil sodium (80 mg in 4 ml saline) was administered as an aerosol to the lung via the endotracheal tube in 6 sensitized and 8 unsensitized pigs. The aerosol was produced by use of an ultrasonic nebulizer and was delivered over 6 min (100 breaths). Twenty min after nedocromil sodium pretreatment, ascaris antigen (14 mg in saline) and capsaicin (10 mg in 60% ethanol) were delivered as an aerosol to the lung over 3 min in a group of 12 sensitized pigs (6 controls and 6 nedocromil sodium-treated). Bradykinin (1.0 mg), histamine (1.5 mg) and PAF (0.6 mg), all in saline, were administered to a group of 16 unsensitized pigs (8 controls and 8 nedocromil sodium-treated). Lung challenges were finished within 75 min or 60 min, respectively, after nedocromil sodium pretreatment in the lung. One hour after lung challenges, nedocromil sodium (50 μg kg^{-1}) was given intra-arterially (i.a.) as a bolus injection and 5–10 min later aerosols of ascaris antigen (7 mg) and capsaicin (10 mg) (sensitized pigs), or histamine (1.5 mg) and bradykinin (0.5 mg) (unsensitized pigs) were delivered to the right nostril over 3 min. The nasal challenges were finished within 75 min or 30 min, respectively, after intra-arterial nedocromil sodium. Bradykinin was also delivered to the lungs and nostrils of 8 capsaicin-treated pigs. Furthermore, bradykinin (1 μg kg^{-1}) was administered i.v. in 8 control and 8 capsaicin-treated pigs. Ethanol itself (60%), administered as an aerosol to the airways, did not change local blood flow, pulmonary calibre, HR or MAP.

Calculations

Blood flow was recorded in ml min^{-1} and vascular resistance (VR), defined as MAP divided by blood flow, was calculated. All data are presented as mean ± s.e.mean of relative changes. The Mann-Whitney test was used for statistical analyses.

Drugs

Atropine sulphate, histamine dihydrochloride, PAF and bradykinin acetate were obtained from Sigma Co., U.S.A. and capsaicin from Fluka Chemie AG, Switzerland, Sodium pentobarbitone was purchased from NordVacc, Stockholm, Sweden, ketamine hydrochloride from Parke Davis, U.S.A. and pancuronium bromide from Organon, The Netherlands. The following agents were kindly donated by the companies indicated: nedocromil sodium (Fisons plc, Loughborough, UK) and ascaris antigen (Pharmacia Diagnostics AB, Uppsala, Sweden).

Results

Pretreatment with nedocromil sodium

Delivery of nedocromil sodium (80 mg) as an aerosol to the lung caused increase in R_{pulm} of 11.7 ± 5.5% and decrease in C_{dyn} of 19.2 ± 4.5% and vasodilatation of the bronchial circulation (decrease in VR of 4.5 ± 1.4%). However, 5–10 min after nebulization of nedocromil sodium, these parameters were back to baseline and no systemic cardiovascular effects were seen with this treatment. When nedocromil sodium (50 μg kg^{-1}) was given i.a. to the nose, no changes in nasal blood flow, pulmonary calibre, MAP or HR were noted.

Allergen and PAF challenge

Ascaris aerosol challenge in the lungs of sensitized, control pigs induced marked pulmonary obstruction of long duration as revealed by increase in R_{pulm} and decrease in C_{dyn} (Figure 1a). Simultaneously, there was a 2 to 3 fold increase in blood flow in the bronchial circulation with a decrease in calculated VR that lasted for 80 ± 10 min (Figure 1b). A similar vasodilatation (decrease in VR) lasting for 84 ± 10 min was seen also in the nasal circulation upon lung challenge with allergen (Figure 1c). In pigs pretreated with nedocromil sodium in the lung, the maximal increase in R_{pulm} upon allergen challenge was reduced by 46%, whereas the maximal decrease in C_{dyn} was not significantly changed (Figure 2a). However, changes in both these parameters were much more transient in nedocromil sodium-treated pigs compared to control animals (Figure 1a), returning to baseline at 16 ± 3 min for R_{pulm}

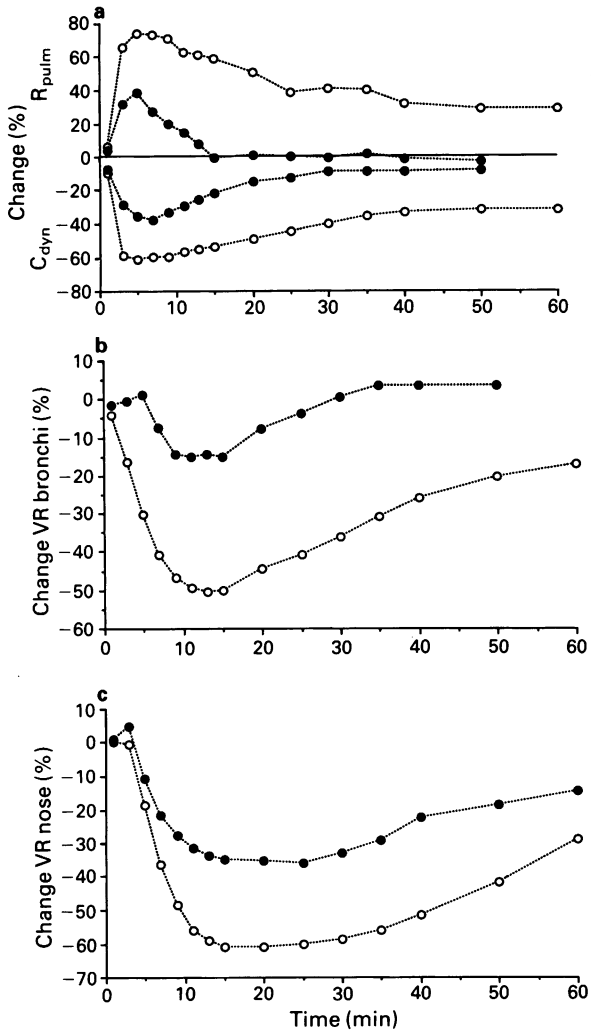

Figure 1 Time course of mean relative changes in (a) R_{pulm} and C_{dyn}, (b) bronchial vascular resistance (VR) and (c) nasal VR upon ascaris (14 mg) aerosol challenge in the lung: (○) control pigs and (●) animals pretreated with nedocromil sodium (80 mg) aerosol in the lung (n = 5–6).

($P < 0.01$) and to within 10% of baseline at 33 ± 11 min for C_{dyn} ($P < 0.01$). Moreover, the maximal decrease in VR in the bronchial circulation in response to allergen challenge was reduced by 58% (Figure 2a) and the vasodilator response was much more transient, lasting for 28 ± 3 min ($P < 0.05$) after nedocromil sodium pretreatment (Figure 1b). The maximal vasodilatation in the nasal circulation upon lung challenge with allergen, however, was not significantly reduced by nedocromil sodium pretreatment (Figure 2a), although the duration of the response was slightly reduced to 56 ± 4 min ($P < 0.05$) (Figure 1c).

Challenge with PAF aerosol (0.6 mg) in the lung induced pulmonary obstruction and vasodilatation in both the bronchial and nasal circulation, lasting for 15–30 min. The increase in R_{pulm}, but not the decrease in C_{dyn}, to PAF aerosol tended to be reduced ($0.05 < P < 0.10$) after nedocromil sodium pretreatment (Figure 2b). Furthermore, nedocromil sodium totally blocked the bronchial vasodilatation induced by PAF aerosol and the vasodilator response in the nasal circulation was reduced by 72% (Figure 2b). Both PAF and ascaris aerosol challenge in the lung caused systemic cardiovascular effects, with increases in MAP and HR (Table 1) peaking at about 5 min and lasting for 10–15 min. Local pretreatment with nedocromil sodium in the lung did not inhibit these systemic changes induced by PAF and allergen (Table 1).

When ascaris aerosol was administered to the nose, a marked vasodilatation lasting for 61 ± 14 min was noticed in

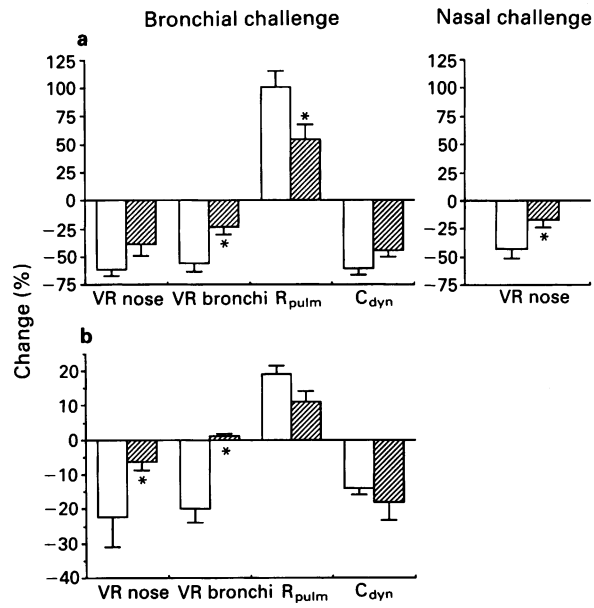

Figure 2 Maximal relative changes in nasal and bronchial vascular resistance (VR), R_{pulm} and C_{dyn} upon (a) ascaris aerosol challenge in the lung (14 mg; left panel) and nose (7 mg; right panel), and (b) PAF (0.6 mg) aerosol challenge in the lung. Open columns represent control pigs and hatched columns animals pretreated locally with nedocromil sodium. Data are presented as mean with s.e.mean shown by vertical bars (n = 5–6). * $P < 0.05$ compared to control (Mann-Whitney).

the nasal circulation (Figure 3). No bronchial or systemic cardiovascular effects were seen upon nasal allergen challenge. Pretreatment with nedocromil sodium i.a. reduced the maximal nasal vasodilatation to allergen by 61% (Figure 2a),

Table 1 Systemic cardiovascular effects (mean arterial pressure (MAP) and heart rate (HR) of allergen and PAF aerosol challenge in the lung

Challenge (pretreatment)	*MAP*	*HR*
Ascaris (control)	12.6 ± 1.5	12.0 ± 3.5
Ascaris (N)	18.9 ± 3.2	19.9 ± 5.1
PAF (control)	12.1 ± 2.6	10.0 ± 3.6
PAF (N)	14.2 ± 6.4	9.5 ± 3.2

Data are presented as change (%) from baseline. Mean ± s.e.mean (n = 5–6). No significant difference between control and nedocromil sodium- (N) treated pigs was noted.

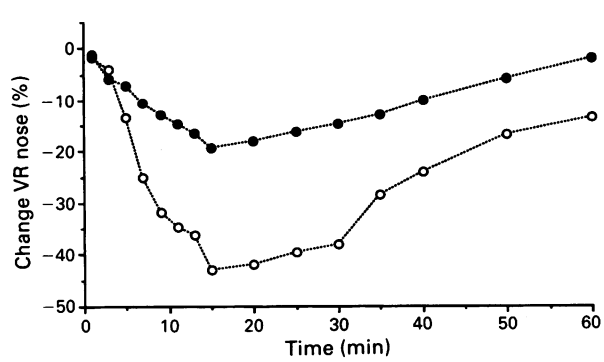

Figure 3 Time course of mean relative changes in nasal vascular resistance (VR) upon ascaris (7 mg) areosol challenge in the nose: (○) control pigs and (●) animals pretreated with nedocromil sodium (50 $\mu g\,kg^{-1}$) i.a. in the nose (n = 5–6).

without significantly changing the duration of the response (56 ± 12 min) (Figure 3).

Capsaicin, histamine and bradykinin challenge

Capsaicin (10 mg) aerosol challenge in the lung and in the nose induced local vasodilatation lasting for about 15 min. Capsaicin aerosol in the lung did not influence the nasal circulation, or *vice versa*, and did not induce any systemic cardiovascular effects or pulmonary obstruction. The vasodilator responses to capsaicin aerosol in the lung and nose were not changed by local nedocromil sodium pretreatment (Figure 4a).

Histamine aerosol (1.5 mg) challenge in the lung produced pulmonary obstruction lasting for about 30 min and bronchial vasodilatation lasting for 10–15 min. Only local responses were seen except in occasional pigs where a transient increase in MAP, HR and nasal blood flow were noticed. Neither of the local bronchial responses were changed by nedocromil sodium pretreatment in the lung (Figure 4b). When histamine aerosol (1.5 mg) was delivered to the nose, vasodilatation lasting for 10–15 min was seen in the nasal circulation. Nedocromil sodium pretreatment locally in the nose inhibited the vasodilator response to nasal challenge with histamine aerosol by 91% (Figure 4b).

Challenge with bradykinin (1.0 mg) aerosol in the lung induced vasodilatation in the bronchial circulation peaking at 3.1 ± 0.3 min and lasting for 10.7 ± 1.6 min, while no bronchoconstriction or systemic cardiovascular effects were seen. Pretreatment with nedocromil sodium in the lung reduced the bronchial vasodilator response to bradykinin aerosol by 59% and systemic pretreatment with capsaicin 2 days before reduced it by 43% (Figure 5a). When bradykinin (0.5 mg) aerosol was delivered to the nose, nasal vasodilatation peaking at 4.8 ± 0.6 min and lasting for 11.5 ± 1.5 min was noticed. Both nedocromil sodium and capsaicin pretreatment markedly reduced this vasodilator response (by 82% and 76%, respectively) (Figure 5a). However, the vasodilatation in the airways after i.v. administration of bradykinin (1 $\mu g\,kg^{-1}$), was not changed by capsaicin pretreatment (Figure 5b). Bradykinin i.v. at this dose also produced some bronchodilatation

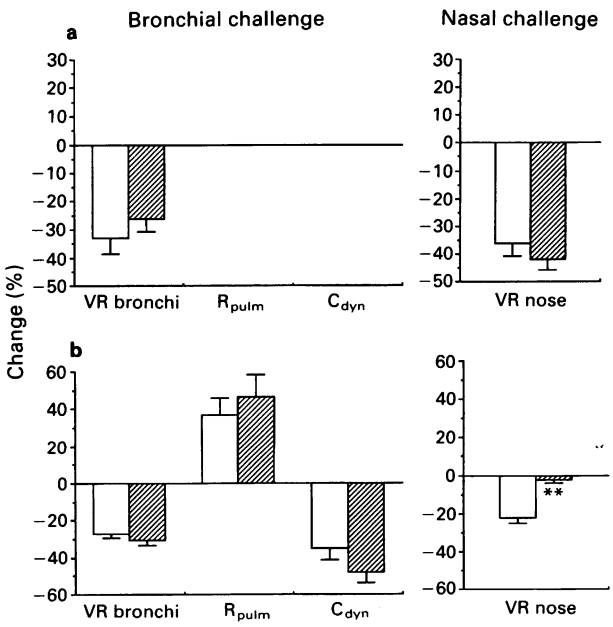

Figure 4 Maximal relative changes in nasal and bronchial vascular resistance (VR), R_{pulm} and C_{dyn} upon (a) capsaicin (10 mg) and (b) histamine (1.5 mg) aerosol challenge in the lung (left panels) and nose (right panels). Open columns represent control pigs and hatched columns animals pretreated locally with nedocromil sodium. Data are presented as mean with s.e.mean shown by vertical bars (n = 7–8). ** $P < 0.01$ compared to control (Mann-Whitney).

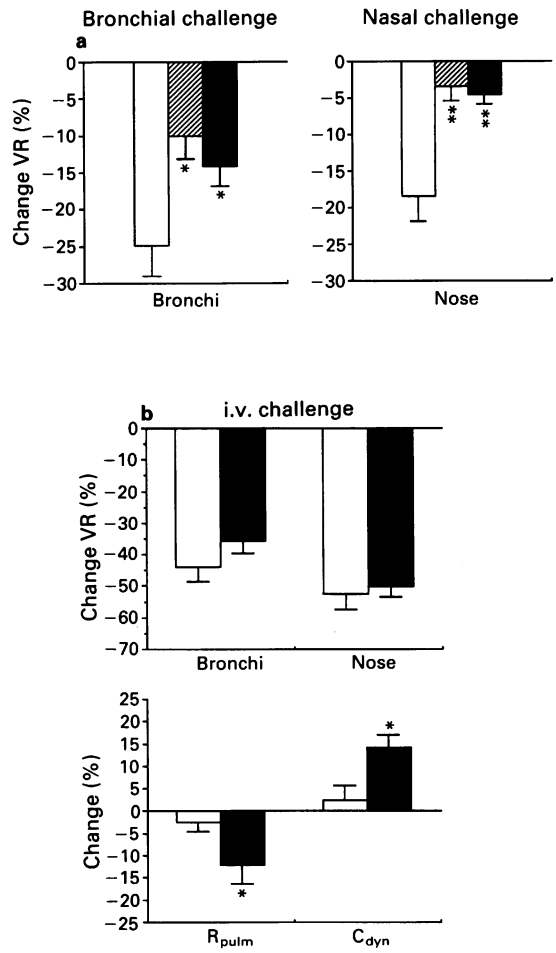

Figure 5 Maximal relative changes in nasal and bronchial vascular resistance (VR), R_{pulm} and C_{dyn} upon (a) bradykinin aerosol challenge in the lung (1.0 mg; left panel) and nose (0.5 mg; right panel), and (b) bradykinin (1 $\mu g\,kg^{-1}$) i.v. injection. Open columns represent control pigs, hatched colums animals pretreated locally with nedocromil sodium and solid columns animals pretreated systemically with capsaicin. Data are presented as mean with s.e.mean shown by vertical bars (n = 7–8). * $P < 0.05$; ** $P < 0.01$ compared to control (Mann-Whitney).

(decrease in R_{pulm} and increase in C_{dyn}), which was increased about 4 to 5 fold in capsaicin-treated animals (Figure 5b).

Discussion

In this study we have examined the effects of nedocromil sodium on vascular and bronchial responses in pig airways, primarily involving either activation of inflammatory cells (e.g. mast cells) with PAF and allergen, or activation of sensory nerves with capsaicin, histamine and bradykinin.

Aerosol challenge with allergen in the lung induced marked bronchoconstriction and vasodilatation in both the bronchial and nasal circulation. The allergen-induced bronchoconstriction and bronchial vasodilatation have been demonstrated to be sensitive to pretreatment with a combination of H_1- and H_2-histamine receptor antagonists, whereas systemic pretreatment with capsaicin mainly affected the bronchial vasodilatation (Alving *et al.*, 1990). The vasodilatation upon allergen challenge, but not the bronchoconstriction, may thus involve activation of sensory nerves. The nasal vasodilator response upon lung challenge with allergen, however, may mainly be caused by a cyclo-oxygenase product entering the systemic circulation since it can be blocked by diclofenac sodium (Alving *et al.*, 1991b). PAF aerosol in the lung produced effects similar to the allergen challenge, including

bronchoconstriction, bronchial and nasal vasodilatation and increases in MAP and HR. The nasal vasodilatation in the pig is consistent with the facial flushing seen in man upon PAF challenge in the lungs (Cuss *et al.*, 1986). PAF seems to have the ability to mimic allergen-induced reactions in human skin (Archer *et al.*, 1984) and lower airways (Barnes & Chung, 1987). This may be caused by the release of arachidonic acid metabolites (Schleimer *et al.*, 1986a) or histamine (Smith *et al.*, 1988) in human airways (see Townley *et al.*, 1989). Allergen challenge in the pig nose also caused nasal vasodilatation of long duration. It was earlier proposed that this effect was partly due to histamine acting through activation of capsaicin-sensitive nerves (Alving *et al.*, 1990). Nasal challenge with PAF was not included in the present study, but local challenge with PAF in the human nose has previously resulted in vasoconstrictor responses which are blocked by the related compound sodium cromoglycate (Karlsson & Pipkorn, 1984).

Pretreatment with nedocromil sodium reduced the increase in local blood flow upon allergen and PAF challenge in the bronchial and nasal mucosae. Furthermore, peak changes in R_{pulm} were reduced, at least upon allergen challenge. The ability of nedocromil sodium to inhibit PAF-induced increases in lung resistance has been demonstrated recently in the sheep (Solèr *et al.*, 1990) and guinea-pig (Pretolani *et al.*, 1990). The inhibitory effect of nedocromil sodium on allergen-induced changes in bronchial VR and R_{pulm} in the pig may be due to its mast cell stabilizing properties (Wells *et al.*, 1986), although nedocromil sodium does not seem to inhibit histamine release from pig isolated lung mast cells induced by anti-pig serum (Leung *et al.*, 1988). The reduction by nedocromil sodium of the nasal vasodilatation induced by lung challenge with PAF but not allergen, suggests that PAF and allergen act via separate mechanisms to induce nasal vasodilatation, although a cyclo-oxygenase product may be the final common mediator (Alving *et al.*, 1991b). In accordance with this, Pretolani *et al.* (1990) showed that nedocromil sodium may inhibit the production of certain arachidonic acid metabolites induced by PAF, but not allergen, in the guinea-pig lung. The residual changes induced by allergen and PAF after nedocromil sodium pretreatment in pigs consisted mainly of increases in MAP and HR as well as some pulmonary obstruction. These changes may, at least in the case of PAF, be due to the action of sulphidopeptide leukotrienes and thromboxanes, which have been shown to be released by PAF from non-mast cell populations in human lung tissue (Schleimer *et al.*, 1986a). Furthermore, it has been demonstrated in the guinea-pig lung that repeated challenges with PAF desensitize against a subsequent allergen challenge but not *vice versa* (Lagente *et al.*, 1988). This indicates that PAF activates other inflammatory cells that are not mast cells and do not respond to allergen. However, such a cell population may still be activated in immediate allergic reactions since PAF seems to be produced in both guinea-pig (Fitzgerald *et al.*, 1986) and human lungs (Schleimer *et al.*, 1986b) upon challenge with antigen or anti-IgE.

A stabilizing effect on sensory nerve function by sodium cromoglycate and nedocromil sodium has been implicated in several studies, including citric acid-induced cough in the dog (Jackson, 1988) and SO_2- (Sheppard *et al.*, 1981; Altounyan *et al.*, 1986), bradykinin- (Barnes, 1986; Fuller *et al.*, 1987) and metabisulphite- (Dixon & Ind, 1990) induced reflex bronchoconstriction in man. However, the reflex bronchoconstriction in man induced by capsaicin, a substance which specifically activates the C-fibre group of sensory nerves (see Buck & Burks, 1986), is not changed by pretreatment with sodium cromoglycate (Fuller *et al.*, 1985) or nedocromil sodium (Hansson *et al.*, 1988). These latter data with capsaicin are in line with the present results, where nedocromil sodium did not inhibit the capsaicin-induced vasodilatation in the mucosa of pig airways.

Histamine aerosol-induced vasodilatation in the pig airways has previously been shown to be at least partly exerted via activation of capsaicin-sensitive mechanisms, especially in the nasal mucosa (Alving *et al.*, 1990). The histamine-induced sensory nerve activation in the airways is probably mediated via H_1-receptors (Secher *et al.*, 1982; Saria *et al.*, 1988). However, the vasodilator response to histamine in the pig bronchial circulation was not affected by an H_1-receptor antagonist, whereas the response in the nasal circulation was reduced. Furthermore, the opposite effect was noticed upon pretreatment with an H_2-antagonist (Alving *et al.*, 1990). This indicates that direct effects by histamine on the vessels via H_2-receptors (which are not sensitive to pretreatment with capsaicin or nedocromil sodium), may be more important in the bronchial than in the nasal mucosa. Alternatively, i.a. pretreatment with nedocromil sodium (nose) may be more efficient than aerosol administration (bronchi) in reaching sensory nerve endings. The bronchoconstriction after histamine aerosol challenge in the pig was not affected by nedocromil sodium pretreatment, which is in accordance with data on capsaicin-treated pigs (Alving *et al.*, 1990).

Bradykinin is another mediator which is thought to be formed during allergic reactions in both the upper (Proud *et al.*, 1983) and lower (Christiansen *et al.*, 1987) airways. In the guinea-pig perfused lung, bradykinin was about a 100 times more potent than histamine in inducing release of tachykinins from capsaicin-sensitive sensory nerves (Saria *et al.*, 1988). The possibility that bradykinin and histamine stimulate different subpopulations of sensory nerves is indicated by the fact that histamine provokes sneezing while bradykinin induces a sore throat upon challenge of human nasal mucosa, and that bradykinin, but not histamine, induces cough when delivered to the lower airways of asthmatics. Sensory nerve activation by bradykinin may be a direct effect via bradykinin BK_2-receptors (Dray & Perkins, 1988), without involving the release of histamine (Proud *et al.*, 1988; Lee & Pearce, 1990) or prostanoids (Fuller *et al.*, 1987; Franco-Cereceda, 1989), at least not to any major extent (Polosa *et al.*, 1990). In the pig airways the vasodilatation induced by bradykinin aerosol was inhibited by capsaicin pretreatment, suggesting dependence on sensory nerve activation. Systemic capsaicin pretreatment of the pig has been demonstrated to reduce markedly the vasodilator response to capsaicin aerosol in the airways (Alving *et al.*, 1990; Matran *et al.*, 1990) and to deplete selectively the mucosal content of calcitonin gene-related peptide and neurokinin A, compared to peptides in sympathetic and parasympathetic nerves which are basically unaffected (Alving *et al.*, 1991a). Bradykinin-induced vasodilatation in the pig bronchial and nasal circulation in the present study was reduced equally by nedocromil sodium and by capsaicin pretreatment, suggesting an inhibitory action on sensory nerves by this anti-asthmatic compound. Bradykinin has been discovered in the nasal fluid during rhinovirus infections (Naclerio *et al.*, 1987) and bradykinin challenge in the human nasal mucosa produces many of the symptoms seen during rhinovirus colds, including a sore throat (Proud *et al.*, 1988). Interestingly, pretreatment with nedocromil sodium markedly reduces both secretion and symptom scores induced by rhinovirus infections in man (Barrow *et al.*, 1990), which thus may be due to inhibition of bradykinin-induced activation of sensory nerves. The vasodilator response in the pig airways to i.v. injections of bradykinin was not affected by capsaicin pretreatment, which is in agreement with previous data on histamine i.v. challenges (Alving *et al.*, 1990). The i.v. bradykinin-induced vasodilatation may, therefore, not involve sensory nerves, but may be the result of a direct effect on the vessels.

The action by nedocromil sodium on the vasodilatation induced by bradykinin, but not that induced by capsaicin, suggests that these agents stimulate sensory nerves via separate mechanisms. It has been suggested that bradykinin activates sensory nerves through pathways involving protein kinase C, whereas capsaicin may not (Dray *et al.*, 1988). Activation of H_1 receptors may also involve phospholipid turnover (and thus protein kinase C) (Subramanian *et al.*, 1980). Capsaicin-induced sensory activation, on the other hand, is inhibited by ruthenium red (Maggi *et al.*, 1988), while

responses to bradykinin are not affected by this inhibitor of transmembrane calcium fluxes (Amann & Lembeck, 1989). Another process, that may involve protein kinase C, is histamine secretion from mast cells (see Chakravarty, 1990). The inhibition of anti-IgE-induced histamine release from rat mast cells by sodium cromoglycate and nedocromil sodium has been shown to be associated with the phosphorylation of a membrane-bound mast cell protein (Wells *et al.*, 1986). Furthermore, the secretion of lysosomal enzyme from neutrophils induced by phorbol ester, which is mediated through protein kinase C, was reduced by nedocromil sodium (Bradford & Rubin, 1986). Taken together, it may be proposed that nedocromil sodium acts on the cell surface to interfere with pathways involving protein kinase C.

Bradykinin and capsaicin aerosol did not produce any bronchoconstriction in the pig, which is in contrast with results from human airways. However, both bradykinin- (Fuller *et al.*, 1987) and capsaicin- (Fuller *et al.*, 1985) induced bronchoconstriction in human subjects are dependent on vagal cholinergic reflexes, and such central reflexes may be inhibited by the pentobarbitone anaesthesia in the pig. When bradykinin was injected i.v. some bronchodilatation was noticed, especially in capsaicin-treated pigs. The mechanism for this effect remains to be established, but is in parallel with what is seen upon capsaicin i.v. challenge in capsaicin-treated pigs (Alving *et al.*, 1991a).

In conclusion, nedocromil sodium reduces the bronchial vasodilatation and increase in R_{pulm} induced by allergen and PAF in the pig, but has only minor effects on peak changes in C_{dyn}, MAP and HR. Bradykinin-induced vasodilatation in the airway mucosa seems to be largely dependent on capsaicin-sensitive sensory nerves. Nedocromil sodium may inhibit sensory nerve activation induced by bradykinin and histamine, but not capsaicin. Thus, nedocromil sodium seems to inhibit both mediator release from certain inflammatory cells and sensory nerve activation induced by endogenous inflammatory mediators.

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Adenosine modulation of potassium currents in postganglionic neurones of cultured avian ciliary ganglia

[1]Max R. Bennett, Rod Kerr & Kerry Nichol

The Neurobiology Research Centre, University of Sydney, Sydney, N.S.W. 2006, Australia

1 Potassium currents in cultured postganglionic neurones of avian ciliary ganglia were analysed under whole-cell voltage clamp and their modulation by adenosine determined.

2 In the presence of tetrodotoxin (200 nM), and with moderate holding potentials (Vh = −40 mV), the steady-state current-voltage (*I/V*) curve was N-shaped over the range from −70 mV to +155 mV. CsCl (1 M) blocked the current, indicating that it was carried by K^+. If Ca^{2+} influx was blocked by $CdCl_2$ (500 μM) then the outward current was reduced and the N-shaped *I-V* curve lost, indicating the presence of a calcium-activated potassium current ($I_{K(Ca)}$); the remaining current, due to the delayed rectifier (I_K), increased with depolarization up to about a conductance of 10 nS near +50 mV. This I_K was 50% activated at about +20 mV and 50% inactivated at about −50 mV. Adenosine (10 μM) had similar affects on the N-shaped *I/V* curve as did $CdCl_2$, indicating that it blocked $I_{K(Ca)}$. However, adenosine had little affect on the steady-state current in the presence of $CdCl_2$, indicating that it did not much affect I_K.

3 In the presence of tetrodotoxin (200 nM), a large inward current occurred for large hyperpolarizations from a Vh = −50 mV. This inward rectifying current (I_{IR}) had a reversal potential near E_K and showed 50% activation at about −130 mV. Adenosine (10 μM) reduced I_{IR}, by as much as 50% at large hyperpolarizations beyond −80 mV.

4 Relaxations of the outward current on hyperpolarization from Vh = −30 mV were blocked by carbachol (10 μM), had a reversal potential near E_K, and an *I/V* curve typical of I_M currents. These currents were little affected by adenosine (10 μM).

5 A fast transient outward current, due to depolarizing pulses from a large Vh = −110 mV was observed in the presence of tetrodotoxin (200 nM). This had the characteristics of an I_A current as it could be blocked with 4-aminopyridine (5 mM) and was 50% activated at about −20 mV and 50% inactivated at about −94 mV. The I_A current was reduced by 42% at a depolarization of −20 mV by adenosine (10 μM).

6 Many neurones possessed a fast transient outward current that was blocked by tetrodotoxin (200 nM). This current could be blocked with 4-aminopyridine (5 mM); it therefore has the characteristics of a sodium-activated potassium current ($I_{K(Na)}$). This $I_{K(Na)}$ was unaffected by adenosine (10 μM).

7 These results are discussed in relation to the role of adenosine in blocking Ca^{2+} channels and thereby modifying calcium-dependent components of K^+ currents.

Keywords: Synapse; potassium current; ciliary neurones; adenosine; postganglionic neurones; Ca^{2+} channels

Introduction

Endogenous adenosine modulates secretion at synapses (Phillis & Wu, 1981), by acting as an autoinhibitory agent which reduces both the mean quantal content of the synaptic potential and its variance (Ribeiro & Sebastiao, 1987; Bennett *et al.*, 1991). The avian ciliary ganglion provides an opportunity to elucidate the mechanism of action of adenosine as transmission is modulated by endogenous purine nucleotides at this synapse (Bennett & Ho, 1991). Furthermore the synapse possesses giant calyciform nerve terminals that are accessible to single electrode voltage clamp (Martin & Pilar, 1963). Such studies have shown that adenosine hyperpolarizes nerve terminals, suggesting that its action increases the outward potassium currents (Bennett & Ho, 1991). Adenosine hyperpolarizes atrial cells by activating I_M potassium channels via GTP-binding proteins (Kurachi *et al.*, 1986). It also hyperpolarizes both hippocampal and striatal neurones through a Ca-independent I_M type K^+ channel that requires guanosine 5'-triphosphate for activation (Trussell & Jackson, 1987). In addition, adenosine increases a 4-amino pyridine-sensitive potassium current in hippocampal neurones (Gerber *et al.*, 1989). In the present work cultured ciliary neurones have been used to analyse the action of adenosine on the large number of macroscopic K^+ currents which are found in these neurones.

[1] Author for correspondence.

Methods

Tissue culture

White leghorn chicks were used for all experiments. Ciliary ganglia from 7–9-day old chick embryos were dissected free in Ca^{2+}, Mg^{2+}-free Hanks Balanced Salt Solution containing 0.3% bovine serum albumin (BSA), and incubated at 37°C for 30 min. No proteolytic enzymes were used. The supernatant was removed and 1 ml of Dulbecco's Modified Eagles' Media (DMEM, Flow, Sydney) supplemented with 2 mM glutamine, 1.00 u ml^{-1} penicillin, 50 $\mu g\,ml^{-1}$ streptomycin, 10% heat-inactivated horse serum (CSL) and 2% chick embryo extract was added. The ganglia were then mechanically dissociated by repeated trituration through a Pasteur pipette. The resultant single cell suspension was plated onto 13 mm glass coverslips previously coated with poly-L-lysine in 24-well plates (Corning) and maintained at 37°C in a 5% CO_2 in air water-saturated atmosphere until required. Neurones were used for whole-cell recording within 12 to 24 h after plating, when they have few processes. These cells were chosen to minimize difficulties involving spatial non-uniformities of membrane potential arising from inadequate space clamp; if these occurred the results were excluded.

Solutions

Electrodes for forming gigaohm seals with the cell membranes of ciliary neurones were fabricated from boro-silicate capillary

tubing and had resistances of 0.5 to 2.0 MΩ when filled with internal solution (Hamill *et al.*, 1981). For the whole-cell recording presented in this work, the pipette solution consisted of (mM): KCl 115, $MgCl_2$ 1, HEPES 40, GTP 2, ATP 1 with KOH added to give a pH of 7.3. The bathing solution consisted of (mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 10, tetrodotoxin 200 nM and NaOH added to give a pH of 7.3. Variations on these solutions involved addition of KEGTA to the pipette solution, addition of $CdCl_2$ to the bath solution or omission of tetrodotoxin or $CaCl_2$ from the bath solution. Such changes are noted in the appropriate legends. All experiments were at room temperature, about 21°C.

Recording

Whole cell patch clamp techniques were used (Fenwick *et al.*, 1982) to voltage-clamp cells. Prior to forming seals the pipette current due to liquid junction potentials within the electrode and bath was nulled and the pipette capacitance compensated. After establishing a seal, the patch membrane was broken under slight negative pressure by a short (0.5 ms) 1.5 V pulse. This negative pressure, which delayed the onset of loss of contact with the cytoplasm, was maintained throughout the course of the experiment. Ion currents were recorded via an Axopatch IC amplifier and a labmaster interface on an IBM-AT using pClamp (Axon Instruments) software. Currents filtered at 2 kHz, were sampled at 0.5 to 6.7 kHz depending on the duration of the voltage clamp command.

Records presented in the figures have had periods of less than 2 ms at the onset and offset of the command pulses removed to eliminate spurious points due to incomplete capacitance compensation. If there were indications of incomplete voltage clamping, due to inadequate space clamp, then the results were rejected. Voltage-clamp control was determined according to several criteria: firstly, smooth voltage-dependent current activation; secondly, lack of excessive delay in onset of current; thirdly, onset and offset kinetics that were dependent on voltage but not on the amplitude of the current. Isopotentiality of the neuronal membrane was determined by testing the exponentiality of the capactive transient.

Adenosine application

Micropipettes with tip diameters of about 10 μm were used to deliver the drug solutions. The pipettes were filled with bath solution together with adenosine to a final concentration of 10 μM to 15 μM. The tips of the micropipettes were positioned 20 to 50 μm from the neurone cell body and adenosine pressure ejected at 5 kPa. During application of the adenosine, currents were evoked every 20 s. Neurones were required to show stable current magnitudes for at least 2 min before application of the adenosine, otherwise the results were rejected.

Drugs

The drugs used and obtained from Sigma, were: tetrodotoxin, tetraethylammonium chloride, adenosine and 8-phenyltheophylline.

Statistical analysis

Values are expressed as mean ± s.e. and n indicates the number of neurones.

Results

The delayed rectifier (I_K) and calcium-activated potassium currents ($I_{K(Ca)}$): effects of adenosine

Depolarizing voltage-command steps of 470 ms duration from a holding potential (Vh) of −40 mV gave outward currents of the kind shown in Figure 1a in all cultured ciliary neurones studied ($n = 20$ neurones). The current at the end of the command step increases up to depolarizations of about +60 mV; thereafter the current declines to reach a minimum at about +100 mV from which it increases again (Figure 1b). This N-shaped current-voltage curve is typical of that for neurones in which a large component of the outward current passes through calcium-activated potassium channels ($I_{K(Ca)}$): as $I_{K(Ca)}$ decreases markedly for depolarizing commands close to the calcium equilibrium potential, a minimum is reached near this potential on the current–voltage relationship (see for example Hermann & Gorman, 1981; Galvan & Sedlmeir, 1984).

The delayed rectifier currents (I_K) were separated from $I_{K(Ca)}$ by blocking the influx of Ca^{2+} ions with 500 μM Cd^{2+}. Figure 2a shows that most of the outward current was blocked by the addition of Cd^{2+}, indicating that most of this current is carried by $I_{K(Ca)}$. The current-voltage relation for the Cd^{2+}-sensitive current and the Cd^{2+}-insensitive current (Figure 2b) show that the Cd^{2+}-sensitive current declines to less than 1 nA about the expected calcium-equilibrium potential (near +100 mV) whereas the Cd^{2+}-insensitive current increases continually with depolarization. If the Cd^{2+}-insensitive current is taken as I_K, then the steady-state conductance for the I_K channel increased from −50 mV depolarization to reach a value of about 8.5 nS (±1.4 nS, $n = 3$) at +50 mV (Figure 2c).

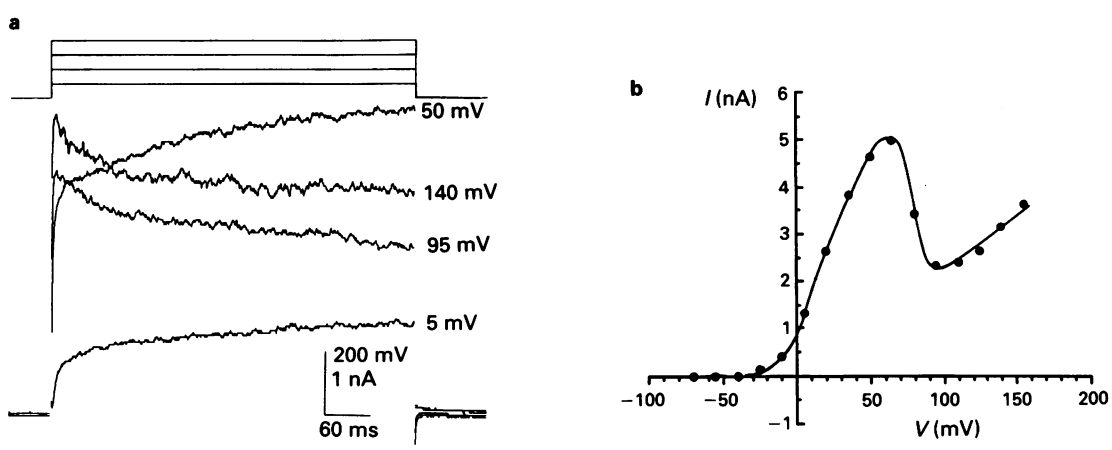

Figure 1 (a) Shows a set of voltage-clamp currents for a ciliary neurone during command pulses at 470 ms duration to the different levels indicated by the rectangular traces; the holding potential was −40 mV; (b) shows the current–voltage relation determined from the currents at the end of the traces for the neurone in (a).

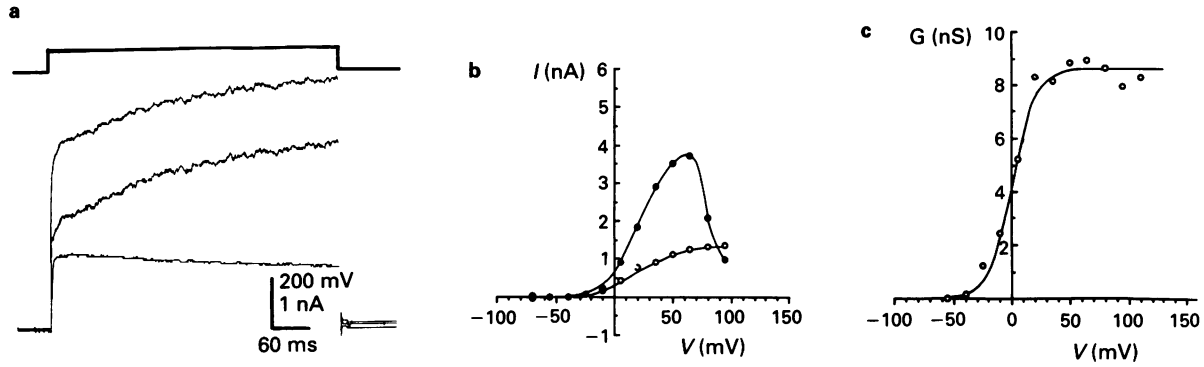

Figure 2 Separation of the total delayed outward current in a ciliary neurone into delayed rectifier (I_K) and calcium-activated potassium ($I_{K(Ca)}$) components: (a) shows the voltage-clamp currents for a ciliary neurone during a depolarizing command pulse of 470 ms duration (indicated by the rectangular trace) before (upper trace) and during the application of Cd^{2+} (500 μM: lower trace) by pressure ejection; holding potential is −40 mV; the remaining (middle) trace gives the difference between the currents before and after Cd^{2+}; (b) gives the current-voltage relation for the current remaining after addition of Cd^{2+} (○, I_K) as well as the current-voltage relation for the current due to subtracting that remaining after Cd^{2+} from the control current (●; $I_{K(Ca)}$). In (c) the steady-state conductance (G_k) for the I_K in (b) according to $G_k = I_K (V - V_K)$, where $V_K = -75$ mV is shown; the curve has been drawn according

to $G_k = G_k\left\{1 + \exp\frac{Ze}{kT}(Vo - V)\right\}^{-1}$ in which $G_k = 8.6$ nS, Vo = 0 mV, Z = 2.5, $\frac{Ze}{kT} = \frac{Zf}{RT} = 0.1$.

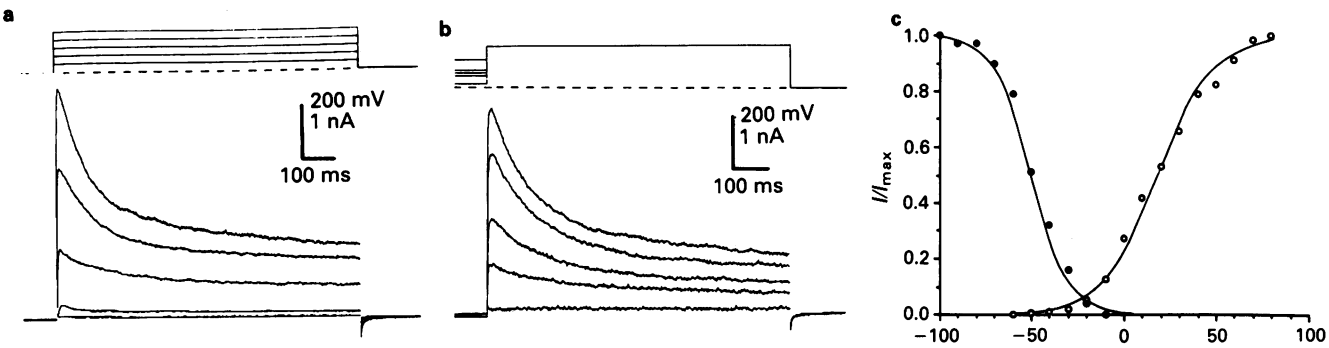

Figure 3 Activation and inactivation of the delayed rectifier current (I_K): (a) gives a set of voltage-clamp currents for the same ciliary neurone during depolarizing commands indicated by the rectangular traces from a holding potential of −80 mV; (b) shows a set of voltage-clamp currents during a depolarizing command to +50 mV from different holding potentials (indicated by the rectangular traces). In (c) the activation curve for the steady-state current (at 940 ms) for this neurone (○; holding potential Vh = −80 mV) is shown, derived from the results in (a), as well as an inactivation curve (●; depolarization to +50 mV) derived from the results in (b); the graph shows the respective current values (I) each normalized to their maximum saturated values (I_{max}). The inactivation curve has been drawn according to $h = (1 + \exp(V - Vh)/V^*)^{-1}$ in which Vh (the potential for h of 0.5) is −50 mV and V* is +10 mV. The activation curve has been drawn according to $h = (1 + \exp(Vh - V)/V^*)^{-1}$ in which Vh is +17 mV and V* is +15 mV. Cd^{2+} (200 μM) present throughout to block $I_{K(Ca)}$. There was no Ca^{2+} in the bath solution and KEGTA (10 mM) substituted for KCl (10 mM) in the pipette solution.

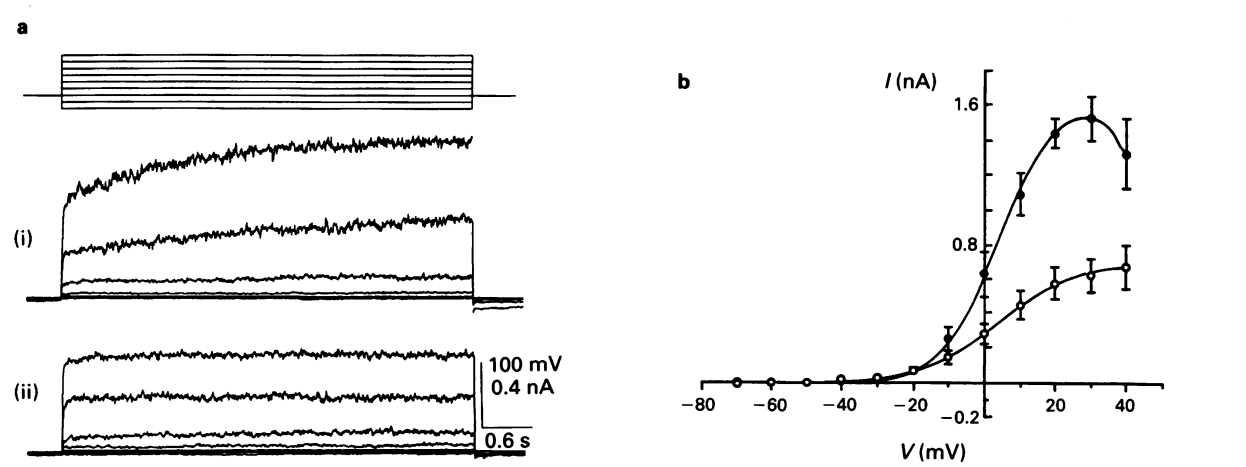

Figure 4 Effects of adenosine on the calcium-activated potassium current $I_{K(Ca)}$. In (a) is shown a set of voltage-clamp currents in a ciliary neurone during command pulses of 5 s duration to the different levels indicated by the rectangular traces from a holding potential of −50 mV both (i) before and (ii) after the addition of adenosine (15 μM) to the bath. In (b) are shown the current-voltage relation determined from the currents at the end of the traces in (a) for control (●) and in the presence of adenosine (○); vertical bars give ±s.e.mean (n = 5 cells).

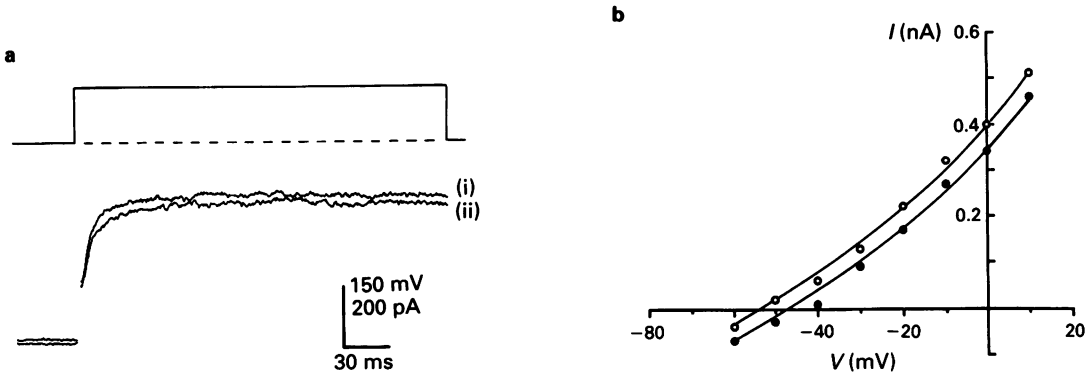

Figure 5 Effects of adenosine on the delayed rectifier current I_K. In (a) are shown voltage-clamp currents in a ciliary neurone during depolarizing command pulses of 250 ms duration to −20 mV from a holding potential of −110 mV before (ii) and during (i) the addition of adenosine (10 μM) by pressure ejection; (b) shows the current-voltage relation determined from the currents at the end of the traces like those for the cell in (a) for control (●) and in the presence of adenosine (○). There was no Ca^{2+} in the bath solution and EGTA (10 mM) substituted for KCl (10 mM) in the pipette solution.

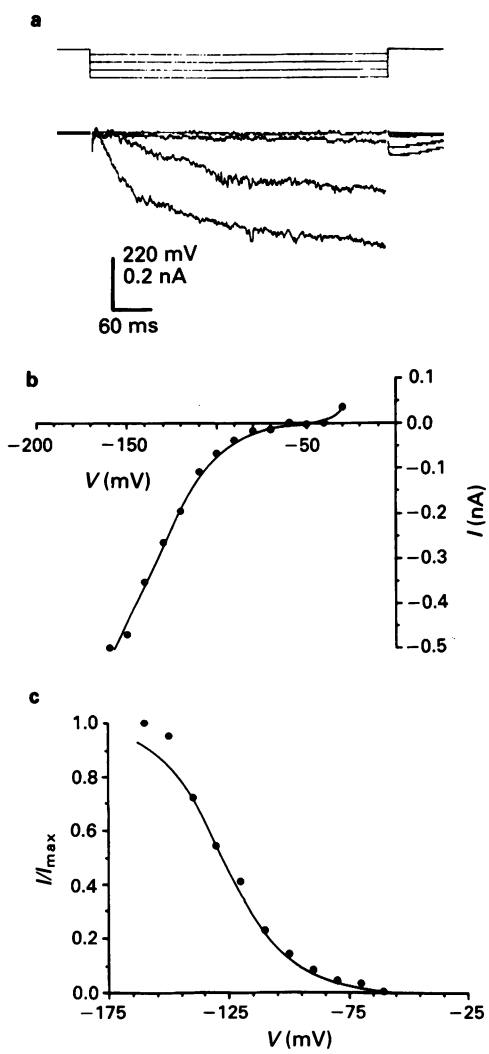

Figure 6 The presence of inward rectifier currents (I_{IR}) in ciliary neurones; (a) a set of voltage-clamp currents in a ciliary neurone during hyperpolarizing command pulses of 470 ms duration to the different levels (mV) indicated by the rectangular traces; the holding potential was −50 mV. The current-voltage relation is shown in (b) determined from the currents at the end of the traces for the neurone in (a). The activation curve for the steady-state current derived from the results in (a) is shown in (c); the graph shows the respective current values (I) each normalized to their maximum saturated values (I_{max}); the curve has been drawn according to $h = (1 + \exp(V - Vh)/V^*)^{-1}$ in which Vh is −127 mV and V^* is = 14 mV. $CdCl_2$ (200 μM) but no Ca^{2+} was present in the bath solution; KEGTA (10 mM) substituted for KCl (10 mM) in the pipette solution.

The activation and inactivation of the steady-state Cd^{2+}-insensitive current, I_K was determined for a cell in which large currents were generated (Figure 3a). Successively larger command depolarizations from a holding potential of −80 mV gave successively larger currents up to about +70 mV when saturation was reached; 50% activation occurred at about +20 mV (±6 mV, $n = 3$) (Figure 3c). An inactivation curve was also determined for this steady-state current by using a depolarizing command of +50 mV from successively more positive holding potentials from −100 mV (see Figure 3b). Figure 3c shows that maximum currents are obtained for holding potentials more negative than −80 mV and that 50% inactivation occurs at about −50 mV (±8 mV, $n = 3$).

Adenosine (10 μM) had similar effects on the steady-state current generated by 5 s depolarizing pulses from moderate-size holding potentials in the 5 neurones studied as did the addition of Cd^{2+} (compare Figure 4a with 2a). Thus the slow increase in outward current in about the first second following depolarization, which is due to $I_{K(Ca)}$, was removed by adenosine (Figure 4b). The current-voltage relation then reverts to that for I_K in the presence of adenosine and is, therefore, similar to that in the presence of Cd^{2+} (compare Figure 4 current-voltage relation with that in Figure 2b). Adenosine had very little effect, if any, on the delayed rectifier, I_K (Figure 5a); this current was increased by, at the most, 40 pA (±10 pA, $n = 5$) over the voltage range from −60 mV to +10 mV (Figure 5b).

Inward rectifier and carbachol-sensitive potassium currents: effects of adenosine

Large hyperpolarizing command voltages from a Vh of −50 mV gave very large inward currents (Figure 6a), indicating the existence of an anomalous inward potassium rectifier (I_{IR}) in all neurones studied. The current-voltage curves for this channel showed a steep rise in the inward current for hyperpolarizations beyond about −90 mV (Figure 6b). Extrapolation of the linear part of this curve intersected the voltage axis at about the potassium reversal potential (Figure 6b). An activation curve for this current showed that 50% activation occurred at −127 mV (±20 mV, $n = 4$) (Figure 6c). Adenosine decreases the currents through the inward rectifier (Figure 7a) by amounts which increase with hyperpolarization in the 4 neurones examined (Figure 7b), so that at about −100 mV over 50% of the current has been reduced.

Potassium currents that are blocked by the addition of carbachol (10 μM) were also present in all ciliary neurones (Figure 8a). These I_M currents can be observed as outward relaxations during hyperpolarizing steps from a holding potential of about −30 mV (Figure 8a). The I_M current, measured as the

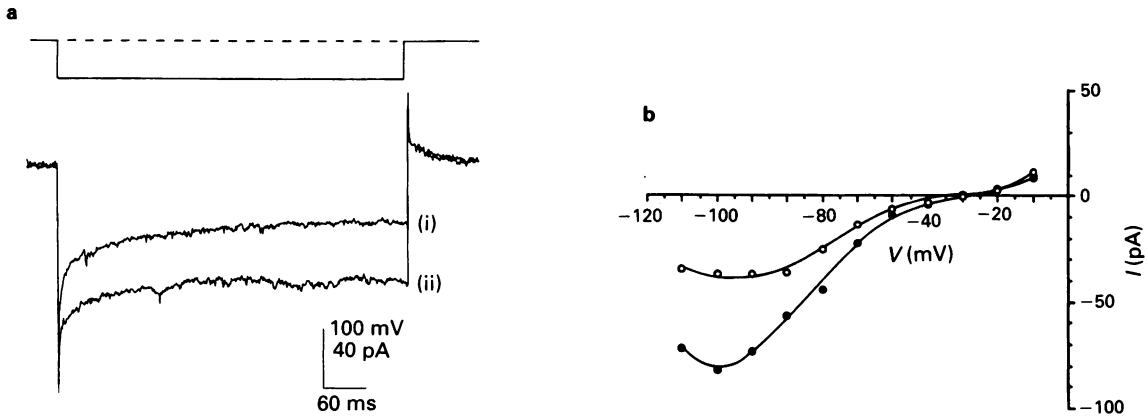

Figure 7 Effects of adenosine on the inward rectifier current I_{IR}: (a) shows voltage-clamp currents during hyperpolarizing command pulses of 490 ms duration to −90 mV from a holding potential of −30 mV before (ii) and during (i) the addition of adenosine (10 μM) by pressure injection. The current-voltage relation is shown in (b) determined from the currents at the end of the traces like those for the cell in (a) for control (●) and in the presence of adenosine (○). There was no Ca^{2+} in the bath solution and KEGTA (10 mM) substituted for KCl (10 mM) in the pipette solution.

difference between the initial current (I_{in}) and steady-state current (I_{SS}) during the hyperpolarizing steps, is relatively unaffected by adenosine ($n = 4$ neurones; Figure 8b).

Transient I_A and sodium-activated potassium currents: effects of adenosine

In the presence of tetrodotoxin (200 nM), an early transient outward current generated by depolarizing pulses to about −35 mV from large holding potentials of about −110 mV is observed in some cells (8 out of 13 neurones; Figure 9a). This current has the inactivation characteristics of the I_A current (Figure 9b) and is blocked by 4-aminopyridine (Figure 9a). Adenosine (10 μM) decreased this current by 42% (±8%, $n = 5$; Figure 9c). Another early transient outward current can also be observed in some cells (7 out of 13 neurones) but only in the absence of tetrodotoxin (Figure 10). This transient was eliminated by 4-aminopyridine. Adenosine had no affect on this sodium activated potassium current ($I_{K(Na)}$) in the 7 neurones in which it was observed.

Discussion

This work shows that cultured postganglionic neurones from nine-day embryonic avian ciliary ganglia possess a large number of different potassium channel types. The N-shaped current-voltage curve for the quasi steady-state current observed at depolarizations from a moderate holding potential after about 500 ms was due to co-activation of two different potassium currents, as is the case in rat sympathetic neurones (Galvan & Sedlmeir, 1984) and molluscan neurones (Hermann & German, 1981). One of these was a delayed rectifier current (I_K), having similar characteristics to that of I_K in sympathetic neurones (Galvan & Seldmeir, 1984), which inactivates over seconds. The other component of the N-shaped current-voltage curve was due to a calcium-activated potassium channel ($I_{K(Ca)}$), as it could be removed by blocking the inward flux of calcium ions with $CdCl_2$. This current had similar characteristics to those of $I_{K(Ca)}$ in bullfrog sympathetic ganglia, in that it activated soon after a depolarizing command (Lancaster & Pennefather, 1987). The channels responsible for $I_{K(Ca)}$ have been identified directly in ciliary neurones by patch-clamp methods (Gardner, 1986), as BK^+ or maxi-K^+ channels (Latorre *et al.*, 1989) and have a large conductance (193 pS) as well as an absolute requirement for $[Ca^{2+}]_i$. Adenosine considerably reduces $I_{K(Ca)}$, probably as a consequence of reducing the inward flux of calcium ions in the same way as adenosine reduces $I_{K(Ca)}$ in dorsal root ganglion cells (Scott & Dolphin, 1987). $I_{K(Ca)}$ contributes significantly to the after-hyperpolarization of the action potential in hippocampal neurones (Wong & Prince, 1981) and to the repolari-

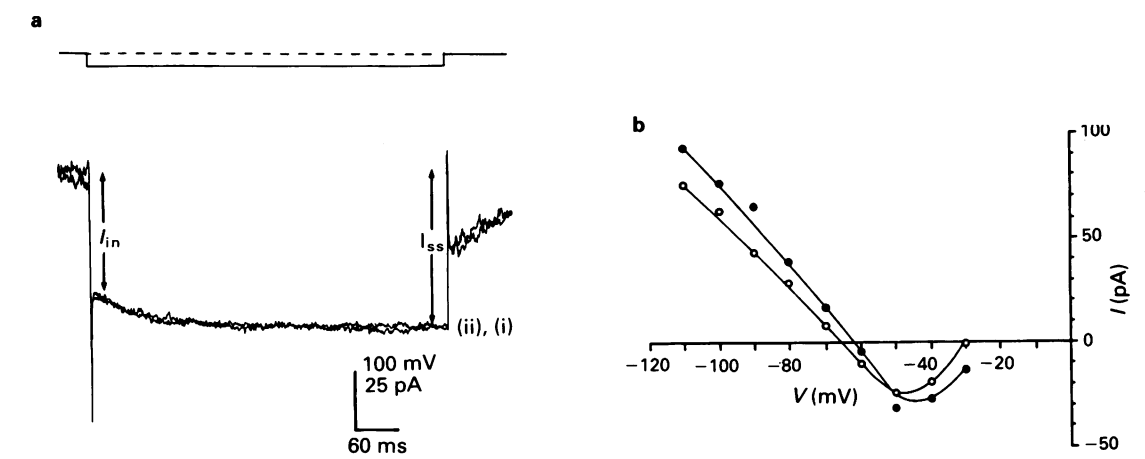

Figure 8 Effects of adenosine on the I_M current: (a) shows voltage-clamp currents during hyperpolarizing command pulses of 490 m duration to −50 mV from a holding potential of −30 mV before (ii) and during (i) the addition of adenosine (10 μM) by pressure ejection. In (b) is shown the current-voltage relation determined from the I_M currents measured as the slow relaxation at the beginning of the pulse ($I_{SS} - I_{in}$); (●) control I_M and (○) I_M in the presence of adenosine. There was no Ca^{2+} in the bath solution and EGTA (10 mM) substituted for KCl (10 mM) in the pipette solution.

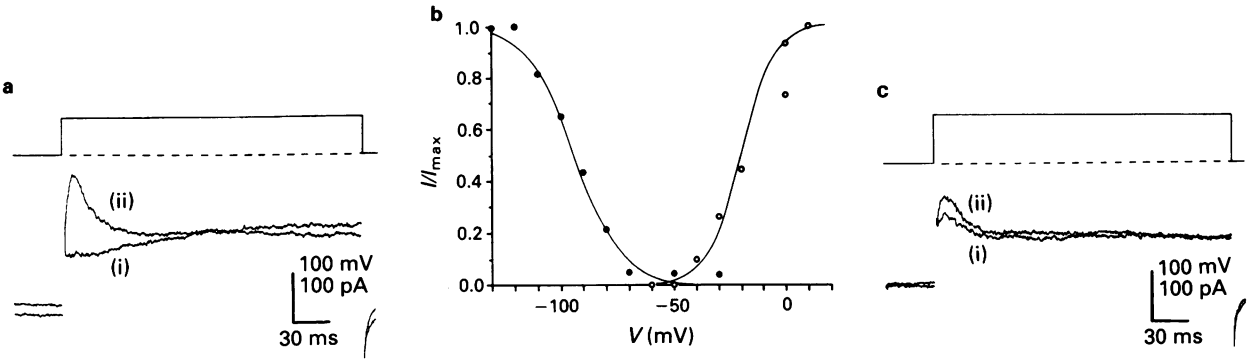

Figure 9 Effects of adenosine on the I_A current. In (a) are shown voltage-clamp currents during depolarizing command pulses of 350 ms duration to −35 mV from a holding potential of −110 mV before (ii) and during (i) the addition of 4-aminopyridine (5 mM) by pressure ejection. Inactivation and activation curves are shown in (b) for the fast transient current derived from results for the cell in (a); the activation graph (●) shows the respective current values (I) each normalized to their maximum saturated values (I_{max}); the curve has been drawn according to $h = (1 + \exp(Vh - Vh)/V^*)^{-1}$ in which Vh is −94 mV and V* is +10 mV; the activation graph (○) shows the respective current values (I) each normalized to their maximum saturated values (I_{max}); the curve has been drawn according to $h = (1 = \exp(Vh - V)/V^*)^{-1}$ in which Vh is −20 mV and V* is +7.5 mV. In (c) are shown voltage-clamp currents during depolarizing command pulses of 300 ms duration to −20 mV from a holding potential of −120 mV before (ii) and after (i) the addition of adenosine (10 μM) by pressure ejection. There was no Ca^{2+} in the bath solution and EGTA (10 mM) substituted for KCl (10 mM) in the pipette solution.

zation of the action potential in sympathetic neurones (Lancaster & Pennefather, 1987). The after-hyperpolarization in hippocampal neurones is greatly reduced by noradrenaline acting to block $I_{K(Ca)}$ (Madison & Nicoll, 1986; Lancaster & Adams, 1986), which adenosine might be expected to do on ciliary neurones.

An anomalous or inward rectifying potassium current (I_{IR}) is present in all the cultured ciliary neurones becoming activated at hyperpolarizing levels near −70 mV, like that of I_{IR} in guinea-pig olfactory cortical neurones (Constanti & Galvan, 1983). Another outward current was revealed during hyperpolarizing steps from a depolarized holding potential. This was distinguishable from the anomalous rectifier by its kinetics and less negative activation range; in addition it was blocked by carbachol and can therefore be identified as an I_M current (Adams *et al.*, 1982; Brown *et al.*, 1982). Adenosine reduced I_{IR} by up to 50% at large hyperpolarizations. This may be contrasted to the effects of opioids in some neurones, which is to increase I_{IR} (Loose & Kelly, 1990). Adenosine had little effect on I_M.

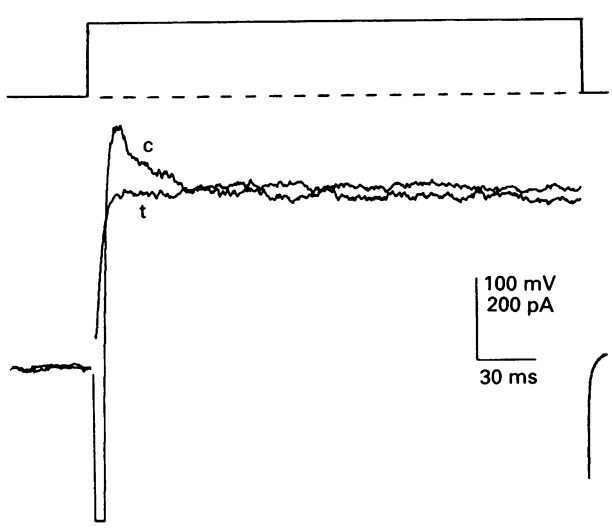

Figure 10 The presence of sodium activated potassium currents $I_{K(Na)}$ in ciliary neurones. Shown are voltage-clamp currents during depolarizing command pulses of 250 ms duration to −20 mV from a holding potential of 110 mV before (c) and during (t) blocking the entry of sodium ions with tetrodotoxin (200 nM) by pressure ejection.

Two fast outward transient currents, peaking in less than 5 ms and then decaying with a time constant of about 20 ms, were also observed for depolarizing steps from large holding potentials. These transients could be distinguished from each other as tetrodotoxin blocked one of them completely, indicating that it was a sodium-activated potassium channel ($I_{K(Na)}$; Hartung, 1985; Dryer *et al.*, 1989). The tetrodotoxin-insensitive transient, which was blocked by 4-aminopyridine and had an activation range from −50 mV to 0 mV, had the characteristics of an I_A current, although somewhat faster than the I_a current transients observed in other excitable cells such as hippocampal neurones (Segal *et al.*, 1984; Zbicz & Weight, 1985). In some neurones only the $I_{K(Na)}$ was observed and in others only I_A, whereas a few neurones possessed both $I_{K(Na)}$ and I_A. Adenosine did not affect $I_{K(Na)}$ but it consistently reduced I_A, in much the same way as it does a similar transient potassium current in dorsal root ganglion cells (Scott & Dolphin, 1987), but in contrast to the effects of adenosine in increasing I_A in hippocampal neurones (Gerber *et al.*, 1989). The rate of action potential firing is regulated by I_A and this rate is higher in tonic-firing sympathetic neurones than in phasic-firing sympathetic neurones (Cassell *et al.*, 1986). It will be of interest to see if adenosine derived from endogenously released adenosine 5'-triphosphate at preganglionic nerve terminals in ciliary ganglia (Bennett & Ho, 1990), has a role in modulating impulse traffic in tonic-firing ciliary neurones.

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Microinjections of 5-HT_{1A} agonists into the dorsal motor vagal nucleus produce a bradycardia in the atenolol-pretreated anaesthetized rat

Simon C.E. Sporton, Sara L. Shepheard, *David Jordan, & [1]Andrew G. Ramage

Academic Departments of Pharmacology and *Physiology, Royal Free Hospital School of Medicine, Rowland Hill St., Hampstead, London NW3 2PF

1 The effects of microinjections (100 nl) into the dorsal motor vagal nucleus of the 5-HT_{1A} receptor agonists 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and flesinoxan, the 5-HT_2 receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), the 5-HT_3 receptor agonist phenylbiguanide (PBG), the α_2-adrenoceptor agonist clonidine and the excitatory amino acid glutamate on heart rate, blood pressure, tracheal pressure and phrenic nerve activity were investigated in atenolol-pretreated rats anaesthetized with sodium pentobarbitone.

2 Microinjections of glutamate (2.5 nmol) caused decreases in blood pressure, heart rate and phrenic nerve activity. In contrast, microinjections of 5-HT (1.2 nmol), 8-OH-DPAT (1.2 nmol) and flesinoxan (1.3 nmol) all caused a bradycardia but had no effect on blood pressure. In addition, 8-OH-DPAT and flesinoxan caused an increase in phrenic nerve activity.

3 Microinjections of DOI, PBG and clonidine had no significant effect on any of the variables recorded. None of the drugs used had any significant effect on tracheal pressure.

4 These results support the hypothesis that activation of 5-HT_{1A} receptors causes excitation of cardiac vagal motoneurones and suggest that these receptors are also important in the control of central respiratory drive.

Keywords: Cardiac vagal motoneurones; phrenic nerve activity; 5-HT_{1A} receptors; 8-OH-DPAT; flesinoxan; brainstem microinjections; anaesthetized rats

Introduction

It has previously been demonstrated that intravenous injections of the 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonists, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), ipsapirone and flesinoxan in the anaesthetized cat cause a large increase in central vagal drive to the heart (Ramage & Fozard, 1987; Ramage *et al.*, 1988). This led to the suggestion that central 5-HT pathways, via 5-HT_{1A} receptors, are involved in the control of vagal tone to the heart. In the cat, the cardiac vagal motoneurones (CVMs) are located mainly in the nucleus ambiguus (see Hopkins, 1987) and microinjection of 8-OH-DPAT into this nucleus produces a bradycardia (Izzo *et al.*, 1988). In addition, autoradiographic studies have shown 8-OH-DPAT binding sites in the nucleus ambiguus (Dashwood *et al.*, 1988) and immunohistochemical studies have demonstrated that 5-HT-immunoreactive boutons make synaptic contact with CVMs in this nucleus (Izzo *et al.*, 1988). Together, these observations strongly support the view that in the cat, there is an excitatory 5-HT-containing pathway that innervates cardiac vagal motoneurones and involves the activation of 5-HT_{1A} receptors.

There is similar evidence for such a pathway in the rat. 5-HT immunoreactivity has been demonstrated in the nucleus ambiguus and the dorsal motor vagal nucleus (DMVN) (Steinbusch, 1981). Both regions contain CVMs in this species (Nosaka *et al.*, 1982) and have been demonstrated to contain 5-HT_{1A} binding sites (Pazos & Palacios, 1985; Manaker & Verderame, 1990). Both intravenous administration of 5-HT_{1A} receptor agonists (Gradin *et al.*, 1985; Cherqui *et al.*, 1988) and intracerebroventricular administration of 5-HT (Dalton, 1986) produces a bradycardia which has been shown to be partly due to an increase in central vagal drive. However, Wolf *et al.* (1981) demonstrated variable changes in heart rate when 5-HT was microinjected into the NTS/DMVN region.

Thus the aim of the present study was to determine by microinjecting the selective and equipotent 5-HT_{1A} receptor agonists 8-OH-DPAT (Middlemiss & Fozard, 1983) and flesinoxan (Wouters *et al.*, 1988; Wijngaarden *et al.*, 1990) into the DMVN whether 5-HT acting via 5-HT_{1A} receptors plays a role in the control of CVMs in the rat. Excitation of CVMs was assessed by measuring changes in heart rate in atenolol-pretreated anaesthetized rats. Atenolol was chosen to block the effects of any changes in sympathetic drive to the heart caused by microinjections of these agonists, as it is a β-adrenoceptor antagonist which does not bind to 5-HT receptors (Middlemiss *et al.*, 1977) and poorly penetrates the central nervous system (Street *et al.*, 1979). Furthermore, as 5-HT_2 (Pazos *et al.*, 1985), 5-HT_3 (See Pratt *et al.*, 1990) and α_2-adrenoceptor (Unnerstall *et al.*, 1984) binding sites have also been demonstrated in the region of the DMVN in the rat, the effects of microinjections of selective agonists at these receptors were also investigated. Finally, the effects of microinjection of these agonists on phrenic nerve activity, a measure of central inspiratory drive, were also investigated since this drive is known to modulate cardiovascular responses and the excitability of CVMs (see Daly, 1986) and 5-HT pathways have been demonstrated to be involved in central respiratory control (Holtman *et al.*, 1986; 1990).

A preliminary account of some of these observations has been given (Sporton *et al.*, 1989).

Methods

Experiments were performed on male Sprague-Dawley rats (250–385 g) anaesthetized with pentobarbitone sodium (75–90 mg kg^{-1}, i.p.). A tracheotomy was performed and both femoral arteries and veins were cannulated. Blood pressure was measured from the left femoral artery by means of a pressure transducer (Gould Statham P23XL) and heart rate was derived electronically from the blood pressure signal. Mean arterial pressure was calculated by adding one third of the pulse pressure to the diastolic pressure. Tracheal pressure was also measured. The animals were artificially ventilated (rate 50 min^{-1}, volume 3–5 ml) with oxygen enriched room air by use of a positive pressure pump and neuromuscular blockade

[1] Author for correspondence.

was produced with decamethonium iodide (1 mg per animal). Blood samples were taken from the right femoral artery and blood gases and pH were monitored with a Corning pH/blood gas analyser. Blood gases were maintained between 90–130 mmHg Po_2, 40–50 mmHg Pco_2 and pH 7.3–7.4. Slow i.v. injections of sodium bicarbonate (1.0 M) or adjustments of the respiratory pump were made as necessary to maintain pH and blood gases within this range. The Pco_2 was kept high in order to produce activity in the phrenic nerve. Once ventilated, the animals were infused (6 ml kg^{-1} h^{-1}) via a femoral vein with a solution containing 50 ml of a plasma substitute (Gelofusine), glucose (2 mg ml^{-1}), $NaHCO_3$ (8.4 mg ml^{-1}), decamethonium iodide (1.5 mg ml^{-1}) and pentobarbitone sodium (3 mg ml^{-1}) made up to a total volume of 100 ml with distilled water. This was to counteract the development of non-respiratory acidosis and to maintain blood volume, anaesthesia and neuromuscular blockade. The other femoral vein was used for the i.v. administration of drugs.

Recordings of phrenic nerve activity

The phrenic nerve was exposed on the left side by retracting the shoulder blade. The nerve was cut or crushed and the central end was placed on bipolar silver hook electrodes for the recording of phrenic nerve activity (PNA). PNA was quantified by counting in 5 s periods the number of events above the noise with a spike processor (Digitimer D 130). In all experiments the bursts of phrenic nerve activity were synchronised with the chest movements caused by the respiratory pump.

Microinjection into the dorsal motor vagal nucleus (DMVN)

The animals were placed in a stereotaxic frame and the dorsal surface of the brainstem was exposed. Microinjections (100 nl) were made into the DMVN on the right or the left sides of the brainstem with five barrelled glass microelectrodes, tip diameter 25–35 μm. Electrode penetrations were made 0–1 mm caudal to obex, 0.1–0.5 mm lateral to the midline and to a depth of 600–1000 μm below the dorsal surface of the brainstem. The electrode tip was judged to be in the DMVN if an instantaneous bradycardia was elicited by microinjection of glutamate. In most animals injection sites were marked with a 100 nl injection of pontamine sky blue dye (2% in 0.15 M sodium chloride (0.9%) solution). At the end of the experiment the brainstem was removed and fixed in a solution of 10% formalin and 1% glutaraldehyde in saline. 50 μm sections were cut and stained with neutral red so that the position of the injection site could be verified histologically.

Drug solutions used in the microelectrode barrels were:- artificial CSF, saline, glutamate (4.3 mg ml^{-1}), 5-HT creatine sulphate (5.0 mg ml^{-1}), 8-OH-DPAT (3.0 mg ml^{-1}), flesinoxan (6.0 mg ml^{-1}), DOI HCl (4.4 mg ml^{-1}), PBG (2.2 mg ml^{-1}) and clonidine HCl (0.5 mg ml^{-1}). Glutamate, 5-HT, and 8-OH-DPAT were dissolved in artificial CSF whereas flesinoxan, DOI, PBG and clonidine were dissolved in saline. In each case control injections were performed with the appropriate vehicle. Drug solutions were adjusted to pH 7.4 where possible, however when using flesinoxan, DOI or PBG, solutions were at pH 4.2–5.6.

Statistical analysis

Measurements of all variables where made just prior to the microinjection of the test substance and then when the fall in heart rate was maximum. For all variables, statistical analysis was performed to compare values before and after the microinjection of drugs by use of Student's paired t test. Values were considered significant at $P < 0.05$.

Drugs

The following drugs were purchased from the companies indicated in parentheses; sodium pentobarbitone and decamethonium HBr (May & Baker Ltd); Gelofusine (Consolidated Chemicals Ltd); L-glutamate and 5-hydroxytryptamine creatine sulphate (Sigma), 8-hydroxy-2-(di-n-propylamino) tetralin HBr (8-OH-DPAT); (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride, (DOI HCl) (Semat Tech., U.K.) and phenylbiguanide (K K Labs. U.S.A.). The following compounds were kindly donated by the companies indicated in parentheses; flesinoxan (Duphar B.V.) and clonidine HCl (Boehringer Ingelheim). All drug concentrations are the salts of the drug except for 8-OH-DPAT and flesinoxan.

Results

Baseline values

Baseline values for heart rate, mean arterial blood pressure and phrenic nerve activity are given in Table 1. Microinjections of vehicle solutions had no effects on any of these variables. Tracheal pressure data has not been quantified as no drug had any obvious effect on this parameter.

Effects of injections of glutamate into the dorsal motor vagal nucleus DMVN

Microinjections of glutamate (2.5 nmol, $n = 26$) into the region of the dorsal motor vagal nucleus caused significant mean decreases in heart rate of -21 ± 3 beats min^{-1}, blood pressure of -32 ± 2 mmHg and phrenic nerve activity of -54 ± 8% (Table 1). An example of the effects of an injection of glutamate is shown in Figure 1a. The effects of glutamate

Table 1 Baseline values and changes (mean ± s.e.mean) in heart rate (HR, beats min^{-1}), mean arterial blood pressure (BP, mmHg) and phrenic nerve activity (PNA, spikes 5 s^{-1}) following microinjection of drugs into the dorsal motor vagal nucleus

		HR (beats min^{-1})		*BP* (mmHg)		*PNA* (spikes 5 s^{-1})		
	n	*Control*	*Change*	*Control*	*Change*	*Control*	*Change*	(%)
Glutamate	26	319 ± 4	−21 ± 3***	100 ± 2	−32 ± 2***	1187 ± 74	−714 ± 104***	(−54 ± 8%)
5-HT	5	328 ± 4	−6 ± 2*	91 ± 6	0 ± 0	838 ± 81	−26 ± 49	(−3 ± 6%)
8-OH-DPAT	14	324 ± 7	−12 ± 1***	100 ± 2	0 ± 0	1048 ± 123	+169 ± 37**	(+20 ± 5%)
Flesinoxan	6	329 ± 4	−10 ± 1***	98 ± 3	−3 ± 2	923 ± 171	+261 ± 49**	(+30 ± 6%)
DOI	7	316 ± 8	+2 ± 1	99 ± 3	−2 ± 1	1611 ± 126	−11 ± 26	(−1 ± 2%)
PBG	5	308 ± 6	+1 ± 2	97 ± 3	0 ± 0	1296 ± 334	−20 ± 18	(−3 ± 3%)
Clonidine	6	334 ± 8	0 ± 1	102 ± 4	−1 ± 1	1453 ± 81	+7 ± 28	(+1 ± 2%)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
8-OH-DPAT = 8-hydroxy-2-(di-n-propylamino)tetralin; DOI = (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; PBG = phenylbiguanide.

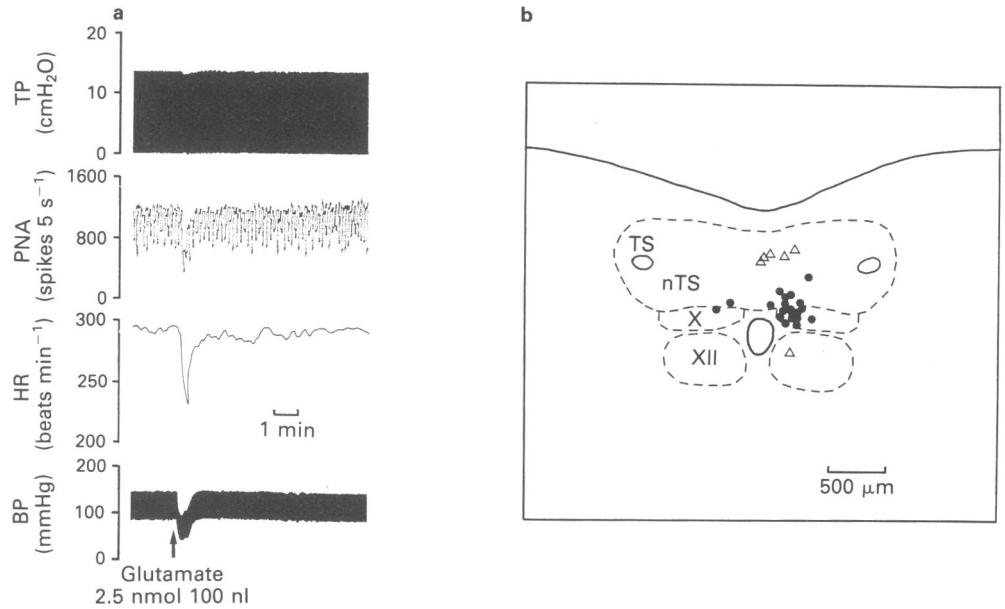

Figure 1 (a) The effects of a microinjection (2.5 nmol, 100 nl) of glutamate into the dorsal motor vagal nucleus on tracheal pressure (TP, cmH_2O), phrenic nerve activity (PNA, spikes $5\,s^{-1}$), heart rate (HR, beats min^{-1}) and blood pressure (BP, mmHg). (b) Diagram showing the position of glutamate injection sites that were successfully labelled on a representative transverse section through the brainstem at a level just caudal to obex. TS = tractus solitarius, nTS = nucleus tractus solitarius, X = dorsal motor vagal nucleus, XII = hypoglossal nucleus. (●) positive glutamate injection sites, (△) negative glutamate injection sites.

were rapid in onset, the mean time to the maximum bradycardia was 16 ± 3 s, and were of short duration (30–60 s). Later histological verification of the injection sites showed that, for sites that were successfully labelled, nearly all were in, or close to, the dorsal motor vagal nucleus (Figure 1b). The effects of glutamate described above were termed a 'positive response' and subsequent injection sites were estimated to be in the region of the DMVN if a positive glutamate response could be obtained. Injections of glutamate ($n = 6$) in areas outside of the DMVN (Figure 1b) had insignificant effects on heart rate, blood pressure and phrenic nerve activity.

Effects of injections of 5-hydroxytryptamine into the dorsal motor vagal nucleus

Microinjections of 5-HT (1.2 nmol, $n = 5$) into the region of the dorsal motor vagal nucleus caused a small but significant

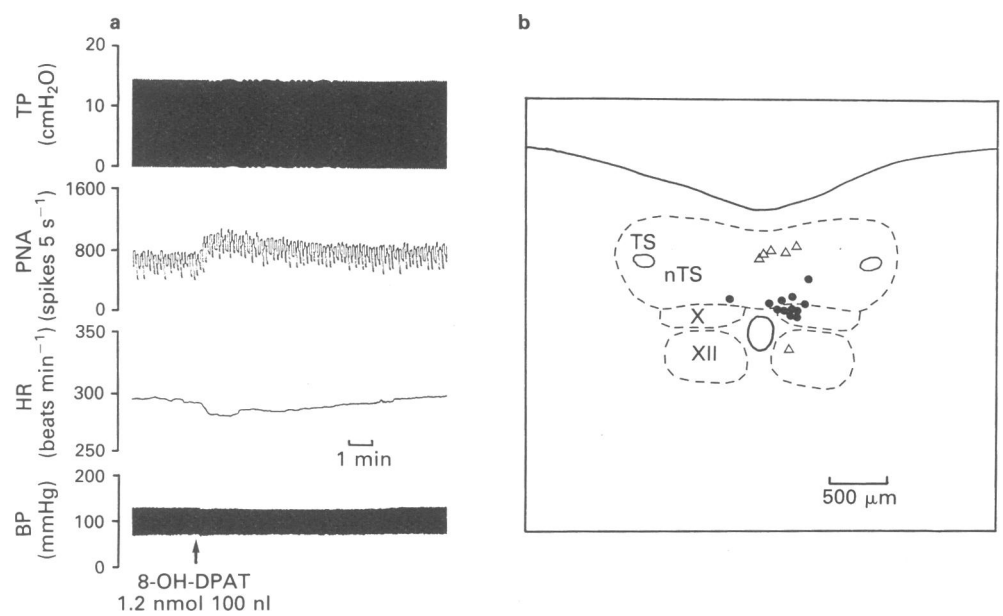

Figure 2 (a) The effects of a microinjection (1.2 nmol, 100 nl) of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) into the dorsal motor vagal nucleus on tracheal pressure (TP, cmH_2O), phrenic nerve activity (PNA, spikes $5\,s^{-1}$), heart rate (HR, beats min^{-1}) and blood pressure (BP, mmHg). (b) Diagram showing the position of 8-OH-DPAT injection sites that were successfully labelled on a representative transverse section through the brainstem at a level just caudal to obex. TS = tractus solitarius, nTS = nucleus tractus solitarius, X = dorsal motor vagal nucleus, XII = hypoglossal nucleus. (●) positive 8-OH-DPAT injections sites, (△) negative 8-OH-DPAT injection sites.

decrease in mean heart rate of -6 ± 2 beats min^{-1} but had no effects on blood pressure or phrenic nerve activity (Table 1). This decrease in heart rate was of long duration; time to the maximum bradycardia was 8.0 ± 0.5 min.

Effects of injections of 8-OH-DPAT and flesinoxan into the dorsal motor vagal nucleus

Microinjections of 8-OH-DPAT (1.2 nmol, $n = 14$) and flesinoxan (1.3 nmol, $n = 6$) into the region of the dorsal motor vagal nucleus caused significant decreases in mean heart rate of -12 ± 1 and -10 ± 1 beats min^{-1} respectively. There were no accompanying effects on blood pressure but there were significant increases in mean phrenic nerve activity of $20 \pm 5\%$ and $30 \pm 6\%$ respectively (Table 1). An example of the effects of injection of 8-OH-DPAT is shown in Figure 2a. For both drugs the effects on heart rate were immediate in onset and mean times to the maximum bradycardia were 6.2 ± 1.0 and 2.0 ± 1.5 min respectively. Since phrenic nerve activity was synchronized with the chest movements caused by the respiratory pump the increase in this activity was seen as an increase in the number of spikes produced during each inspiratory burst, rather than as an increase in inspiratory rate.

Injections ($n = 6$) of 8-OH-DPAT made into areas outside the region of the DMVN (Figure 2b) had no effects on heart rate, blood pressure or phrenic nerve activity. These sites were also those at which glutamate did not produce a positive response (Figure 1b).

Effects of injections of DOI, phenylbiguanide and clonidine into the dorsal motor vagal nucleus

Microinjections of DOI (1.2 nmol, $n = 7$), PBG (1.2 nmol, $n = 5$) and clonidine (0.2 nmol, $n = 6$) into the region of the dorsal motor vagal nucleus had no effects on any of the variables recorded (Table 1). Microinjection of glutamate at these same sites before and following the injection of these compounds produced a positive response. Histology confirmed that these sites were all within or close to the dorsal motor vagal nucleus.

Discussion

These results show that microinjections into the dorsal motor vagal nucleus of glutamate, 5-HT and the structurally unrelated 5-HT$_{1A}$ agonists, 8-OH-DPAT, and flesinoxan produce a decrease in heart rate in the atenolol pretreated rat. In addition, the selective agonists for 5-HT$_2$ and 5-HT$_3$ receptors DOI and phenylbiguanide (see Wijngaarden *et al.*, 1990) respectively, failed to have any effect on heart rate. These results, taken together with previous observations (see introduction) and the ability of 5-HT$_{1A}$ antagonists to block synaptic activation of cardiac vagal motoneurones via cardiopulmonary afferents (Bogle *et al.*, 1990), strongly support the hypothesis that 5-HT causes increased activity in cardiac vagal motoneurones by an action on 5-HT$_{1A}$ receptors. Although both 8-OH-DPAT and flesinoxan are known to be highly selective for 5-HT$_{1A}$ receptors (Middlemiss & Fozard, 1983; Wouters *et al.*, 1988) at increased concentrations all drugs tend to lose their selectivity. Evidence from binding studies suggests that at higher concentrations, 8-OH-DPAT may have some action on α_2-adrenoceptors (Wijngaarden *et al.*, 1990) and these receptors are known to be present in the region of the DMVN (Unnerstall *et al.*, 1984). However, flesinoxan has no affinity for α_2-adrenoceptors (Wouters *et al.*, 1988; Wijngaarden *et al.*, 1990) but had an identical effect to 8-OH-DPAT in the present experiments indicating that it is highly likely that these drugs are causing the above action by activating 5-HT$_{1A}$ receptors.

The precise anatomical site at which these 5-HT$_{1A}$ receptors are located is more difficult to assess. Although the injection sites were either in the DMVN, or were very close to the DMVN in the commissural region of the nucleus tractus solitarius (NTS), it would seem likely that the injected drugs may have spread to, and possibly had their actions at, sites surrounding the DMVN. An indication of the spread of these drugs can be assessed by the size of the dye spots produced by 100 nl injections of pontamine sky blue though the tissue volume receiving an effective concentration of drug cannot be determined. However, these recovered dye spots were all fairly compact, usually only up to 100 μm in diameter. Injections of 8-OH-DPAT into the hypoglossal nucleus and the dorsal part of the NTS had no effects on heart rate but injections into the NTS/DMVN border were effective (see Figure 2). Histological studies in rats have shown that vagal motoneurones in the DMVN which innervate the stomach have extensive dendritic fields which spread into the medial and commissural NTS (Shapiro & Miselis, 1985). If cardiac vagal motoneurones have a similar morphology then it is possible that injections into these regions of the NTS may still directly activate cardiac vagal motoneurones if there are 5-HT$_{1A}$ receptors on the dendrites of these motoneurones. However, the present results do not rule out the possibility that the effects of 5-HT$_{1A}$ agonists are mediated via local interneurones. In this respect Manaker & Verderame (1990) have shown 5-HT$_{1A}$ binding in the NTS as well as the DMVN of the rat.

These results also show that microinjections of 8-OH-DPAT and flesinoxan into the DMVN caused an increase in phrenic nerve activity suggesting that activation of 5-HT$_{1A}$ receptors in the DMVN results in an increase in central respiratory drive. In the rat, the ventral and ventrolateral subnuclei of the NTS are involved in respiratory control (see Feldman, 1986). Electrical stimulation of the ventral regions of the caudal NTS in the rat have been shown to produce hypopneas (Barraco & El-Ridi, 1989) similar to those produced by injections of glutamate in the present study. However, 8-OH-DPAT and flesinoxan both produced an increase in central respiratory drive. In this respect, in the cat (Holtman *et al.*, 1986) and the rat (Dreteler *et al.*, 1991), chemical stimulation of the raphe obscurus causes a similar increase in phrenic nerve activity and raphe neurones containing 5-HT are known to project to the NTS (Schaffar *et al.*, 1988; Holtman *et al.*, 1990). The present results would suggest that these 5-HT pathways activate 5-HT$_{1A}$ receptors to cause an increase in central inspiratory drive. The failure of 5-HT to have any effect on phrenic nerve activity may be due to the fact that the 5-HT is unable to spread to the respiratory regions of the NTS due to rapid neuronal uptake.

DOI and PGB failed to have any effect when given at the same molar concentrations as 8-OH-DPAT and flesinoxan. Clonidine, at the concentration used, is known to cause a sympathetically mediated decrease in heart rate when injected into the NTS (Gurtu *et al.*, 1982). The lack of effects demonstrated in this present study suggests that activation of 5-HT$_2$ and 5-HT$_3$ receptors and α_2-adrenoceptors in the region of DMVN does not result in the activation of CVM's or affect central inspiratory drive.

The present results support the view that 5-HT pathways produce excitation of CVMs and phrenic motoneurones through the activation of 5-HT$_{1A}$ receptors.

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The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors

Mohammed Shahid, *Ronald G.M. van Amsterdam, Jacob de Boer, *Ronald E. ten Berge, [1]C. David Nicholson & *Johan Zaagsma

Organon Laboratories Limited, Newhouse, Lanarkshire ML1 5SH, United Kingdom and *Department of Pharmacology and Therapeutics, University of Groningen, A Deusinglaan 2, 9713 AW Groningen, The Netherlands

1 The profile of cyclic nucleotide phosphodiesterase (PDE) isoenzymes and the relaxant effects of isoenzyme selective inhibitors were examined in bovine tracheal smooth muscle. The compounds examined were the non-selective inhibitor 3-isobutyl-1-methylxanthine (IBMX), zaprinast (PDE V selective), milrinone and Org 9935 (4,5-dihydro-6-(5,6-dimethoxy-benzo[b]thien-2-yl)-5-methyl-1(2H)-pyridazinone; both PDE III selective), rolipram (PDE IV selective) and Org 30029 (N-hydroxy-5,6-dimethoxy-benzo[b]-thiophene-2-carboximidamide HCl a dual PDE III/IV inhibitor).

2 Ion exchange chromatography showed three main peaks of PDE activity. The first peak was stimulated by Ca^{2+}/calmodulin (PDE I), the adenosine 3':5'-cyclic monophosphate (cyclic AMP) hydrolytic activity of the second peak was stimulated by guanosine 3':5'-cyclic monophosphate (cyclic GMP) (PDE II) whilst that of the third peak was not significantly modified by any regulator (PDE IV). Calmodulin affinity chromatography revealed the additional presence of cyclic GMP-specific PDE (PDE V) in the first peak. A clearly distinct peak of cyclic GMP-inhibited PDE (PDE III) was not observed. However, Org 9935 inhibited the third activity peak more effectively in the presence, than in the absence, of rolipram ($3\,\mu mol\,l^{-1}$), indicating the presence of PDE III activity.

3 Rolipram was the most potent inhibitor of PDE IV. The mean $-\log_{50}$ IC_{50} values for rolipram, IBMX, milrinone, Org 30029, Org 9935 and zaprinast were 5.9 ± 0.1, 4.9 ± 0.1, 4.7 ± 0.1, 4.6 ± 0.1 and 4.6 ± 0.1, respectively.

4 Rolipram was a potent relaxant of both histamine ($1\,\mu mol\,l^{-1}$) and methacholine ($0.03\,\mu mol\,l^{-1}$) precontracted preparations; (pD_2 values; histamine 7.1 ± 0.1, methacholine 6.8 ± 0.2 and 4.5 ± 0.1, biphasic relaxation). IBMX also relaxed all preparations (pD_2 values; histamine 5.6 ± 0.1, methacholine 5.6 ± 0.1) whilst zaprinast (pD_2 values; histamine 5.2 ± 0.1, methacholine 4.4 ± 0.3), milrinone (pD_2 values; histamine 5.2 ± 0.1, methacholine 4.3 ± 0.3) and Org 9935 (pD_2 values; histamine 4.1 ± 0.1, methacholine 4.1 ± 0.2) did not completely relax preparations at concentrations up to $100\,\mu mol\,l^{-1}$. Org 30029 (pD_2 values; histamine 6.2 ± 0.1, methacholine 5.4 ± 0.1) was a more effective relaxant than can be explained on the basis of PDE IV inhibition alone.

5 We conclude that bovine tracheal smooth muscle contains five distinct PDE isoenzymes. PDE IV appears to be more important in the modulation of tissue function than PDE III and PDE V.

Keywords: Cyclic nucleotide phosphodiesterase isoenzymes; bovine tracheal smooth muscle relaxation; rolipram; IBMX; zaprinast; Org 9935; Org 30029; milrinone

Introduction

The presence of multiple cyclic nucleotide phosphodiesterase (PDE) isoenzymes in mammalian cells and their role in the metabolism of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) has been extensively described (Appleman *et al.*, 1982; Reeves *et al.*, 1987; Beavo, 1988; Nicholson *et al.*, 1991). The identification of five families of PDE in cells has been paralleled by the synthesis of isoenzyme selective inhibitors. By use of these agents as pharmacological tools, the effects of selective isoenzyme inhibition on tissue function have been examined. It has become apparent that the presence of a particular isoenzyme within a cell does not necessarily mean that its inhibition will initiate a response normally associated with cyclic nucleotide elevation (Weishaar *et al.*, 1987; Gristwood *et al.*, 1986; Shahid & Nicholson, 1990). Accordingly, the role played by particular PDE isoenzymes in cells can only be determined by the elucidation of both the isoenzyme profile within a particular tissue as well as the functional activity of isoenzyme selective inhibitors.

The aim of the present study was to analyse the PDE isoenzymes present in bovine tracheal smooth muscle and to evaluate the functional effects of isoenzyme inhibitors in this tissue. PDE inhibitors differ in their ability to inhibit contractions of airways preparations induced by different agonists (Heaslop *et al.*, 1986; Torphy *et al.*, 1988). The ability of non-selective PDE inhibitors to relax airways smooth muscle is well known (Fredholm *et al.*, 1979; Lohmann *et al.*, 1977). Thus we investigated the effects of a range of isoenzyme-selective inhibitors on both histamine and methacholine-induced contractions. The inhibitors used were 3-isobutyl-1-methylxanthine (IBMX), a non-selective PDE inhibitor (Davis 1984; Nicholson *et al.*, 1989), the PDE III selective inhibitors milrinone (Bristol *et al.*, 1984; Shahid & Nicholson, 1990) and Org 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thien-2-yl- 5-methyl-3(2H)-pyridazinone; Shahid *et al.*, 1991), rolipram, a PDE IV selective inhibitor (Reeves *et al.*, 1987), Org 30029 (N-hydroxy-5,6-dimethoxy-benzo[b]thiophene-2-carboximidamide HCl) a dual PDE III/PDE IV inhibitor (Shahid & Nicholson, 1990) and zaprinast, a PDE V selective inhibitor (Weishaar *et al.*, 1986).

Methods

Separation of cyclic nucleotide phosphodiesterase isoenzymes

Fresh bovine tracheae were obtained from a local slaughterhouse and transported to the laboratory in chilled Krebs-Henseleit solution (pH 7.4) pre-gassed with 95% O_2: 5%

[1] Author for correspondence.

CO_2. All subsequent steps were performed at 4°C. Tracheal smooth muscle was carefully dissected and cut into smaller strips which were then trimmed free of mucosal, connective and fat tissue in Krebs-Henseleit solution as described by Roffel et al. (1988). Two ion-exchange chromatography procedures were used for the separation of PDE isoenzymes.

Ion-exchange chromatography on Q-sepharose fast flow

Bovine tracheal smooth muscle (12–20 g) was chopped into small cubes and homogenized (Polytron: PT20 probe, 2 × 10 s bursts at speed setting 5 and 2 × 10 s bursts at speed setting 7) in 6 volumes of buffer (pH 6.5) containing (mmol l^{-1}): Bistris 20, dithiothreitol 1, benzamidine 2, EDTA 2, sodium acetate 50 and phenylmethanesulphonyl fluoride (PMSF) 0.1. The homogenate was filtered through two layers of cheese-cloth and centrifuged at 12,000 *g* for 10 min. The supernatant fraction (40–80 ml) was applied (60 ml h^{-1}) to a Q-sepharose fast flow column (1 × 28 cm) pre-equilibrated in homogenization buffer. The column was then washed with at least two bed volumes of homogenization buffer to remove unbound material. Proteins bound to the Q-sepharose were eluted (30 ml h^{-1}) with a linear sodium acetate gradient approximately 5 bed volumes in size. Fractions (2 ml) were collected and initial characterization of PDE activity performed within 24 h. Ethylene glycol was added to a final concentration of 30% (v/v) and the active fractions frozen in liquid nitrogen before long term storage at −70°C. Under these conditions, the enzyme activity was stable for at least 3 months.

Ion-exchange chromatography on Mono-Q

This procedure was essentially the same as that described by Shahid & Nicholson (1990). The use of a Mono-Q column attached to a fast protein liquid chromatography system enabled rapid separation of PDE isoenzymes, reduced proteolysis to a minimum and optimised isoenzyme resolution.

Membrane-bound phosphodiesterase

The pellet obtained after the 12,000 *g* spin of the tracheal smooth muscle extract was resuspended (Potter S homogeniser; 4 passes at 400 r.p.m.) in 2–5 ml of homogenization buffer and was used as the source of membrane-bound PDE.

Affinity chromatography on calmodulin-agarose

The first peak of PDE activity obtained after ion-exchange chromatography was further purified on calmodulin-agarose to separate calmodulin-sensitive and insensitive activities. A combined sample (1.3 ml) of fractions representing peak 1 was prepared and the Ca^{2+} and Mg^{2+} concentrations increased to 2 mmol l^{-1} before application to a calmodulin-agarose column (1.6 × 4 cm) which had been pre-equilibrated in at least 10 bed volumes of buffer A (pH 6.5) containing (mmol l^{-1}): Bistris 20, dithiothreitol 1, benzamidine 2, NaCl 50, $MgCl_2$ 3, $CaCl_2$ 0.1 and PMSF 0.1. Penetration into the calmodulin-agarose was ensured by applying 2–4 ml of buffer A after sample loading. To allow complete adsorption, the enzyme sample was left on the column for 30 min before serial stepwise elution with: (a) 20 ml of buffer A, (b) 20 ml of buffer B (buffer A plus 1 M NaCl) and (c) buffer C (buffer B plus 1 mM EGTA). Fractions (1 ml) were collected and assayed for PDE activity within 24 h.

Cyclic nucleotide phosphodiesterase assay

PDE activity was measured as described by Shahid & Rodger (1989). The standard assay mixture contained $MgCl_2$ 3 mmol l^{-1}, 5'adenosine monophosphate or 5'guanosine monophosphate 1 mmol l^{-1}, ^{3}H-labelled/unlabelled cyclic nucleotide (150,000–200,000 d.p.m.) 1 μmol l^{-1}, dithiothreitol 1 mmol l^{-1}, bovine serum albumin 0.05% (w/v) in Tris HCl 50 mmol l^{-1} (pH 7.4) in a final volume of 200 μl. Inhibitors were tested in the concentration range 0.1–250 μmol l^{-1} with cyclic AMP (1 μmol l^{-1}) as substrate except for PDE V where cyclic GMP (1 μmol l^{-1}) was used. Inhibitory effects on PDE I and PDE II activity were examined in the presence of Ca^{2+} (1 mmol l^{-1})/calmodulin (1.5 μg ml^{-1}) and cyclic GMP (1 μmol l^{-1}), respectively, to enhance basal activity. The drug concentration producing 50% inhibition of PDE activity (IC_{50} value) was determined with a non-linear regression curve fitting programme. All experiments were repeated between 4 and 12 times with fractions from 2 to 3 preparations.

Milrinone (2.0 mmol l^{-1}), IBMX (4.5 mmol l^{-1}) and zaprinast (1.0 mmol l^{-1}) were dissolved in Tris-HCl 50 mmol l^{-1} (pH 7.4) whereas Org 30029 (69 mmol l^{-1}) and rolipram (73 mmol l^{-1}) were dissolved in dimethylsulphoxide (DMSO) and subsequently diluted in Tris-HCl 50 mmol l^{-1}. The final concentrations of DMSO, for these compounds, in the assay was less than 0.5% (v/v) and did not significantly affect basal PDE activity. Org 9935 (66 mmol l^{-1}) was dissolved in DMSO and subsequently diluted in 10% DMSO/Tris HCl. The final concentration of DMSO in the assay was 2.4% (v/v) which inhibited PDE activity by 10–20%, control tubes contained the same quantity of DMSO. Protein content was determined by the biuret method.

Determination of kinetic constants

In these experiments, 7 to 9 different substrate concentrations ranging from 0.1 μmol l^{-1} to 1 mmol l^{-1} were used. The concentration of radiolabelled cyclic nucleotide was kept constant and the concentration of unlabelled nucleotide varied. Incubation time was also varied (15–45 min) to adjust enzyme activity such that approximately 25% of the substrate was hydrolysed. The data were analysed with a commercial non-linear regression curve fitting programme (ENZFITTER, Elsevier-Biosoft) to determine K_m and V_{max} values. All experiments were performed in duplicate 2 to 3 times using at least 2 different separations.

Relaxation of tracheal smooth muscle

Bovine tracheal smooth muscle strips (2 × 2 × 10 mm) were mounted for isotonic recording in 20 ml organ baths. A 300 mg load was used which ensured stable and reproducible responses. After equilibration for 30 min in Krebs-Henseleit solution (37°C), (−)-isoprenaline, 0.1 μmol l^{-1}, was applied to establish basal tone. The tissue was washed immediately and later contracted by cumulative administration of methacholine 10 and 90 μmol l^{-1} followed by 60 min of repeated washings. Subsequently, contraction was induced with either histamine, 1 μmol l^{-1}, or methacholine, 0.03 μmol l^{-1}; 15 min later cumulative relaxation curves were constructed with either IBMX, rolipram, zaprinast, milrinone, Org 30029 or Org 9935. After completion, (−)-isoprenaline, 10 μmol l^{-1}, was added to re-establish basal tone. All experiments were performed 4 to 6 times.

Chemicals

The following substances were used: 3-isobutyl-1-methylxanthine, calmodulin, calmodulin-agarose (Sigma, Poole, Dorset), rolipram (a gift from Schering, Berlin, F.R.G.), milrinone, Org 30029 and Org 9935 (Chemistry Department, Organon Laboratories Ltd, Newhouse, Lanarkshire), [^{3}H]-cyclic AMP, [^{3}H]-cyclic GMP (Amersham International, Bucks) and Q-sepharose (fast-flow), Mono Q (Pharmacia, Milton Keynes). All other chemicals were obtained either from Sigma Chemical Co or BDH Chemicals (both of Poole, Dorset) and were of Analar or equivalent grade. Water purified on a Milli-Q (Millipore, Herts) system was used throughout.

Results

Analysis of PDE activity in bovine tracheal homogenate (H), supernatant (S) and pellet (P) showed that 2.3% and 0.60% of

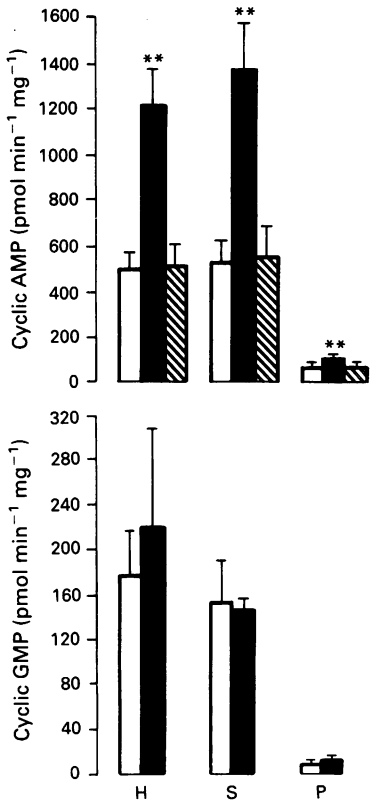

Figure 1 The hydrolysis of cyclic AMP (a) and cyclic GMP (b) by homogenate, supernatant and pellet fractions prepared from bovine tracheal smooth muscle in the absence (open columns) and presence of either Ca^{2+} (20 μmol l^{-1})/calmodulin (1.5 μg ml^{-1}) (solid columns) or cyclic GMP (1 μmol l^{-1}) (hatched columns). Supernatant and particulate fractions were obtained by centrifugation from the homogenate (values are means with s.e.mean shown by vertical bars, $n = 3$–6; see text for details). ** $P < 0.01$ (paired t test).

the total cyclic AMP- and cyclic GMP-hydrolytic activity was present in the membrane fraction. The effects of the regulators Ca^{2+}/calmodulin and cyclic GMP on H, S and P fractions are shown in Figure 1. The cyclic AMP-hydrolytic activity of all three fractions was stimulated by Ca^{2+}/calmodulin. Cyclic GMP hydrolysis tended towards an increase but this did not reveal the level of statistical significance. EGTA (1 mmol l^{-1}) inhibited the basal PDE activity of H, S and P fractions by 33%, 41% and 18%, respectively, indicating activation by endogenous Ca^{2+}/calmodulin. Cyclic GMP did not significantly affect the cyclic AMP-hydrolytic activities of any fraction.

Separation of phosphodiesterase isoenzymes

Elution profiles obtained with both Q-sepharose and Mono-Q FPLC were similar. The resolution was superior with the latter method, but greater sample capacity was achieved with Q-sepharose. Approximately 82–93% of the applied PDE activity was recovered. Three major peaks of PDE activity eluting at 0.23 M, 0.34 M and 0.44 M NaCl were identified (Figure 2). According to the nomenclature of Beavo & Reifsnyder (1990), these peaks could be tentatively designated PDE I, PDE II and PDE IV, respectively. The cyclic AMP-hydrolytic activity of PDE I and PDE II was stimulated by Ca^{2+}/calmodulin and cyclic GMP, respectively. The hydrolytic activity of the third activity peak was not significantly modulated by either regulator (Figure 2). A further minor activity peak eluting at 0.69 M NaCl was also observed. As analysed in one separation, cyclic AMP-hydrolytic activity was not inhibited by cyclic GMP but was potently inhibited by rolipram ($-\log IC_{50}$ 6.52) suggesting that it may be an isoform of PDE IV. This peak was of low activity and although reproducibly obtained, it was not further characterized. The elution profile in the absence and presence of cyclic GMP did not reveal PDE III activity.

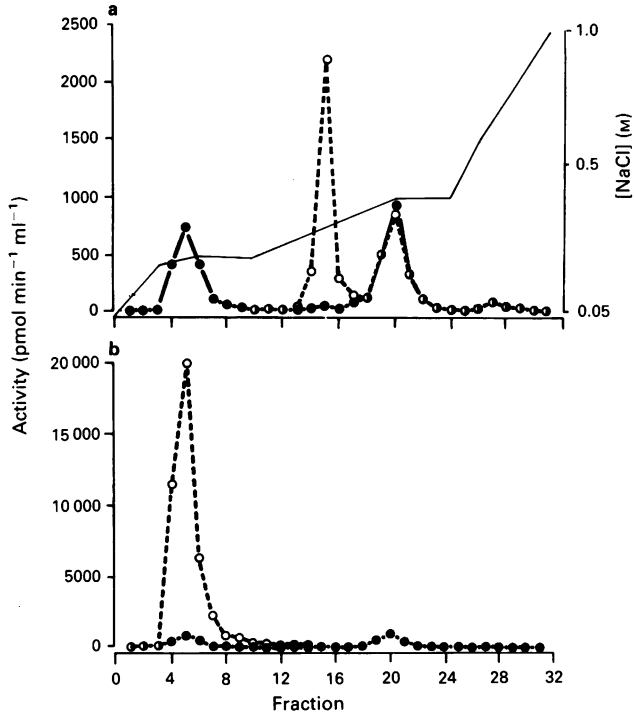

Figure 2 Elution profiles of cyclic nucleotide phosphodiesterase (PDE) activities from bovine tracheal smooth muscle after separation on a Mono-Q ion-exchange column. Low speed (12,000 g) supernatant fractions from fresh bovine tracheal smooth muscle (4–6 g) were prepared as described for the Q-sepharose method, except that the sodium acetate in the homogenization buffer was replaced with NaCl, 50 mmol l^{-1}. Each sample was filtered (0.22 μm filters) and 10–27 ml applied (1 ml min^{-1}) to a 1 ml Mono-Q column, pre-equilibrated in homogenization buffer. The column was washed with 6 ml of homogenization buffer to remove unbound material. PDE activity was then eluted at 1 ml min^{-1} with a 32 ml gradient of NaCl (0.05–1.0 M) in homogenization buffer kept on ice. Fractions (1 ml) were collected in tubes kept on ice and assayed for cyclic AMP (1 μmol l^{-1}) PDE activity either in the absence of any known regulator (●) or in the presence of cyclic GMP (1 μmol l^{-1}; a; ○) or Ca^{2+} (20 μmol l^{-1})/calmodulin (1.5 μg ml^{-1}; b; ○).

To analyse further the PDE isoenzymes present in peak 1, the combined fractions of this peak obtained with Q-sepharose were applied to a calmodulin-agarose column. As shown in Figure 3, when Ca^{2+} containing wash buffer was used, the PDE activity obtained in the eluate was not stimulated by Ca^{2+}/calmodulin and was specific for cyclic GMP and hence could be classified as PDE V (Beavo & Reifsnyder, 1990). The Ca^{2+}/calmodulin dependent PDE could be released from the column by buffer containing EGTA.

Kinetic parameters of phosphodiesterase isoenzymes

The kinetic characteristics (K_m and V_{max} values) of bovine tracheal PDE isoenzymes are presented in Table 1. PDE V had a high affinity and activity for cyclic GMP. PDE I had a high affinity for cyclic AMP. PDE II shows a low affinity but high activity against both cyclic nucleotides; cyclic GMP hydrolysis by this enzyme displayed positive cooperativity. In the presence of cyclic GMP, cyclic AMP hydrolysis followed Michealis Menten kinetics. The kinetics of cyclic AMP hydrolysis in the absence of cyclic GMP were not evaluated, but it is known that the presence of cyclic GMP reduces the degree of cooperativity in the kinetics of cyclic AMP hydrolysis by PDE II from canine trachealis (Torphy & Cieslinski, 1990). PDE IV had a high affinity for cyclic AMP and a low cyclic GMP hydrolytic activity. The kinetic parameters of cyclic AMP for PDE V and cyclic GMP for PDE I and IV were not precisely determined, as basal activity was low.

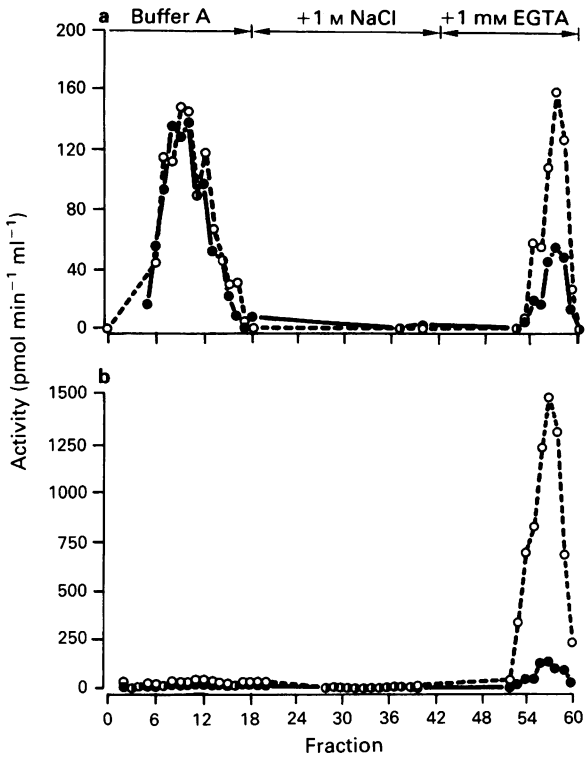

Figure 3 Subfractionation of cyclic nucleotide phosphodiesterase (PDE) activities contained within the first activity peak obtained after ion-exchange chromatography of the supernatant fractions from bovine tracheal smooth muscle. PDE activity was eluted from a calmodulin-agarose column with either Ca^{2+} or EGTA containing buffers (see text for details). Fractions were assayed for cyclic GMP (a) or cyclic AMP (b) hydrolytic activities ($1\,\mu mol\,l^{-1}$ substrate concentration) in the absence (●) or presence of Ca^{2+} ($1\,mmol\,l^{-1}$)/ calmodulin ($1.5\,\mu g\,ml^{-1}$; ○).

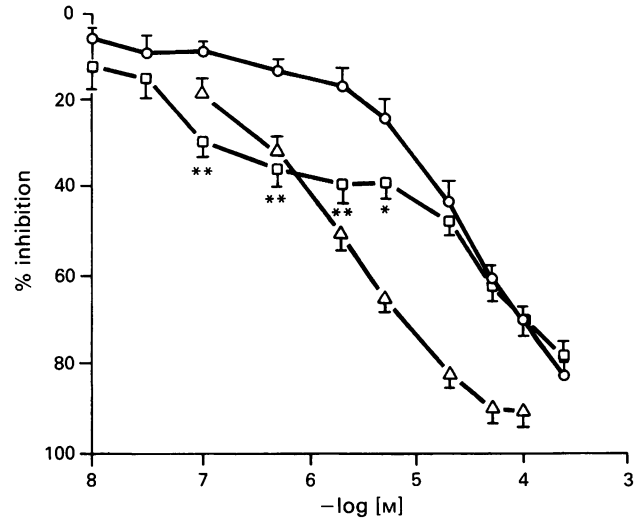

Figure 4 The inhibition of the third peak of cyclic AMP hydrolytic activity obtained from the supernatant fraction of bovine tracheal smooth muscle by Org 9935 (○), rolipram (△) and Org 9935 in the presence of $3\,\mu mol\,l^{-1}$ rolipram (□). Rolipram $3\,\mu mol\,l^{-1}$ inhibited activity by $52 \pm 7\%$; in experiments examining PDE inhibition by Org 9935 in the presence of rolipram, the activity remaining in the presence of rolipram was normalised to 100%. All points represent mean (with s.e.mean shown by vertical bars) of at least six determinations performed with 2–3 separations. Asterisks denote significant difference (Student's t test, $^*P < 0.05$, $^{**}P < 0.01$) from inhibition produced by Org 9935 alone.

The effects of phosphodiesterase inhibitors on phosphodiesterase isoenzyme activity

The effects of PDE isoenzyme inhibitors on bovine tracheal PDE isoenzymes are summarized in Table 2. IBMX non-specifically inhibited all isoenzymes. Zaprinast showed

Table 1 Kinetic characteristics of bovine tracheal phosphodiesterase (PDE) isoenzymes

		K_m ($\mu mol\,l^{-1}$)		V_{max} ($nmol\,min^{-1}\,ml^{-1}$)	
PDE	*Isoenzyme family*	*Cyclic AMP*	*Cyclic GMP*	*Cyclic AMP*	*Cyclic GMP*
I	Ca^{2+}/calmodulin-dependent	2.9[1]	ND	4.0[1]	ND
II	Cyclic GMP-stimulated	105[2]	85[3]	131[2]	72
IV	Cyclic GMP-insensitive	2.9	ND	4.1	ND
V	Cyclic GMP-specific	ND	2.6	ND	694

ND, Low activity at $1\,\mu mol\,l^{-1}$, precise value not determined.
[1] In the presence of Ca^{2+} ($1\,mmol\,l^{-1}$) and calmodulin ($1.5\,\mu g\,ml^{-1}$).
[2] In the presence of cyclic GMP ($1\,\mu mol\,l^{-1}$).
[3] Non linear kinetics (value given is the substrate concentration giving 50% of maximal activity.
All values are means of 2–3 determinations performed in duplicate from at least 2 different separations.

Table 2 Inhibition of bovine tracheal smooth muscle phosphodiesterase (PDE) isoenzymes (I, II, IV, V) and rabbit heart PDE III

	$-log\ IC_{50}$[1]				
	Ca^{2+}/calmodulin[2] -dependent PDE I	*Cyclic GMP[3] -stimulated PDE II*	*Cyclic GMP[4] -insenstiive PDE IV*	*Cyclic GMP[5] -specific PDE V*	*Cyclic GMP[4,6] -inhibited PDE III*
IBMX	4.7 ± 0.1	4.3 ± 0.1	4.9 ± 0.1	4.5 ± 0.1	5.1 ± 0.1
Zaprinast	4.8 ± 0.1	4.5 ± 0.2	4.6 ± 0.1	6.6 ± 0.1	3.9 ± 0.1
Rolipram	<3.60 (24)	<3.60 (11)	5.9 ± 0.1	<3.60 (23)	4.3 ± 0.2
Milrinone	<3.60 (48)	<3.60 (22)	4.7 ± 0.1	<3.60 (34)	5.5 ± 0.1
Org 30029	<3.60 (32)	<3.60 (14)	4.6 ± 0.1	<3.60 (19)	4.5 ± 0.1
Org 9935	<3.60 (39)	<3.60 (6)	4.6 ± 0.1	5.01 ± 0.15	7.0 ± 0.2

[1] Mean ± s.e.mean of 4–12 assays performed with 2–3 separations; figures in parentheses indicate % inhibition at $250\,\mu mol\,l^{-1}$.
[2] Cyclic AMP ($1\,\mu mol\,l^{-1}$) as substrate and in the presence of Ca^{2+} ($1\,mmol\,l^{-1}$) and calmodulin ($1.5\,\mu g\,ml^{-1}$).
[3] Cyclic AMP ($1\,\mu mol\,l^{-1}$) as substrate and in the presence of cyclic GMP ($1\,\mu mol\,l^{-1}$).
[4] Cyclic AMP ($1\,\mu mol\,l^{-1}$) as substrate.
[5] Cyclic GMP ($1\,\mu mol\,l^{-1}$) as substrate.
[6] Inhibition of rabbit heart PDE III (Shahid & Nicholson, 1990).
IBMX = 3-isobutyl-1-methylxanthine.

approximately 100 fold selectivity for the inhibition of PDE V. Milrinone, Org 30029, Org 9935 and rolipram inhibited PDE IV, rolipram being the most potent inhibitor. Comparison with cardiac PDE III data shows rolipram to be the sole truly selective PDE IV inhibitor, milrinone and Org 9935 are both more potent PDE III inhibitors, with Org 9935 showing the greater selectivity. Org 30029 has a similar ability to inhibit both PDE III and PDE IV.

To evaluate further the third peak of PDE activity, the ability of cyclic GMP and Org 9935 to attenuate hydrolytic activity was examined in the presence and absence of rolipram ($3\,\mu mol\,l^{-1}$). Org 9935 was chosen for these experiments because it has the greatest selectivity for PDE III amongst the compounds examined in the present study. Rolipram ($3\,\mu mol\,l^{-1}$) produced a $52 \pm 7\%$ inhibition of total activity in these assays. Part of the remaining activity was potently inhibited by Org 9935 (Figure 4). The concentration-inhibition curve was clearly biphasic, the $-\log IC_{50}$ for the initial section of the curve (7.4 ± 0.3) was similar to the $-\log IC_{50}$ for cardiac PDE III (Table 2). Cyclic GMP ($1\,\mu mol\,l^{-1}$) inhibited 7% and 12% of the hydrolytic activity in the absence and presence of rolipram, respectively.

Functional activity of phosphodiesterase inhibitors

The concentrations of histamine ($1\,\mu mol\,l^{-1}$) and methacholine ($0.03\,\mu mol\,l^{-1}$) employed produced $55.8 \pm 7.0\%$ and $57.7 \pm 2.3\%$ of the methacholine maximum contractions, respectively. All PDE inhibitors tested produced concentration-dependent relaxant responses (Figure 5); the $-\log EC_{50}$ values are summarised in Table 3. The most effective relaxant of both histamine and methacholine-induced contractions was rolipram; with methacholine as the agonist, the relaxation curve was biphasic. IBMX and Org 30029 were active but less potent. Zaprinast, milrinone and Org 9935 were not effective at concentrations maintaining inhibitory selectivity for PDE V and PDE III, respectively, although Org 9935 did produce a small relaxation of methacholine-induced contractions at low concentrations.

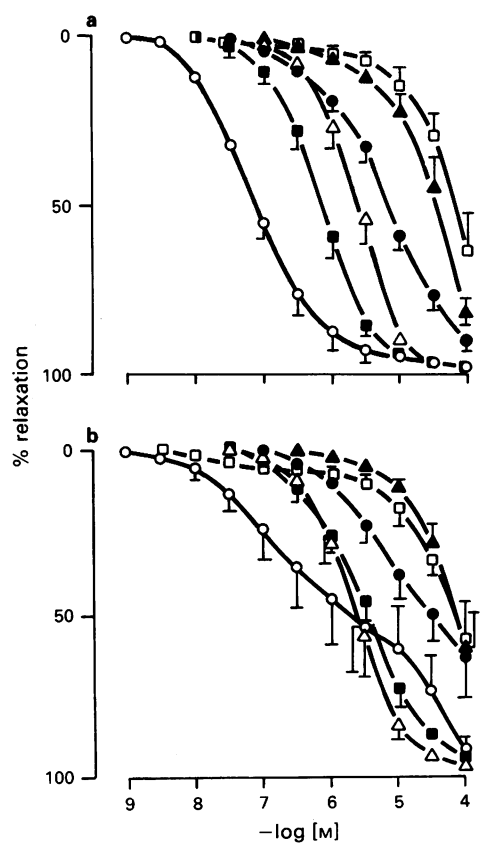

Figure 5 Relaxation of histamine- (a; $1\,\mu mol\,l^{-1}$) and methacholine- (b; $0.03\,\mu mol\,l^{-1}$) contracted bovine tracheal smooth muscle by cyclic nucleotide phosphodiesterase inhibitors (○, rolipram; ■ Org 30029; △ isobutylmethylxanthine; ● zaprinast, ▲ milrinone; □ Org 9935). Data are the mean of between 4 and 6 experiments; s.e.mean shown by vertical bars.

Table 3 Relaxation of bovine tracheal smooth muscle by cyclic nucleotide inhibitors in the presence of methacholine ($0.03\,\mu mol\,l^{-1}$) or histamine ($1.0\,\mu mol\,l^{-1}$)

		Methacholine	*Histamine*
IBMX		5.6 ± 0.1	5.6 ± 0.1
Zaprinast		4.4 ± 0.3	5.2 ± 0.1
Rolipram	1st phase	6.8 ± 0.2	7.1 ± 0.1
	2nd phase	4.5 ± 0.1	
Milrinone		4.1 ± 0.2	4.5 ± 0.1
Org 30029		5.4 ± 0.1	6.2 ± 0.1
Org 9935		4.1 ± 0.2	4.1 ± 0.2

Data represent $-\log EC_{50} \pm$ s.e.mean from 4–6 experiments. IBMX = 3-isobutyl-1-methylxanthine.

Discussion

The present study demonstrated bovine tracheal smooth muscle to contain representatives of all five presently identifiable PDE isoenzymes in the cell cytosol. As such it is qualitatively comparable to canine tracheal smooth muscle (Torphy & Cieslinski, 1990). The finding of three major peaks of PDE activity in the supernatant of bovine tracheal smooth muscle homogenates is in agreement with published data (Elliott *et al.*, 1991). As previously described for canine (Torphy & Cieslinski, 1990), but not for bovine preparations, PDE V coelutes with PDE I, hence calmodulin affinity chromatography is necessary to separate these isoenzymes.

The high affinity of PDE I in bovine trachealis for cyclic AMP indicates that this isoform of PDE I is different from that found in cerebral tissue (Strada *et al.*, 1984) and is similar to that found in cardiac muscle (Reeves *et al.*, 1987) and canine tracheal smooth muscle (Torphy & Cieslinski, 1990). However, in these latter tissues PDE I has a comparable activity against cyclic GMP, therefore, the identity of PDE I in bovine tracheal smooth muscle awaits further clarification. PDE V in bovine tracheal smooth muscle has characteristics similar to those previously reported for this isoenzyme in lungs (Beavo, 1988) and canine tracheal smooth muscle (Torphy & Cieslinski, 1990). It is a different isoform from that found in the retina of the eye which has a lower affinity for cyclic GMP (Beavo, 1988). The characteristics of PDE II were similar to those observed in canine tracheal smooth muscle (Torphy & Cieslinski, 1990) and other tissues such as cardiac muscle (Reeves *et al.*, 1987; Nicholson *et al.*, 1989) and brain (Nicholson *et al.*, 1989).

The major Ca^{2+}/calmodulin-independent low K_m cyclic AMP PDE activity peak initially appeared to meet the criteria (Beavo, 1988) for being PDE IV-like in character. However, previous studies have found PDE III and PDE IV to co-elute from guinea-pig (Harris *et al.*, 1989) and canine (Torphy & Cieslinski, 1990) trachea. The inhibitory activity of Org 9935 and cyclic GMP in the presence of rolipram revealed the presence of PDE III within the third major activity peak, accounting for 12–18% of activity. The activity of PDE III relative to PDE IV was less than that found in guinea-pig (Harris *et al.*, 1989) and canine (Torphy & Cieslinski, 1990) tracheal smooth muscle. The importance of PDE IV for cyclic AMP hydrolysis in bovine trachea has previously been suggested by Hall *et al.* (1989) who found rolipram to elevate cyclic AMP more effectively than the PDE III selective inhibitor, SK& F 94120.

As previously reported for rat kidney (Hoey & Houslay, 1990) and liver (Lavan et al., 1989), the present study indicated the presence of two distinct PDE IV isoforms in bovine trachea that could be separated by ion-exchange chromatography. Whether one has been displaced from membranes during homogenization or if the two isoforms represent distinct cytosolic activities, as opposed to one being a proteolytic breakdown product, requires clarification.

As in canine trachea (Torphy & Cieslinski, 1990), the membrane-bound PDE activity observed in bovine tracheal smooth muscle contains PDE I. Cyclic GMP neither inhibited nor stimulated cyclic AMP hydrolysis, hence, neither PDE III nor PDE II activity seem to be concentrated in a particulate fraction, although PDE IV may be present.

The most significant finding of the functional experiments of the present study was that rolipram was the most effective relaxant of both histamine and methacholine-induced tone. The selective inhibition of PDE IV by rolipram indicates that PDE IV is of great functional significance in bovine trachealis. However, a high affinity binding site has been identified at which rolipram has a higher affinity than for the catalytic site of PDE IV (Schneider et al., 1986). Until the nature of rolipram binding site is clarified, all the pharmacological activity of rolipram cannot unequivocally be attributed to PDE inhibition. The rolipram-induced relaxation of methacholine-induced tone was clearly biphasic. In guinea-pig trachealis, rolipram produces a biphasic relaxation of carbachol and histamine-induced contractions (Harris et al., 1989), which was converted into a monophasic relaxation in the presence of a PDE III inhibitor. Apparently, combined PDE III and PDE IV inhibition more effectively relaxes guinea-pig trachea than selective inhibition of either of these isoenzymes alone. As described above, in bovine trachea PDE IV constitutes a greater proportion of the Ca^{2+}/calmodulin independent low K_m cyclic AMP PDE than in guinea-pig tracheal smooth muscle. This may explain why rolipram is a more effective relaxant of histamine-induced contractions in bovine trachea. PDE III is not only present in small quantities in bovine tracheal smooth muscle it also appears to be functionally relatively unimportant. PDE III inhibitors do not effectively elevate cyclic AMP in bovine trachea (Hall et al., 1989) and the present study has shown them to be singularly ineffective in causing relaxation. The small relaxation of methacholine-induced contractions produced by low concentrations of Org 9935 may be a reflection of the presence of some PDE III activity.

Zaprinast was weakly active as a relaxant, indicating the relative lack of functional significance of PDE V, at least when guanylate cyclase tone is low, in bovine tracheal smooth muscle. Previously, zaprinast has been reported to be completely ineffective in relaxing methacholine-induced contractions of bovine tracheal smooth muscle preparations (Hall et al., 1990). However, in this earlier study a higher concentration of methacholine was used to contract the preparations than in the present study. With the exception of Org 9935 and IBMX, all the PDE inhibitors tested more effectively relaxed histamine – than methacholine – contracted preparations. The preferential relaxation of histamine-contracted preparations is in agreement with previous findings (Torphy et al., 1988). The ability of PDE inhibitors to relax tracheal preparations is crucially dependent upon the degree of muscarinic agonist-induced tone (Torphy et al., 1988). This may explain the activity of zaprinast and also why Org 9935 and IBMX were equieffective relaxants of both methacholine and histamine in the present study.

Org 30029, despite showing similar potency to milrinone as a PDE IV inhibitor, was more effective as a tracheal relaxant. This compound is not only a dual PDE III/IV inhibitor (Shahid & Nicholson, 1990) it also produces Ca^{2+} sensitization in skinned cardiac muscle (Miller & Steele, 1990). Additional properties may explain the potency of this compound as a tracheal relaxant.

In summary, the present study has shown bovine tracheal smooth muscle to contain representatives of all five presently identifiable PDE isoenzyme families. In comparison with guinea-pig and canine trachealis, PDE III has a very low activity in bovine trachealis. Whereas the PDE IV inhibitor rolipram is a potent relaxant of bovine tracheal preparations, PDE III inhibitors are relatively inactive. This contrasts with tracheal preparations from other species in which PDE III and PDE IV inhibitors are approximately equieffective relaxants. In view of these species differences and because PDE inhibitors have potential utility as drugs for the treatment of asthma, clarification of both the relative activity levels and functional significance of PDE isoenzymes in human airways is important.

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Noradrenaline release in the rat vena cava is inhibited by γ-aminobutyric acid via $GABA_B$ receptors but not affected by histamine

Dörthe Schneider, [1]Eberhard Schlicker, [2]Barbara Malinowska & Gerhard Molderings

Institut für Pharmakologie und Toxikologie, Rheinische Friedrich-Wilhelms-Universität Bonn, Reuterstr. 2 b, D-5300 Bonn 1, Germany

1 Segments of the rat vena cava preincubated with [^{3}H]-noradrenaline were superfused with [^{3}H]-noradrenaline-free solution containing desipramine and corticosterone and the effects of γ-aminobutyric acid (GABA) receptor ligands and of histamine on tritium overflow evoked by transmural electrical stimulation were studied.

2 GABA inhibited, and histamine failed to affect, the electrically (0.66 Hz) evoked tritium overflow both in the absence and presence of rauwolscine (which was present in the superfusion medium in the subsequent experiments). The effect of GABA was less pronounced at a stimulation frequency of 2 Hz.

3 The inhibitory effect of GABA (pIC_{35} 5.83) on the electrically (0.66 Hz) evoked overflow was mimicked by the $GABA_B$ receptor agonist, **R**-(−)-baclofen (6.07) and less potently by **S**-(+)-baclofen (3.30) and the $GABA_A$ receptor agonist, muscimol (3.70). The concentration-response curve of GABA was shifted to the right by the $GABA_B$ receptor antagonist, CGP 35348 (P-(3-aminopropyl)-P-diethoxymethyl-phosphinic acid; apparent pA_2 4.76), but not affected by the $GABA_A$ receptor antagonist, (−)-bicuculline methiodide 100 μmol l^{-1}. Given alone, (−)-bicuculline methiodide slightly increased, and CGP 35348 did not affect, the evoked overflow.

4 The effect of GABA in veins from rats treated for 14 days with **RS**-baclofen (10 mg kg^{-1}, i.p. once daily) did not differ from that in veins from rats which received the vehicle instead. The effect of GABA also did not differ in veins from rats treated once either with **RS**-baclofen or with its vehicle.

5 The results suggest that the postganglionic sympathetic nerve fibres in the rat vena cava are endowed with presynaptic $GABA_B$ receptors, but not with receptors for histamine. (−)-Bicuculline methiodide increases noradrenaline release by a mechanism not related to $GABA_A$, $GABA_B$, α_2-receptors or noradrenaline uptake.

Keywords: Rat vena cava; postganglionic sympathetic neurones; noradrenaline release; γ-aminobutyric acid; presynaptic $GABA_B$ receptors; histamine; baclofen; (−)-bicuculline methiodide; CGP 35348; muscimol

Introduction

Presynaptic $GABA_B$ receptors have been shown to be present on postganglionic sympathetic nerve fibres innervating vessels of the rabbit (Starke & Weitzell, 1980; Anwar & Mason, 1982; Manzini *et al.*, 1985) and the goat (Miranda *et al.*, 1989). Recently, we found that the neurogenic vasopressor response in the pithed rat is inhibited by γ-aminobutyric acid (GABA) via $GABA_B$ receptors (Kohlenbach & Schlicker, 1990) and that the maximum extent of inhibition (50%) is at least as high as that in the studies on rabbit and goat vessels quoted above. Thus, it was tempting to examine the possible occurrence of $GABA_B$ receptors also in an *in vitro* vascular preparation of the rat. We used the superfused vena cava in which a series of presynaptic receptors has been characterized in previous papers from our group (Göthert & Kollecker, 1986; Göthert *et al.*, 1986; Molderings *et al.*, 1987), thus allowing a direct comparison between the effects of GABA and other neurotransmitters on noradrenaline release.

In addition, the effect of histamine on noradrenaline release in the superfused rat vena cava was studied. So far, an H_3 receptor-mediated effect of histamine on noradrenaline release from blood vessels has not been described. However, the existence of presynaptic H_3 receptors on postganglionic sympathetic nerve fibres innervating vessels is suggested by the findings of Ishikawa & Sperelakis (1987) who showed that histamine, via H_3 receptors, inhibited the amplitude of the electrically evoked excitatory junction potentials in the guinea-pig mesenteric artery.

Preliminary accounts of the present results were given to the 7th International Symposium on Vascular Neuroeffector Mechanisms (Schlicker *et al.*, 1990a) and to the 32nd Spring Meeting of the Deutsche Gesellschaft für Pharmakologie und Toxikologie (Schneider, 1991).

Methods

The cranial segment of the inferior vena cava of male Wistar rats was dissected and ligated at both ends. Subsequently, the vein was incubated for 30 min in 1.5 ml physiological salt solution (37°C, composition see below) containing (−)-[ring-2, 5, 6-^{3}H] noradrenaline 0.1 μmol l^{-1} (specific activity 42.1–43.7 Ci mmol^{-1}) and then mounted vertically in an organ bath (tension adjusted to 0.5 g) between two parallel platinum electrodes (1.5 cm long). The adventitial surface of the vein was superfused with [^{3}H]-noradrenaline-free physiological salt solution of 37°C at a rate of 2 ml min^{-1}. The composition of the solution was (mmol l^{-1}): NaCl 118, Na_2HPO_4 1.2, $NaHCO_3$ 25.0, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, glucose 11, ascorbic acid 0.3, Na_2EDTA 0.03 (aerated with 95% O_2 and 5% CO_2). Throughout superfusion, the solution contained desipramine 0.6 μmol l^{-1} and corticosterone 40 μmol l^{-1} for inhibition of neuronal and extraneuronal noradrenaline uptake, respectively. Five periods of electrical stimulation (240 rectangular pulses of 100 mA and 0.3 ms at 0.66 or 2 Hz) were applied to each vein after 93 (S_1), 117 (S_2), 141 (S_3), 165 (S_4) and 189 (S_5) min of superfusion. The superfusate was collected

[1] Author for correspondence.
[2] Permanent address: Zakład Farmakodynamiki, Akademia Medyczna, Mickiewicza 2 C, 15-222 Białystok 8, Poland.

in 3 or 6 min fractions. The radioactivity in the superfusate samples and veins was determined by liquid scintillation counting.

Calculations and statistics

Tritium efflux was expressed as a fraction of the tritium present in the vein at the start of the respective collection period (fractional rate of tritium efflux). Basal ^3H efflux was expressed as the ratio of the fractional rate during the collection period immediately before S_3 (t_3), S_4 (t_4) or S_5 (t_5) over that immediately before S_2 (t_2). Stimulation-evoked overflow of tritium was calculated by subtraction of the basal efflux (assumed to decrease linearly from the collection period before to that 12–15 min after onset of stimulation) from the total efflux during the 12 min subsequent to the onset of stimulation. Evoked ^3H overflow was given as a percentage of tissue tritium at the start of stimulation, and the ratios of the overflow evoked by S_3, S_4 or S_5 over that evoked by S_2 were determined. In order to determine the effects of histamine and GABA receptor ligands (*in vitro*) on basal and stimulation-evoked tritium overflow, the ratios t_3/t_2, t_4/t_2 or t_5/t_2 and S_3/S_2, S_4/S_2 or S_5/S_2 obtained in the presence of the drug under study were compared to the corresponding ratios obtained in its absence. For determination of the effects of rauwolscine (*in vitro*) and of **RS**-baclofen (*in vivo*), the fractional rate of tritium efflux during t_2 or the tritium overflow evoked by S_2 in veins exposed to the drug under study was compared to the respective parameter in untreated veins.

Results are given as means ± s.e.mean. Student's *t* test for unpaired data was used for comparison of mean values. If two or more experimental series were compared to the same control series, the *t* test was subjected to Bonferroni's procedure. The apparent pA_2 value of CGP 35348 was calculated according to formula 4 of Furchgott (1972).

Drugs used

γ-Aminobutyric acid (GABA), (−)-bicuculline methiodide, corticosterone, muscimol (Sigma, Munich, F.R.G.); **R**-(−)-, **S**-(+)-baclofen hydrochloride, **RS**-baclofen, CGP 35348 (P-(3-aminopropyl)-P-diethoxymethyl-phosphinic acid), desipramine hydrochloride (CIBA-Geigy, Wehr, F.R.G. or Basle, Switzerland); histamine dihydrochloride (Merck, Darmstadt, F.R.G.); rauwolscine hydrochloride (Roth, Karlsruhe, F.R.G.).

Results

Basal tritium efflux

For quantification of the basal tritium efflux, the fractional rate of ^3H efflux during t_2 or the ratios t_3/t_2, t_4/t_2 and t_5/t_2 (t_n/t_2) were determined (for further details, see Methods). The fractional rate of ^3H efflux during t_2 was not affected by rauwolscine 1 μmol l^{-1} *in vitro* or by *in vivo* treatment of rats with **RS**-baclofen (or its vehicle; Table 1). The ratios t_3/t_2, t_4/t_2 and t_5/t_2 were 0.96 ± 0.03, 0.88 ± 0.02 and 0.85 ± 0.03, respectively, in control experiments (i.e. GABA receptor ligands and histamine absent) performed on vascular segments exposed to rauwolscine (n = 8–9); these ratios were not changed by omission of rauwolscine from the superfusion medium or by **RS**-baclofen (or its vehicle) administered *in vivo* (results not shown). The ratio t_n/t_2 was not affected by histamine or the GABA receptor ligands in the whole concentration-range (results not shown).

Electrically evoked tritium overflow

To quantify the electrically evoked tritium overflow, the ^3H overflow evoked by S_2 or the ratios S_3/S_2, S_4/S_2 and S_5/S_2 (S_n/S_2) were determined (for further details, see Methods). Tritium overflow evoked by S_2 was markedly increased by rauwolscine 1 μmol l^{-1}, but not affected by *in vivo* treatment of rats with **RS**-baclofen (or its vehicle; Table 1). The ratios S_3/S_2, S_4/S_2 and S_5/S_2 were 1.03 ± 0.04, 1.01 ± 0.06 and 0.93 ± 0.07, respectively, in 7–9 controls (histamine and GABA receptor ligands absent) superfused in the presence of rauwolscine 1 μmol l^{-1}. Similar values were obtained for control experiments on vascular segments superfused in the absence of rauwolscine or on preparations obtained from rats which were pretreated with **RS**-baclofen (or its vehicle; Table

Table 1 Effects of drugs administered *in vivo* (**RS**-baclofen) or *in vitro* (rauwolscine) on the basal and electrically (0.66 Hz) evoked tritium overflow and on the GABA-mediated inhibition of the latter in rat vena cava segments preincubated with [^3H]-noradrenaline

Compound administered in vivo	*Rauwolscine* (μmol l^{-1}) *administered* in vitro	*Basal ^3H efflux* (t_2)[a] *expressed as fractional rate* (% × min^{-1})[b]	*Evoked ^3H overflow* (S_2) *expressed as % of tissue tritium*[c]	*Evoked ^3H overflow* (S_5/S_2) *No GABA*	*GABA* (10 μmol l^{-1})
—	—	0.20 ± 0.01	1.42 ± 0.11	1.15 ± 0.08	0.51 ± 0.06†
—	1[d]	0.19 ± 0.01[e]	3.65 ± 0.27[f,*]	0.93 ± 0.07	0.40 ± 0.04†
Vehicle, 1 injection[g]	1	0.16 ± 0.01	4.81 ± 0.62	0.96 ± 0.10	0.41 ± 0.05†
RS-baclofen (10 mg kg^{-1}) 1 injection[g]	1	0.19 ± 0.01	3.74 ± 0.45	1.08 ± 0.05	0.43 ± 0.06†
Vehicle, 14 injections[h]	1	0.18 ± 0.02	3.65 ± 0.26	0.93 ± 0.05	0.46 ± 0.04†
RS-baclofen (10 mg kg^{-1}) 14 injections[h]	1	0.18 ± 0.01	4.57 ± 0.40	0.91 ± 0.07	0.51 ± 0.03†

The veins were superfused with [^3H]-noradrenaline-free physiological salt solution containing desipramine 0.6 μmol l^{-1} and corticosterone 40 μmol l^{-1} throughout superfusion and GABA in three increasing concentrations 9 min before and during S_3, S_4 and S_5. Five 6 min periods of transmural electrical stimulation (S_1–S_5) were applied in each preparation (after 93, 117, 141, 165 and 189 min of superfusion) and the ratios of the ^3H overflow evoked by S_3, S_4 and S_5 over that evoked by S_2 were determined. Only the ratios S_5/S_2 obtained in the absence or presence of GABA 10 μmol l^{-1} are shown. Means ± s.e.mean of 4–28 experiments.

* $P < 0.001$, compared to the value without rauwolscine, † $P < 0.005$, compared to the corresponding control ('NO GABA').

[a] t_2 represents the 3-min period of superfusate sampling immediately before S_2.

[b] Efflux of tritium per min, expressed as fraction of tissue tritium.

[c] Evoked overflow in excess of basal efflux.

[d] Present in the superfusion fluid from 90 min of superfusion onward.

[e] Corresponding to 0.18 ± 0.02 nCi min^{-1}.

[f] Corresponding to 2.14 ± 0.27 nCi.

[g] Rats received one i.p. injection of **RS**-baclofen or its vehicle (water) 24 hours before decapitation.

[h] Rats received i.p. injections of **RS**-baclofen or its vehicle (water) for 14 days (once daily); the last injection was administered 24 h before decapitation.

1 or not shown). In the following paragraphs, the S_3/S_2, S_4/S_2 and S_5/S_2 values obtained in the presence of GABA receptor ligands and histamine relative to the respective control values are considered.

GABA inhibited the electrically (0.66 Hz) evoked tritium overflow in a concentration-dependent manner; the extent of inhibition was virtually the same when rauwolscine 1 μmol l^{-1} was present in, or omitted from, the superfusion medium (Figure 1a). Histamine failed to affect the evoked overflow both in the absence and presence of the α_2-adrenoceptor antagonist (Figure 1a). Rauwolscine was present in each of the experiments to be described now. The inhibitory effect of GABA was less pronounced when the frequency of stimulation used to evoke tritium overflow was 2 instead of 0.66 Hz (Figure 1b).

The inhibitory effect of GABA on the electrically (0.66 Hz) evoked tritium overflow was mimicked by the $GABA_B$ receptor agonist, R-(−)-baclofen and, less potently, by its S-(+)-enantiomer and by the $GABA_A$ receptor agonist, muscimol

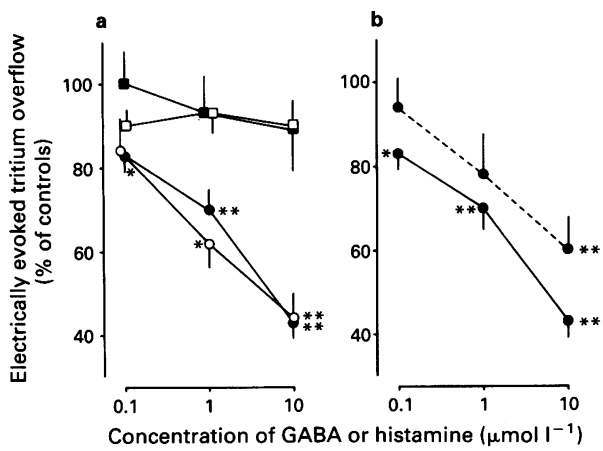

Figure 1 Effect of γ-aminobutyric acid (GABA) and/or histamine on the electrically evoked tritium overflow from rat vena cava segments preincubated with [^{3}H]-noradrenaline (a) in the presence or absence of rauwolscine and (b) with different frequencies of stimulation. The veins were superfused with [^{3}H]-noradrenaline-free physiological salt solution containing desipramine 0.6 μmol l^{-1} and corticosterone 40 μmol l^{-1}. Five periods of transmural electrical stimulation (S_1–S_5; 240 pulses each) were applied at 0.66 or 2 Hz. The ratios of the ^{3}H overflow evoked by S_3, S_4 or S_5 over that evoked by S_2 are given, expressed as percentages of the ratios obtained in the respective control experiments (not shown). GABA or histamine was added to the superfusion fluid at increasing concentrations 9 min before and during S_3, S_4 and S_5; rauwolscine 1 μmol l^{-1} was present in the superfusion fluid from 3 min before S_1 onward. Explanation of symbols: circles, GABA; squares, histamine; open symbols, no rauwolscine; filled symbols, rauwolscine 1 μmol l^{-1}; solid line, 0.66 Hz; broken line, 2 Hz. Means of 4–18 experiments with s.e.mean shown by vertical lines. *$P < 0.05$; **$P < 0.005$ (compared to the corresponding ratios in the respective control experiments).

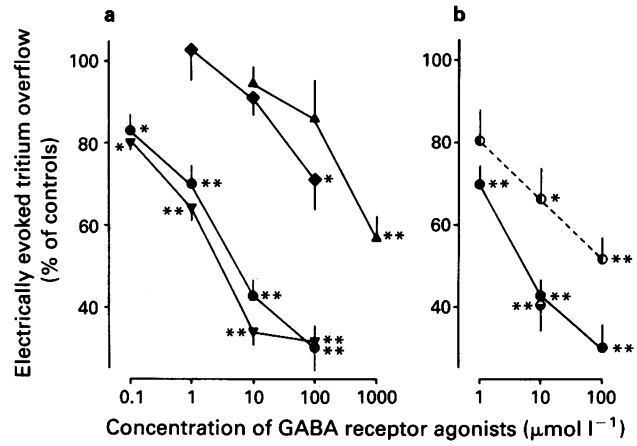

Figure 2 Effect of γ-aminobutyric acid (GABA) receptor agonists on the electrically (0.66 Hz) evoked tritium overflow from rat vena cava segments preincubated with [^{3}H]-noradrenaline (a) and interaction of GABA with GABA receptor antagonists (b). The veins were superfused with [^{3}H]-noradrenaline-free physiological salt solution containing desipramine 0.6 μmol l^{-1} and corticosterone 40 μmol l^{-1}. Five 6 min periods of transmural electrical stimulation were applied (S_1–S_5). The ratios of the ^{3}H overflow evoked by S_3, S_4 or S_5 over that evoked by S_2 are given, expressed as percentages of the ratios obtained in the respective control experiments (not shown). Rauwolscine 1 μmol l^{-1} and, if relevant, the $GABA_A$ receptor antagonist, (−)-bicuculline methiodide 100 μmol l^{-1} or the $GABA_B$ receptor antagonist, CGP 35348 100 μmol l^{-1} were present in the superfusion fluid from 3 min before S_1 onward. GABA (◒: in the presence of bicuculline, ◐: in the presence of CGP 35348, ●: in the absence of either antagonist), R-(−)-baclofen (▼), S-(+)-baclofen (▲) or muscimol (◆) was added to the superfusion fluid at increasing concentrations 9 min before and during S_3, S_4 and S_5. Means of 4–18 experiments; vertical lines show s.e.mean. *$P < 0.05$; **$P < 0.001$ (compared to the corresponding ratios in the respective control experiments).

(Figure 2a). The maximum degree of inhibition obtainable with GABA or R-(−)-baclofen was about 70%. Since an inhibition by 35% may, therefore, represent the half-maximum effect, IC_{35} values were determined from Figure 2a to quantify the potencies of the four agonists (in the case of muscimol, the IC_{35} value was extrapolated). IC_{35} values for R-(−)-baclofen, GABA, muscimol and S-(+)-baclofen were 0.85, 1.48, 200 and 500 μmol l^{-1}, respectively. The effect of GABA 10 μmol l^{-1} was not affected by the $GABA_A$ receptor antagonist, (−)-bicuculline methiodide 100 μmol l^{-1}, whereas the $GABA_B$ receptor antagonist, CGP 35348 100 μmol l^{-1} shifted to the right the concentration-response curve of GABA (Figure 2b; apparent pA_2 value: 4.76). Given alone, (−)-bicuculline methiodide 30 and 100 μmol l^{-1} increased the evoked overflow by 35 and 36%, respectively, whereas CGP 35348, in the same concentration-range, had no effect (Table 2).

In another series of experiments, we examined whether acute or chronic treatment of rats with RS-baclofen affects the

Table 2 Effects of (−)-bicuculline methiodide and CGP 35348 on the electrically (0.66 Hz) evoked tritium overflow from rat vena cava segments preincubated with [^{3}H]-noradrenaline

	Electrically evoked ^{3}H overflow (% of controls)		
GABA receptor antagonist	S_3/S_2	S_4/S_2	S_5/S_2
(−)-Bicuculline methiodide[a]	118 ± 11	135 ± 12*	136 ± 11*
CGP 35348[a]	103 ± 3	98 ± 5	95 ± 4

The veins were superfused with [^{3}H]-noradrenaline-free physiological salt solution containing desipramine 0.6 μmol l^{-1} and corticosterone 40 μmol l^{-1}. Five 6 min periods of transmural electrical stimulation were applied to each preparation (S_1–S_5). The ratios of the ^{3}H overflow evoked by S_3, S_4 or S_5 over that evoked by S_2 are given, expressed as percentages of the ratios obtained in the respective controls (not shown). Rauwolscine 1 μmol l^{-1} was present in the superfusion medium from 3 min before S_1 onward; the GABA receptor antagonist under study was added to the medium in three increasing concentrations 9 min before and during S_3, S_4 and S_5. Means ± s.e.mean of 6–13 experiments.

* $P < 0.02$ (compared to the corresponding control).

[a] Concentration during S_3, S_4 and S_5: 10, 30 and 100 μmol l^{-1}, respectively.

effect of GABA 0.1–10 μmol l^{-1} on the electrically (0.66 Hz) evoked tritium overflow. Treatment of animals with **RS**-baclofen for 14 days did not change the effect of GABA on tritium overflow in isolated vena cava segments compared to preparations from animals which had received the vehicle instead (Table 1 or not shown; $n = 7$–10). There was also no difference in the effect of GABA between veins from animals which received only one injection of **RS**-baclofen or of its vehicle (Table 1 or not shown; $n = 4$–6).

Discussion

The electrically evoked tritium overflow from segments of the rat vena cava (which represents quasi-physiological noradrenaline release; Göthert *et al.*, 1986) was inhibited by GABA, but not affected by histamine. When the effects of both drugs were studied in the presence of rauwolscine, virtually the same results were obtained as in its absence. Thus, the α_2-adrenoceptor antagonist could be included in the superfusion fluid in order to increase stimulation-evoked noradrenaline release. With respect to the experiments with histamine, there was also another reason to add a drug with α_2-adrenolytic properties to the superfusion medium. Thus, the H_3-receptor-mediated inhibitory effect of histamine on noradrenaline release in superfused rat brain cortex slices and pig retina discs was unmasked or at least increased by rauwolscine or the unselective α-adrenoceptor antagonist, phentolamine but not by the α_1-adrenoceptor antagonist, prazosin (Schlicker *et al.*, 1989; 1990b; unpublished results). The failure of histamine to inhibit noradrenaline release in the present model suggests that the postganglionic sympathetic nerve fibres innervating the rat vena cava are not endowed with presynaptic H_3 receptors.

Both the data obtained with GABA receptor agonists and antagonists demonstrate that the effect of GABA is mediated via $GABA_B$ receptors. Thus, the relative potencies of the GABA receptor agonists in the present model closely resemble their relative affinities for $GABA_B$ binding sites on rat whole brain synaptic membranes (Bowery *et al.*, 1983). The relative potencies (IC_{35} value of **R**-(−)-baclofen divided by the IC_{35} value of the respective GABA receptor agonist) for **R**-(−)-baclofen, GABA, muscimol and **S**-(+)-baclofen were 1, 0.57, 0.004 and 0.002, respectively, and the relative affinities (IC_{50} of **R**-(−)-baclofen divided by IC_{50} of the respective GABA receptor agonist) were 1, 1.02, 0.003 and 0.001, respectively. The effect of GABA in the present model was not affected by a high concentration of the $GABA_A$ receptor antagonist, (−)-bicuculline methiodide, whereas the recently characterized $GABA_B$ receptor antagonist, CGP 35348 (Olpe *et al.*, 1990) produced a rightward shift of the concentration-response curve of GABA. The apparent pA_2 value (4.76) closely resembles its pIC_{50} value for the inhibition of $GABA_B$ binding in rat brain cortex membranes (4.47; Olpe *et al.*, 1990; determined with the agonist ligand [^{3}H]-aminopropylphosphinic acid); it was almost identical to its pA_2 value against **RS**-baclofen for its inhibitory effect on the field stimulation-induced twitch response in the rat vas deferens (5.0; Hills *et al.*, 1991a) and only slightly lower than its pA_2 value against the $GABA_B$ receptor agonist, 3-aminopropylphosphinic acid, for its inhibitory effect on the electrically induced contraction in the rat anococcygeus muscle (5.38; Hills *et al.*, 1991b). The $GABA_B$ receptor-mediated effect of GABA in the rat vena cava was attenuated when the frequency of stimulation was increased from 0.66 to 2 Hz; a frequency-dependence was also obtained for effects mediated via $GABA_B$ autoreceptors (Raiteri *et al.*, 1989; Baumann *et al.*, 1990) and heteroreceptors (mediating inhibition of 5-hydroxytryptamine (5-HT) release; Schlicker *et al.*, 1984) in the rat brain cortex.

The inhibitory effect of GABA on noradrenaline release in the rat vena cava could be directly compared to the effects of two other neurotransmitters which have been examined under almost identical conditions (stimulation frequency of 0.66 Hz). Thus, the maximum extent of inhibition produced by GABA (by 70%) was only slightly less than that produced by noradrenaline (by 80%; effect mediated via α_2-adrenoceptors; Göthert & Kollecker, 1986; Molderings & Göthert, unpublished results) and markedly higher than that produced by 5-HT (by 30%; effect mediated via 5-HT_{1B} receptors; Göthert *et al.*, 1986; Molderings *et al.*, 1987). The maximum effect shown by **R**-(−)-baclofen in the present model was identical to that at the $GABA_B$ autoreceptor in rat brain cortex slices (stimulation frequency: 0.5 Hz; Waldmeier *et al.*, 1988). Furthermore, the potency of **R**-(−)-baclofen in the present model (pIC_{35} 6.07) was only slightly lower than at the $GABA_B$ autoreceptor in the rat brain (pIC_{50} 6.43; stimulation frequency: 0.25 Hz; Baumann *et al.*, 1990). With respect to the marked effect of GABA in the vena cava both in terms of the maximum degree of inhibition and the potency, the question arises whether GABA might also act on these receptors *in vivo*. However, the results obtained with CGP 35348 are not in favour of the idea that the $GABA_B$ heteroreceptors in the present model are activated tonically by endogenous GABA. In this case, one would have expected that CGP 35348, given alone, would increase noradrenaline release by interruption of the inhibition produced by endogenous GABA (e.g., a facilitation of noradrenaline release in the present model was obtained with rauwolscine suggesting that the presynaptic α_2-adrenoceptors are tonically activated by endogenous noradrenaline). Although a physiological function of the $GABA_B$ receptors in the rat vena cava cannot be postulated on the basis of the present study, one has to consider that, besides the sympathetic nervous system, there are other sources for GABA (e.g., blood cells, endocrine and exocrine cells; for review, see Erdö & Wolff, 1990) which might provide GABA *in vivo*.

Unlike CGP 35348, (−)-biculline methiodide increased noradrenaline release. This effect is not related to the blockade of $GABA_A$ receptors which could not be detected in the present study. The facilitatory effect of (−)-bicuculline methiodide on noradrenaline release is also not due to blockade of neuronal or extraneuronal uptake or α_2-adrenoceptors since the relevant experiments were carried out in the presence of desipramine, corticosterone and rauwolscine, respectively. The effect of (−)-bicuculline methiodide is reminiscent of its facilitatory effect on the electrically induced contractions in the guinea-pig ileum (Bartolini *et al.*, 1990) and might be related to direct nonsynaptic actions on neuronal membranes shown for high concentrations of bicuculline (Heyer *et al.*, 1981).

Finally, we examined whether long-term treatment of rats with **RS**-baclofen produces a down-regulation of the $GABA_B$ receptors. However, treatment of rats with RS-baclofen 10 mg kg^{-1} (i.p., once daily) for 14 days (or for 1 day) failed to affect the inhibitory effect of GABA in the vena cava. In two previous studies on mice, chronic treatment with **RS**-baclofen according to the same protocol had reduced the inhibitory effect of baclofen on 5-HT release (Gray *et al.*, 1986) and the number of $GABA_B$ binding sites in preparations of the cerebral cortex (Suzdak & Gianutsos, 1986).

In conclusion, the present study extends our previous one in the pithed rat (Kohlenbach & Schlicker, 1990) in several respects. First, release of the neurotransmitter was determined instead of end organ responses; second, the present study shows that the $GABA_B$ receptors are located presynaptically on the postganglionic sympathetic nerve fibres; third, $GABA_B$ receptors are not restricted to resistance vessels, but also occur in the vena cava. These $GABA_B$ receptors do not appear to be activated tonically by endogenous GABA and did not show down-regulation after chronic treatment of rats with **RS**-baclofen.

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Characterization of the interaction of R 56865 with cardiac Na- and L-type Ca channels

[1]D. Wilhelm, *H. Himmel, *U. Ravens & T. Peters

Janssen Research Foundation, Raiffeisenstraße 8, D-4040 Neuss 21 and *Pharmakologisches Institut, Universität-Gesamthochschule Essen, Hufelandstraße 55, D-4300 Essen 1, Germany

1 In isolated cardiac muscle, submicromolar concentrations of R 56865 (N-[1-[4-(4-fluorophenoxy)-butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine) have been shown to attenuate the toxicity of cardiac glycosides.

2 We studied the influence of R 56865 on calcium and sodium currents in single isolated ventricular cardiomyocytes. The effect of R 56865 on action potential and contractile force in the presence of increased sodium load was also tested by exposing papillary muscles to veratridine or *Anemonia sulcata* toxin ATX II.

3 The calcium current was not affected by R 56865 as assessed in slow action potentials of papillary muscles and current measurements in ventricular cardiomyocytes.

4 In papillary muscles, R 56865 (1 μmol l^{-1}) abolished veratridine-induced aftercontractions and afterdepolarizations without affecting the profound prolongation of the action potential. When pretreated with R 56865, the occurrence of afterdepolarizations was prevented and the decline of the resting membrane potential was attenuated.

5 Pretreatment with R 56865 (1 μmol l^{-1}) did not counteract the ATX II-induced prolongation of the action potential.

6 The sodium current (Na_o 30 mmol l^{-1}) was concentration-dependently decreased by R 56865 (0.1–10 μmol l^{-1}). The blocking effect was more pronounced at less negative holding potentials.

7 Our results demonstrate that the protective effect of R 56865 against veratridine-induced electrical and mechanical oscillations is not due to a direct effect on the calcium current. A potential-dependent inhibition of the sodium current may contribute. Additional sites of action, like interference with intracellular calcium release and inhibition of potassium currents, remain to be investigated.

Keywords: Ca and Na currents; R 56865; veratridine; guinea-pig heart

Introduction

R 56865 (N-[1-[4-(4-fluorophenoxy)-butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine) has been found to prevent electrical and mechanical signs of cardiac glycoside toxicity in the guinea-pig papillary muscle (Vollmer *et al.*, 1987). The compound attenuates the glycoside-induced shortening of the action potential duration and the decrease in action potential amplitude and prevents the occurrence of delayed afterdepolarizations. With respect to changes of mechanical parameters induced by a toxic concentration of ouabain, R 56865 hinders the development of a 'contracture' (rise in diastolic and decline in systolic tension) and the occurrence of arrhythmias and aftercontractions. These effects of R 56865 cannot be explained by an interference with the Na-K-ATPase or by a direct interaction with binding of ouabain at the receptor level (Heers *et al.*, 1988). From these results it may be speculated that R 56865 inhibits the cardiac glycoside-induced gain in sodium and calcium. In order to test this hypothesis the influence of R 56865 was studied on calcium-dependent slow action potentials and on the calcium current in ventricular myocytes. The possible interference of R 56865 with the sodium influx was tested indirectly by exposing the cardiac tissue to either veratridine or *Anemonia sulcata* toxin ATX II, compounds known to increase the intracellular sodium load (Honerjäger & Reiter, 1975; Ravens, 1976). Furthermore, the effect of R 56865 on the sodium current was assessed in isolated ventricular myocytes of the guinea-pig.

[1] Author for correspondence.

Methods

Measurements in isolated papillary muscles

Experimental set-up Right ventricular papillary muscles of about 1–2 mm in diameter were obtained from guinea-pig hearts. The muscles were fixed horizontally in a 2.5 ml tissue chamber between the lever of a force transducer (Gould U 2 cell) and a teflon clamp. In order to initiate action potentials, the muscles were electrically stimulated at 1 Hz with square wave pulses of 1 ms duration and 20% above threshold voltage via two platinum electrodes close to the muscle base. The organ bath was continuously perfused with modified Tyrode solution (8 ml min^{-1}), the temperature was maintained at 35 ± 0.2°C. The composition of the Tyrode solution (in mmol l^{-1}) was as follows: NaCl 124.0, KCl 4.0, $CaCl_2$ 0.9, $MgCl_2$ 1.1, $NaHCO_3$ 25.0, NaH_2PO_4 0.42, glucose 11.0 gassed with 95% O_2 and 5% CO_2 (pH 7.3–7.5). Tension was measured via a force transducer and documented on a pen recorder (WeKaGraph 1100 R). Resting tension was adjusted to about 5 mN in order to obtain maximal contractile force. The intracellular potential was measured against a silver/silver chloride electrode in the organ bath with conventional glass electrodes (10–20 MOhm), filled with KCl 3 mol l^{-1}. The potential difference was measured by a preamplifier (Hugo Sachs Elektronik) with a capacitance compensated, high impedance input and displayed on the screen of a storage oscilloscope (Tektronix 7D20). The recordings were collected by an analog-digital converter and transferred to an IBM host computer. The parameters resting membrane potential (RMP), action potential amplitude (APA), action potential duration (APD), maximal rate of rise ($\dot{V}_{max}$) and contractile

force (CF) were calculated by means of computer programmes developed in our department.

Experimental protocol After an equilibration period of 90 min in normal Tyrode solution, the perfusion solution was changed to veratridine-containing Tyrode solution (0.8 or 2.5 μmol l^{-1}). The influence of veratridine on electrical and mechanical parameters was observed for 60 min before R 56865 (1 μmol l^{-1}) was added in the continuous presence of veratridine and 45 min allowed for incubation. Additionally, experiments were carried out with veratridine in the absence of R 56865 for 105 min to correct for the effect of veratridine over time.

In another set of experiments, papillary muscles were pretreated with R 56865 (1 μmol l^{-1}) for 45 min during the equilibration period and then veratridine (2.5 μmol l^{-1}) was added for 105 min.

Furthermore, the effect of ATX II (0.01 μmol l^{-1}) was studied in the papillary muscle for 60 min. In case of pretreatment, R 56865 (1 μmol l^{-1}) was added to the Tyrode solution during the equilibration period for 45 min and then ATX II (0.01 μmol l^{-1}) was administered for 60 min.

Slow action potentials were induced by the following experimental protocol: after an equilibration period of at least 90 min during which normal action potentials were recorded, the extracellular KCl concentration was increased to 25 mmol l^{-1}. In the presence of isoprenaline (1 μmol l^{-1}) calcium-dependent slow action potentials were recorded. R 56865 was added to the superfusion medium at concentrations ranging from 0.01 to 10 μmol l^{-1}.

The data are expressed as means ± s.e.mean ($n = 6$). Statistical significance was evaluated by the Mann-Whitney U test and is indicated with an asterisk; $P < 0.05$ was taken as significant.

Measurements in single cardiomyocytes

Cell isolation and experimental set-up Guinea-pig ventricular cardiomyocytes were isolated by enzymatic dissociation according to the method described by Bendukidze *et al.* (1985). In order to measure the membrane currents by means of the 'whole-cell patch-clamp' technique (Hamill *et al.*, 1981), with a List EPC-7 amplifier, isolated cardiomyocytes were transferred into a small superfusion chamber mounted on the stage of an inverted microscope (details in Cragoe *et al.*, 1987). The liquid junction potential was corrected for electronically before the seal was established. After disruption of the membrane, the series resistance was compensated to a level just before 'ringing' (> 50%), the current signals were filtered at 3 kHz. At the beginning of each experiment, a depolarizing ramp pulse (4 V s^{-1}, 5 ms duration) was applied to determine the membrane capacity. Assuming a specific membrane capacitance C_s of 1 μF cm^2 (Marty & Neher, 1983), the surface area A of the cell was calculated according to $A = I/[(dU/dt)C_s]$. All membrane currents were normalized to the cell surface.

Measurement of calcium current I_{Ca} Cardiomyocytes with a mean surface area (± s.e.mean) of 11,792 ± 1596 μm^2 ($n = 6$) were chosen. The microelectrodes used had a mean resistance of 3.3 ± 0.3 MOhm, the mean series resistance was 9.7 ± 0.9 MOhm. The pipette solution consisted of (in mmol l^{-1}) CsCl 140.0, $MgCl_2$ 4.0, EGTA 10.0, HEPES 10.0, Na_2ATP 4.0.

The superfusion solution contained (in mmol l^{-1}): NaCl 150.0, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 2.5, HEPES 10.0 and glucose 10.0. With a frequency of 0.5 Hz, clamp pulses of 400 ms duration from −40 mV to +10 mV were applied subsequent to a 100 ms prepulse from the holding potential of −80 mV to −40 mV to inactivate the sodium channels. After 6 min of stabilization, each cell was exposed to one concentration of R 56865 for 6 min followed by a wash-out period of at least 6 min. At the end of each period, we determined the current-voltage relation in the range of −40 to +50 mV.

Measurement of sodium current I_{Na} The cardiomyocytes chosen ($n = 24$) had a mean surface area (± s.e.mean) of 10,140 ± 1940 μm^2. The microelectrodes used had a mean resistance of 2.2 ± 0.3 MOhm, the mean series resistance was 6.0 ± 1.2 MOhm. The experiments were carried out at room temperature (20–22°C) using medium-size cardiomyocytes and microelectrodes with large tip openings in order to keep the voltage errors of the clamp as low as possible (Marty & Neher, 1983). The pipette solution consisted of (in mmol l^{-1}): CsCl 140.0, $MgCl_2$ 4.0, EGTA 10.0, HEPES 10.0 and Na_2ATP 4.0. The superfusion solution contained (in mmol l^{-1}): NaCl 30.0, CsCl 107.0, $CaCl_2$ 0.5, $CdCl_2$ 0.05, $MgCl_2$ 2.5, HEPES 10.0, glucose 10.0. Clamp pulses were applied at a frequency of 0.5 Hz from a holding potential of −80 mV to −20 mV (pulse duration 16 ms). These pulses activated the inwardly directed, TTX-sensitive I_{Na}. Each cell was maintained for 5 min under control conditions, then exposed to one concentration of R 56865 for 5 min, followed by a washout period. At the end of each of these periods, the dependence of I_{Na} on the holding potential V_H was determined by running the following pulse protocol: a 500 ms conditioning pulse from the holding potential of −80 mV to various conditioning potentials V_{con} ranging from −90 to −70 mV with a subsequent test clamp step to −20 mV (16 ms); in time-matched controls, this protocol was run in intervals of 2 min.

Data processing of I_{Ca} *and* I_{Na} The data shown are expressed as mean values ± s.e.mean of n experiments. Concentration-response curves were roughly estimated by fitting a four parameter logistic equation to the mean values (computer program GraphPAD, Motulsky, 1985). Thereby three of the four parameters were set constant, i.e. maximum = 1, minimum = 0, and slope = −1. The concentration at which half-maximum effects occur were expressed as pD_2-values. The non-parametric H-test was used in order to detect significant differences ($P < 0.05$) between grouped observations (in: Sachs, 1984).

Compounds and stock solutions

Analytical grade chemicals (Merck, Darmstadt, F.R.G.), dissolved in demineralized water, were used throughout. Compounds tested in the present study were as follows: veratridine (Sigma Chemie GmbH, Deisenhofen, F.R.G.) and R 56865 (=(N-[1-[4-(4-fluorophenoxy)-butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine) (Janssen Pharmaceutica, Beerse, Belgium). Stock solutions of veratridine and R 56865 (both 1 mmol l^{-1}) were prepared by using acidified water (10 ml l^{-1}, 1 N-HCl).

ATX II was kindly provided by Dr Béress (Inst. f. Toxokologie, Kiel, F.R.G.). The toxin was dissolved in demineralized water.

Results

Effect of R 56865 on slow action potentials of guinea-pig isolated papillary muscles

In order to investigate a possible interference of R 56865 with the slow inward current, experiments were performed in guinea-pig right ventricular papillary muscles partially depolarized by exposure to KCl 25 mmol l^{-1} which inactivates the fast sodium current. In the presence of isoprenaline (1 μmol l^{-1}), slow action potentials could be elicited from a resting potential of −45 ± 5 mV. Addition of R 56865 in concentrations ranging from 0.01–10 μmol l^{-1} did not influence the slow action potentials. The respective parameters are listed in Table 1.

Table 1 Slow action potentials in the presence of 25 mmol l^{-1} KCl and of isoprenaline (control) and the influence of R 56865

	Control	*R 56865* (10^{-8} mol l^{-1})	*Control*	*R 56865* (10^{-7} mol l^{-1})	*Control*	*R 56865* (10^{-6} mol l^{-1})	*Control*	*R 56865* (10^{-5} mol l^{-1})
RMP (−mV)	45 ± 4	48 ± 5	42 ± 5	45 ± 2	47 ± 3	45 ± 4	44 ± 1	50 ± 5
APA (mV)	54 ± 2	58 ± 4	56 ± 5	63 ± 7	63 ± 5	64 ± 7	65 ± 1	65 ± 1
APD_{50} (ms)	151 ± 13	161 ± 9	146 ± 6	159 ± 8	147 ± 8	157 ± 8	154 ± 10	155 ± 15
APD_{90} (ms)	174 ± 12	177 ± 8	175 ± 7	185 ± 5	171 ± 6	179 ± 6	175 ± 8	171 ± 6

RMP, resting membrane potential; APA, action potential amplitude; APD_{50} and APD_{90}, action potential duration at 50% and 90% repolarization. Mean values ± s.e.mean of 4–5 experiments.

Effect of R 56865 on the calcium current I_{Ca} in isolated ventricular cardiomyocytes of the guinea-pig

I_{Ca} was separated from other ionic currents by prepulse inactivation of the sodium channels and by replacement of K^+ by Cs^+ in order to reduce the potassium currents. Because of the lack of effect on slow action potentials in guinea-pig papillary muscles, only one concentration of R 56865 (10 μmol l^{-1}) was used in the single cell experiments. Figure 1a shows original current tracings obtained during a clamp step from −40 to +10 mV for 400 ms in the absence and presence of R 56865.

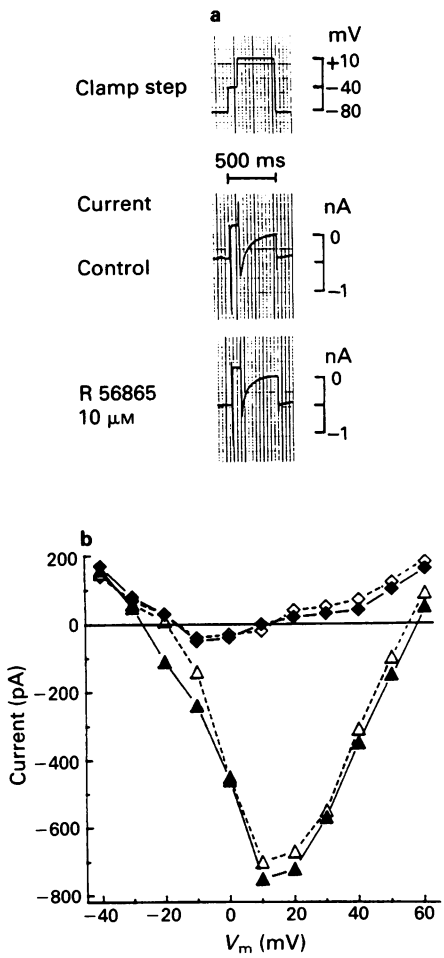

Figure 1 Effects of R 56865 on the calcium current I_{Ca}. (a) Original current tracings from a representative experiment. The clamp step was from −40 mV to +10 mV (400 ms) subsequent to a prepulse from the holding potential of −80 mV to −40 mV (100 ms). The current tracings were recorded at the end of the control period and after 6 min of exposure to R 56865 (10 μmol l^{-1}), respectively. (b) Current-voltage relation for I_{Ca} under control conditions (filled symbols) and in the presence of 10 μmol l^{-1} of R 56865 (open symbols). Peak currents (triangles) and currents measured at the end of the 400 ms clamp step (diamonds) from the same experiment as in (a) and plotted as a function of the clamp potential V_m. Similar results were obtained in 2 other experiments with cardiomyocytes from different hearts.

Neither peak currents nor currents measured at the end of the clamp step were affected by R 56865 as shown in the current-voltage relationship in Figure 1b.

Effect of R 56865 on veratridine-induced electrical and mechanical alterations in guinea-pig papillary muscles

A possible influence of R 56865 on the cellular sodium load was investigated by experiments with two compounds known to lead to an increased sodium influx, ATX II and veratridine. After 105 min of exposure, veratridine (2.5 μmol l^{-1}) caused a profound prolongation of APD_{90} from 195 ± 8 ms to 715 ± 35 ms (n = 6), a depolarization from −82 ± 4 mV to −62 ± 6 mV, a decrease in APA from 99 ± 5 mV to 64 ± 5 mV, and the occurrence of early afterdepolarizations associated with aftercontractions (Figure 2a). Veratridine (2.5 μmol l^{-1}) strongly increased force of contraction from 0.2 ± 0.1 mN to 1.7 ± 0.3 mN after 60 min of exposure (Figure 3). Toxicity manifested itself in the occurrence of extrasystoles and aftercontractions, by an increase in diastolic tension by 0.4, 0.6, and 0.4 mN in 3 out of 6 experiments, and by a decline in systolic tension (final value: 1.3 ± 0.2 mN; Figures 3 and 4).

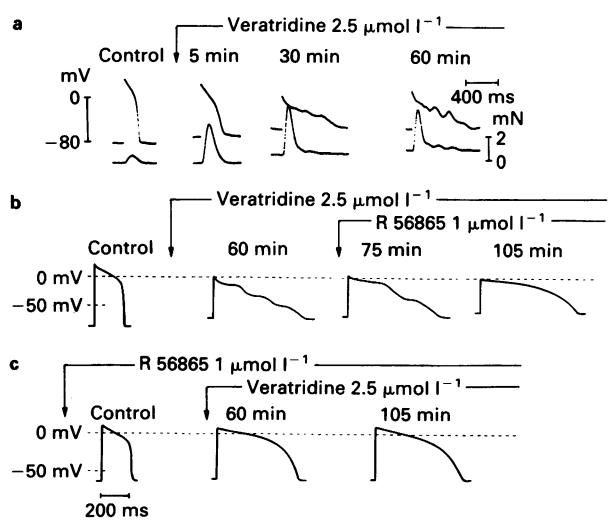

Figure 2 Redrawn recordings of action potentials (a–c) and contractile force (a) of a guinea-pig papillary muscle. Stimulation rate was 1 Hz: (a) shows alterations of the action potential configuration and force of contraction in the presence of veratridine (2.5 μmol l^{-1}). Action potential duration is increased accompanied by a rise in force of contraction. After 60 min of exposure to veratridine, electrical and concomitant mechanical oscillations and a shift of the resting membrane potential are prominent. The inhibitory effect of R 56865 (1 μmol l^{-1}) on veratridine-induced afterdepolarizations is shown in (b). Pretreatment with R 56865 (1 μmol l^{-1}) prevents the development of afterdepolarizations and the shift of the resting membrane potential (c). R 56865 has no effect on the prolongation of the action potential duration by veratridine.

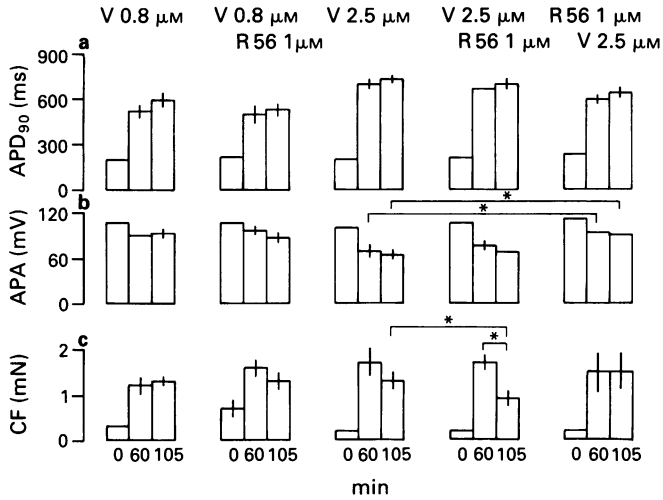

Figure 3 Effect of 60 and 105 min of exposure to veratridine (V 0.8 μmol l^{-1}, 1st and 2nd data set arranged vertically) on action potential duration (APD_{90}, a), action potential amplitude (APA, b), and contractile force (CF, c). The influence of R 56865 (R 56 1 μmol l^{-1}) given post or prior veratridine is shown in the 2nd and 4th, and the 5th data set arranged vertically, respectively. Statistical significance of differences versus veratridine value is indicated by asterisks (*$P < 0.05$). The veratridine-induced significant differences versus control value are not indicated for sake of clarity.

Addition of R 56865 (1 μmol l^{-1}) on top of veratridine after 60 min resulted in the abolition of the early afterdepolarizations and a decline in force of contraction (final value: 0.9 ± 0.2 mN; $P < 0.05$) (Figure 2–Figure 4). Early afterdepolarization occurred in all experiments with veratridine (2.5 μmol l^{-1}) and were abolished by R 56865 in 5 out of 6 experiments. R 56865 was not able to counteract the veratridine-induced alterations in APD_{90} and APA (Figure 3). A lower concentration of veratridine (0.8 μmol l^{-1}) exerted a sustained positive inotropic effect accompanied by a prolongation of the action potential duration from 195 ± 11 ms to 592 ± 40 ms after 105 min ($n = 6$). The action potential amplitude declined over the time course of the experiment (Figure 3). No change in resting membrane potential occurred: control: -87 ± 1 mV versus veratridine: -87 ± 5 mV (105 min). Addition of R 56865 (1 μmol l^{-1}) on top of veratridine (0.8 μmol l^{-1}) did not affect the alterations by veratridine of the action potential duration and action potential amplitude. R 56865 did not impair the positive inotropic response to veratridine (0.8 μmol l^{-1}) (Figure 3).

R 56865 alone (1 μmol l^{-1}) did not affect the APD_{90} (control: 195 ± 8 ms; R 56865: 207 ± 14 ms; $n = 6$) the APA (control: 99 ± 4 mV; R 56865: 104 ± 1 mV) or the rate of rise (control: 270 ± 4 V s^{-1}; R 56865: 260 ± 8 V s^{-1}). In myocytes, 1 μmol l^{-1} R 56865 reduced I_{Na} to 70% of the control at a holding potential of -90 mV. This seeming discrepancy can be explained by differences in Na_0 and drug concentration and distribution in myocytes and papillary muscles. Furthermore, inhibition of I_{Na} and reduction of the rate of rise are not strictly parallel (Walton & Fozzard, 1979). Exposure to R 56865 prior to veratridine (2.5 μmol l^{-1}) prevented the occurrence of early afterdepolarizations and attenuated the decline in action potential amplitude (Figure 2). R 56865 did not influence the effect of veratridine on action potential duration (Figures 2 and 3). Force of contraction of untreated papillary muscles was 0.2 ± 0.1 mN which was not affected by R 56865 (1 μmol l^{-1}; 0.2 ± 0.1 mN). Pretreatment with R 56865 allowed the development of the positive inotropic response to veratridine without signs of extrasystoles and aftercontractions (1.5 ± 0.4 mN; 105 min) (Figure 3 and 4).

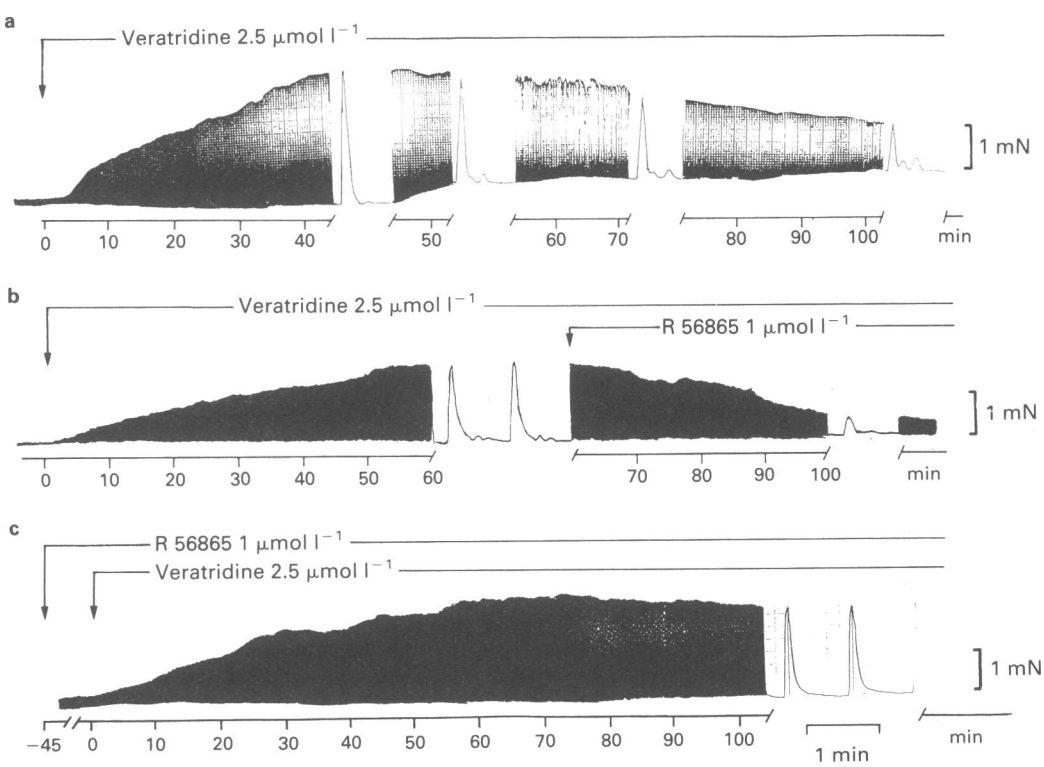

Figure 4 Force of contraction of the guinea-pig papillary muscle (stimulation rate: 1 Hz). (a) Mechanical recordings showing the positive inotropic effect of veratridine (2.5 μmol l^{-1}) accompanied by extrasystoles, aftercontractions, and an increase in diastolic tension. (b) R 56865 (1 μmol l^{-1}), given after 60 min of exposure to veratridine (2.5 μmol l^{-1}), causes a decline of contractile force accompanied by a marked reduction of aftercontractions. (c) Effect of pretreatment with R 56865 (1 μmol l^{-1}) on the veratridine-induced increase of contractile force. Aftercontractions did not occur. Time scale in lower right corner for recordings at higher paper speed which were redrawn for clarity.

Table 2 Electrophysiological characteristics of guinea-pig papillary muscles under the influence of *Anemonia sulcata* toxin (ATX II) and R 56865

	Control	*ATX II* (10^{-8} mol l^{-1})	*R 56865* (10^{-6} mol l^{-1}) *plus ATX II* (10^{-8} mol l^{-1})
RMP (−mV)	87 ± 5	88 ± 4	89 ± 5
APA (mV)	106 ± 7	110 ± 4	113 ± 5
APD_{50} (ms)	192 ± 4	221 ± 4*	227 ± 5*
APD_{90} (ms)	222 ± 5	251 ± 7*	265 ± 8*

RMP, resting membrane potential; APA, action potential amplitude; APD_{50} and APD_{90}, action potential duration at 50% and 90% repolarization.
Mean values ± s.e.mean of 4–5 experiments. *$P < 0.05$ to control.

Effect of R 56865 on ATX II-induced electrical alterations in guinea-pig papillary muscles

The *Anemonia sulcata* polypeptide ATX II (0.01 μmol l^{-1}) increased the action potential duration and augmented force of contraction (Ravens, 1976). Neither action potential amplitude nor resting membrane potential were altered by application of ATX II and pretreatment with R 56865 (1 μmol l^{-1}) did not counteract the effect of ATX II on action potential duration (Table 2).

Effect of R 56865 on the sodium current I_{Na} in isolated ventricular cardiomyocytes of the guinea-pig

The sodium current I_{Na} was isolated by blocking Ca^{2+} channels with Cd^{2+} (Rosenberg *et al.*, 1988) and by using Cs^+ instead of K^+ in order to inhibit the K^+ currents (Isenberg, 1976). Furthermoré, the extracellular sodium concentration was reduced to 30 mmol l^{-1} in order to facilitate potential control. At the end of the control period, the mean amplitude of I_{Na} was 3.5 ± 0.9 nA ($n = 24$), and the mean sodium current density was 343 ± 75 mA m^{-2}.

In control experiments ($n = 6$), we could demonstrate that I_{Na} was subject to a substantial, time-dependent decline in amplitude. The rate of this 'run-down' depended on the conditioning potential V_{con} (data not shown).

R 56865 (1 μmol l^{-1}) clearly reduced I_{Na} (inset, Figure 5). The extent of this reduction depended on both the concentration of R 56865 and the conditioning potential V_{con}: the sodium channel blocking effect was more pronounced at less negative conditioning potentials (see Figure 5).

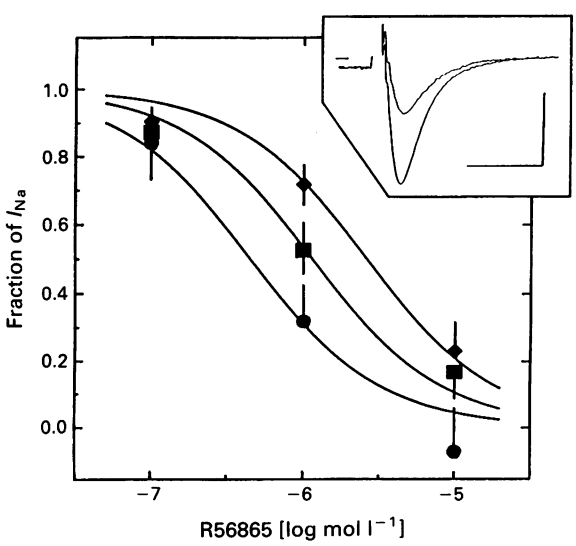

Figure 5 Potential- and concentration-dependence of the effects of R 56865 on the sodium current I_{Na}. Concentration-response relation for the effect of R 56865 on I_{Na} measured from various conditioning potentials V_H. The test clamp steps to −20 mV were preceded by 500 ms clamp steps to −90 mV (◆), −80 mV (■), and −70 mV (●), respectively. The currents were normalized to their respective amplitudes at the end of the control period (5 min) and corrected for the mean time-dependent decrease in I_{Na} occurring in time-matched controls. The application of a four parameter logistic equation to the data resulted in pD_2-values of 6.4 (V_H − 70 mV), 6.0 (V_H − 80 mV), and 5.6 (V_H − 90 mV) with the three other parameters assumed to be constant (maximum = 1, minimum = 0, slope = −1). The amplitudes of I_{Na} in the presence of 1 μmol l^{-1} R 56865 measured with clamp steps from −70 mV, −80 mV, or −90 mV do not originate from one single totality (H-test, $P < 0.05$). Mean from 5–6 experiments per concentration; vertical lines show s.e.mean. Inset: Original superimposed tracings of I_{Na} under control conditions and after 5 min exposure to R 56865 (1 μmol l^{-1}). Holding potential was −80 mV between stimuli (0.5 Hz); calibration bars: 3 ms, 3 nA; the small horizontal bar indicates the zero current level.

Discussion

The benzothiazolamine derivative R 56865 has been found to protect cardiac muscle against cardiac glycoside intoxication (Vollmer *et al.*, 1987; Schneider *et al.*, 1988), the electrical signs of which, e.g. action potential shortening and afterdepolarizations, and mechanical signs, e.g. rise in diastolic tension and aftercontractions, are thought to be due to intracellular sodium and/or calcium accumulation (Wit & Rosen, 1986). Therefore, R 56865 could elicit its protective effect by several mechanisms, including an interaction with the inhibitory effect of ouabain on the Na-K-ATPase; an influence on the Na-Ca-exchanger; a direct inhibition of the sodium and/or calcium influx via potential-dependent channels; or an interference with the calcium release from cellular stores. An interaction of R 56865 with ouabain at the Na-K-ATPase could be excluded since R 56865 does not modify the enzyme activity of a crude cardiac membrane Na-K-ATPase preparation (Heers *et al.*, 1988), nor does it influence the ouabain-induced inhibition of the sodium pump in human red blood cells (Finet *et al.*, 1989). In sarcolemmal vesicles prepared from bovine cardiac tissue, a direct influence of R 56865 on the Na-Ca-exchange was observed only with concentrations above 10 μmol l^{-1} (Heers, unpublished results). Theoretically, R 56865 could reduce intracellular calcium accomulation in the presence of a toxic concentration of cardiac glycosides (Wier & Hess, 1984) by directly inhibiting the calcium current. However, we have no experimental evidence for this possibility, even at the high concentration of 10 μmol l^{-1}.

In rat left atria, pretreatment with R 56865 prevented a rise in the cellular sodium content after exposure to a toxic concentration of ouabain (Wilhelm *et al.*, 1990). An inhibitory action of R 56865 on the sodium current could indirectly relieve the sodium load in an ouabain-intoxicated cardiac muscle. In our experiments with cardiomyocytes, R 56865 reduced the sodium current; however, the concentrations necessary were 2 to 3 orders of magnitude larger than those previously found to be protective (Vollmer *et al.*, 1987). The inhibition of the sodium current by R 56865 can nevertheless contribute to the protective effect because of its marked potential dependence. In the papillary muscle, R 56865 did

not affect the enhanced action potential duration in the presence of a non-toxic concentration of veratridine or ATX II. Under both conditions the resting membrane potential was unaltered. In the cardiomyocytes, a conditioning potential, less negative to the normal resting potential by only 20 mV, shifted the concentration-response curve of sodium current reduction to the left by almost one order of magnitude. If such a shift continues at even less negative potentials, it may be speculated that in the potential range of the action potential plateau, R 56865 may become effective at nanomolar concentrations. However, in this case, one would expect that veratridine could not maintain a prolonged plateau. This discrepancy suggests that possibly additional effects on potassium currents are involved. In a previous study, however, no effect of R 56865 on quasi steady-state potassium current was found (Himmel *et al.*, 1990). In any case, further experiments are needed to clarify this point.

One of the most prominent findings of our present study was the abolition of afterdepolarizations and aftercontractions by R 56865. In order to explain this effect, a brief outline of the mechanism of oscillations seems useful. Early and late afterdepolarizations occur under conditions that induce a large increase in the cytosolic calcium concentration with which the sarcoplasmic reticulum is unable to cope. Both ouabain and veratridine induce a marked sodium load albeit by different actions. The elevation of the intracellar sodium activity leads to an increase in the intracellular calcium concentration via the Na-Ca-exchange (Reuter & Seitz, 1968). This extra calcium is taken up by cellular stores from which it can be released upon following excitations. If the capacity limit of the stores to sequester calcium is reached, they become unstable and may release calcium spontaneously by an as yet unknown mechanism. The subsequent rise in the intracellular calcium concentration may induce a contractile response and a transient depolarization due to electrogenic calcium efflux via the sodium-calcium exchange (e.g. Noble, 1984; Arlock & Katzung, 1985; Mechmann & Pott, 1986; Wettwer & Ravens, 1991). It should also be mentioned that the contribution of non-specific leaky channels has been discussed (e.g. Kass *et al.*, 1978 a,b; Colquhoun *et al.*, 1981; Cannell & Lederer, 1986; Ehara *et al.*, 1988). According to this model, R 56865 could suppress potential and contractile oscillations by interference with the intracellular release mechanism of calcium; however, at present, we have no experimental evidence for a positive proof.

In addition to suppression of veratridine-induced early afterdepolarizations, R 56865 shifted the plateau of the action potential to more positive potentials indicating additional changes in net membrane current. Since inward currents were either inhibited (I_{Na}) or unaffected (I_{Ca}), this finding strongly suggests that R 56865 inhibits outward current. We have shown previously that the quasi steady-state potassium current is not altered (Himmel *et al.*, 1990), but no further positive evidence as to which potassium channel may be involved is at present available. A likely candidate is the sodium-activated potassium channel (Luk & Carmeliet, 1990).

Contractile force of untreated guinea-pig papillary muscles is not impaired by R 56865. Likewise, if veratridine is applied in a low concentration leading to a positive inotropic effect and a prolongation of the action potential duration without changing the resting membrane potential, R 56865 does not diminish the augmented force of contraction when added after veratridine. In contrast, the positive inotropism brought about by veratridine in the concentration of 2.5 $\mu mol\,l^{-1}$ is reduced when R 56865 is given on top of veratridine. The reason for the decline of contractile force is not clear; it may be speculated that a high concentration of veratridine changes the process of contraction, making it more susceptible to attenuation by R 56865.

In conclusion, our results demonstrate that the protective effect of R 56865 against veratridine-induced electrical and mechanical oscillations is not caused by a direct inhibitory effect on the calcium current. A potential-dependent inhibition of the sodium current may, however, contribute. As additional sites of action, the compound may interfere with the calcium release mechanism and inhibit potassium currents, but direct evidence for the latter two mechanisms awaits further investigations.

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Inhibition of the myocardial Ca^{2+}-current (I_{Ca}) by the enantiomers of DPI 201-106 and BDF 8784

[1]Ursula Ravens, Thomas Pfeifer, Erich Wettwer & Martin Grundke

Department of Pharmacology, University of Essen, Hufelandstraße 55, D-4300 Essen 1, Germany

1 We studied the stereoselectivity of the effects of the enantiomers of the cardiotonic agent DPI 201-106 (4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile, DPI) and its methyl-for-carbonitrile analogue BDF 8784 on cardiac calcium currents (I_{Ca}) of guinea-pig ventricular myocytes. The actions of the S- and R-enantiomers were compared with those of the racemate.

2 Racemic, S- and R-DPI depressed I_{Ca} in a concentration-dependent manner, the IC_{50} values were 1.1 μmol l^{-1} for racemic and S-DPI, and 1.2 μmol l^{-1} for R-DPI, respectively. Racemic, S- and R-BDF 8784 also reduced I_{Ca}, the respective IC_{50} values were 3.6 μmol l^{-1} for racemic BDF 8784, 1.3 μmol l^{-1} for S-BDF 8784 and 1.1 μmol l^{-1} for R-BDF 8784.

3 Neither the DPI nor the BDF 8784 enantiomers alter the time course of inactivation of I_{Ca}. The steady-state inactivation curve for I_{Ca} was shifted along the voltage axis to negative membrane potentials. The block of I_{Ca} was dependent on the membrane potential and increased with membrane depolarization.

4 Our findings indicate that DPI and BDF 8784 inhibit I_{Ca} in a non-stereoselective manner, which contrasts the opposite effects of the DPI enantiomers on Na^+ channels.

Keywords: Calcium channels; sodium channel modifying agents; stereoselectivity of effects; DPI 201-106; BDF 8784; cardiac myocytes; whole cell membrane currents; potential dependence of block

Introduction

The compounds DPI 201-106 (4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile, DPI) and BDF 8784 (4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-2-methyl-1H-indole) were originally studied because of their sodium channel modifying action and the concomitant changes in cardiac contractility (Scholtysik *et al.*, 1985; Buggisch *et al.*, 1985; Kohlhardt *et al.*, 1986). BDF 8784 differs from the DPI-molecule by the substituent in position 2 of the indol moiety (Figure 1). This structural modification leads to a pronounced change in the pharmacological profile. In guinea-pig papillary muscles, the racemate of BDF 8784 reverses the cardiotonic and action-potential prolonging effects of the racemate of DPI (Armah *et al.*, 1989). Furthermore, it depresses sodium currents in guinea-pig heart cells (Honerjäger *et al.*, 1988).

Although the enantiomers of DPI and BDF 8784 bind with similar affinities to the investigated sites in sodium channels (DPI: guinea-pig brain synaptosomes, Romey *et al.*, 1987; DPI and BDF 8784: guinea-pig brain membranes, Glossmann, personal communication), only DPI affects the gating of the channels in a stereoselective manner: it is the S-enantiomer which enhances Na^+ influx by inhibiting sodium channel inactivation, whereas the R-enantiomer blocks Na^+ influx (Scholtysik *et al.*, 1986; Romey *et al.*, 1987; Wang *et al.*, 1990). In contrast, both enantiomers of BDF 8784 block sodium channels (Honerjäger *et al.*, 1988).

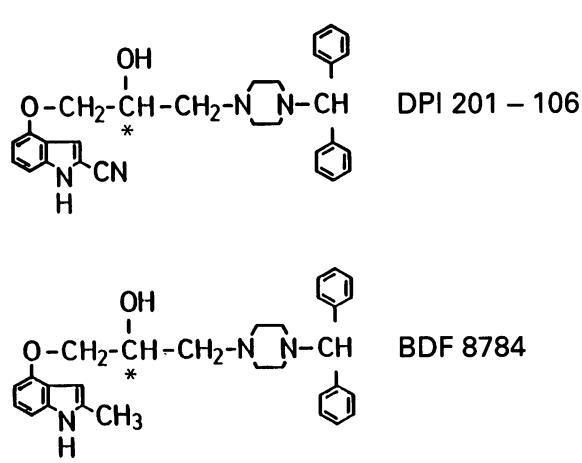

Figure 1 Chemical structure of DPI 201-106 and BDF 8784. The asterisk denotes the asymmetric carbon atom.

In addition to modifying sodium channels, the racemic mixtures of both DPI and BDF 8784 block cardiac calcium channels (Buggisch *et al.*, 1985; Siegl *et al.*, 1988; Holck & Osterrieder, 1988; Armah *et al.*, 1989). Furthermore, they displace radioligands from L-type calcium channels of skeletal muscle membranes (Zech *et al.*, 1989). This effect is highly stereoselective for BDF 8784. At the phenylalkylamine binding site, the affinity of the R-enantiomer is about 40 times higher than for the S-enantiomer, at the benzothiazepine binding site the affinity of the R-enantiomer is only 4 times higher than for the S-enantiomer (Zech *et al.*, 1989). For the DPI enantiomers, no differences in affinities were found at this site (Zech *et al.*, 1989).

From the binding characteristics it may be expected that the BDF 8784 but not the DPI enantiomers inhibit I_{Ca} stereoselectively. In spite of the potential clinical usefulness of this new class of inotropic agents (Thormann *et al.*, 1986), details about the stereoselectivity of their pharmacological effects on L-type Ca^{2+} channels are still lacking. We therefore have addressed this point by comparing the effects of the enantiomers of DPI and BDF 8784 on I_{Ca} in guinea-pig isolated cardiac myocytes. The respective racemates were investigated for comparative purposes. Some of the results have been presented in preliminary form (Pfeifer *et al.*, 1989).

Methods

Single myocytes from guinea-pig hearts were prepared by enzymatic dissociation as described previously (Borchard & Ravens, 1986) and stored at room temperature (20–25°C) until used. A small drop of cell suspension was added to the investigation chamber filled with modified Tyrode solution and placed on the stage of an inverted microscope (Olympus IMT-2). The majority of the experiments was carried out at

[1] Author for correspondence.

room temperature, with the exception of 9 experiments measuring the steady-state availability of I_{Ca} (see Results). The cells were superfused at a constant rate of 2 ml min^{-1}. In the modified Tyrode solution, CsCl was substituted for KCl in order to reduce K^+ currents (Isenberg, 1976). The solution (Cs-Tyrode) had the following composition (mmol l^{-1}): NaCl 137, CsCl 5.4, $MgCl_2$ 2.5, $CaCl_2$ 1.8, HEPES (hydroxyethylpiperazinylethane-sulphonic acid) 10 and glucose 10. The pH was adjusted with NaOH to 7.4. Stock solutions of racemic DPI 201-106 and BDF 8784 as well as their enantiomers (1 mmol l^{-1}) were prepared in dimethylsulphoxide (DMSO) and were further diluted by addition of Cs-Tyrode for each concentration investigated (0.3, 3 and 10 μmol l^{-1}). The DPI and BDF 8784 compounds are poorly soluble in aqueous solutions and strongly adhere to surfaces; thus, as in our previous work (Buggisch *et al.*, 1985; Armah *et al.*, 1989), nominal drug concentrations are given. In order to avoid contamination of the chamber with agent from the previous experiment, a freshly cleaned chamber and new connective tubings were used in each experiment. The investigation chambers were cleaned over night in detergent (Mucasol, Merz & Co, Frankfurt, Germany) followed by several times of rinsing with distilled water.

DPI was a gift from the Sandoz Company Basel, Switzerland (Prof. Dr G. Scholtysik); the BDF 8784 compounds were kindly provided by Dr B. I. Armah, Beiersdorf AG, Hamburg, Germany. All other chemicals were purchased from Merck, Darmstadt, Germany.

Membrane currents were recorded with the whole cell patch-clamp technique (Hamill *et al.*, 1981). Patch electrodes were pulled from filament borosilicate glass (Hilgenberg, Malsfeld, Germany). The tip resistances were in the range of 2–4 MΩ when filled with a solution of the following composition (mmol l^{-1}): CsCl 140, $MgCl_2$ 4, EGTA (ethylene-glycol-*bis*(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid) 10, HEPES 10 and Na_2-ATP 4. The pH was adjusted to 7.3 with CsOH.

The membrane potential was controlled by a single electrode voltage clamp circuit, using a LIST EPC-7 patch-clamp amplifier (LIST medical instruments, Darmstadt, Germany). Membrane currents were monitored and stored on a digital storage oscilloscope (4094A, Nicolet Instrument, Madison, Wisconsin, U.S.A.) and registered in parallel on a chart recorder (GOULD Brush 220, Gould Inc., Cleveland, Ohio, U.S.A.) for later evaluation. For the study of Ca^{2+}-currents, cells were kept at a holding potential (V_h) of −40 mV to inactivate the Na^+-current and were depolarized by clamp pulses of 300 ms duration to 0 mV at a frequency of 0.2 Hz. Five minutes after establishing the seal, a current-voltage relationship (*I-V* curve) was determined by increasing the test pulse amplitude in 10 mV steps between the holding potential and a final clamp potential of +70 mV at the same frequency and duration as the test pulses. The cells were then depolarized again regularly to 0 mV. Drug was added 8 min after the start of the experiment, and the time course of the effect on I_{Ca} (measured as the difference between peak inward and late outward current) was observed for further 5 min before a second *I-V* curve was obtained. All experiments were terminated after 15 min. In order to estimate the spontaneous decline of I_{Ca} with time ('run-down'), control recordings were obtained at time-matched intervals after establishing the seal. Results from cells showing more than 30% of run-down of I_{Ca} during the initial 5 min of the experiment were discarded. All data are expressed as mean values ± s.e.mean from at least 4 cells.

Results

Time course of the drug effects

With regular depolarizing clamp steps, the peak amplitude of I_{Ca} in control cells declined spontaneously ('run-down'). When I_{Ca} was normalized to the value at the end of the 8 min control period (=100%) it decreased to 87.2 ± 6.1% (n = 6) during the subsequent 7 min (Figure 2). The inset of Figure 2a shows a typical tracing of I_{Ca} from a cell before and after 7 min of exposure to R-DPI (10 μmol l^{-1}). The agent reduced peak I_{Ca} to a mean value of 12.8 ± 1.7% (n = 4) without affecting the time-course of inactivation. However, the current at the end of the 300 ms clamp pulse was also reduced. Essentially similar results were obtained with R-BDF 8784 (10 μmol l^{-1}) which decreased I_{Ca} to 11.5 ± 7.4% (Figure 2b). The peak amplitude of I_{Ca} also declined after exposure to racemic DPI and S-DPI (Figure 2a) and the respective compounds of BDF 8784 (Figure 2b, 10 μmol l^{-1} each).

Current-voltage relationship (I-V curves) after exposure to DPI and BDF 8784

In order to investigate the drug effects on I_{Ca} over a larger voltage range, the currents were measured in response to clamp pulses to different potentials (−40 mV to +70 mV). The difference current obtained by substracting the amplitude of the current at the end of the clamp pulse from the peak inward current amplitude was taken as I_{Ca} and plotted in Figure 3. *I-V* curves obtained in control cells after 5 and 13 min differed by 16.2 ± 5.2% (n = 6) in their maximum current amplitude ('run-down'). The potential of maximum activation of I_{Ca} was +10 mV in control cells and did not change with time. The effects of S- and R-DPI and S- and

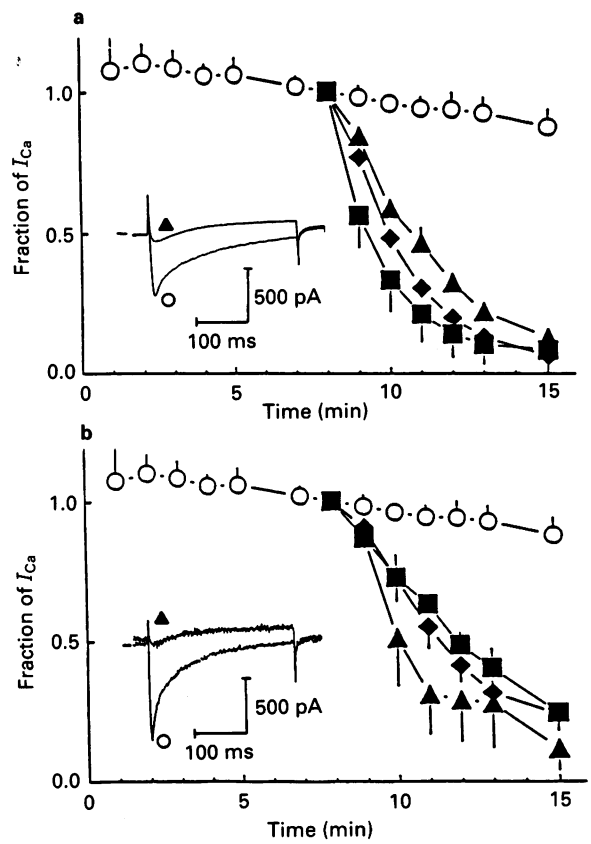

Figure 2 Time courses of I_{Ca} under control conditions and after exposure to the racemate and the enantiomers of DPI 201-106 or BDF 8784 (10 μmol l^{-1}). Clamp pulses were applied at a frequency of 0.2 s^{-1}, the holding potential was −40 mV, the clamp step potential 0 mV, duration 300 ms. Temperature 22°C. The amplitude of I_{Ca} 8 min after seal establishing and rupture of the cell membrane was normalized to 1. (a) Time courses after addition of racemic DPI 201-106 (■), R-DPI 201-106 (▲) and S-DPI 201-106 (◆), and of controls with solvent only (○). (b) Time courses after addition of racemic BDF 8784 (■), R-BDF 8784 (▲) and S-BDF 8784 (◆), same controls as in (a). Mean values from 4–6 cells; vertical lines show s.e.mean. Insets: Current tracings in response to standard clamp pulses before and 5 min after addition of R-DPI 201-106 and R-BDF 8784 (a and b, respectively). I_{Ca} was measured as the difference between the peak inward current amplitude and the current amplitude at the end of the 300 ms clamp step.

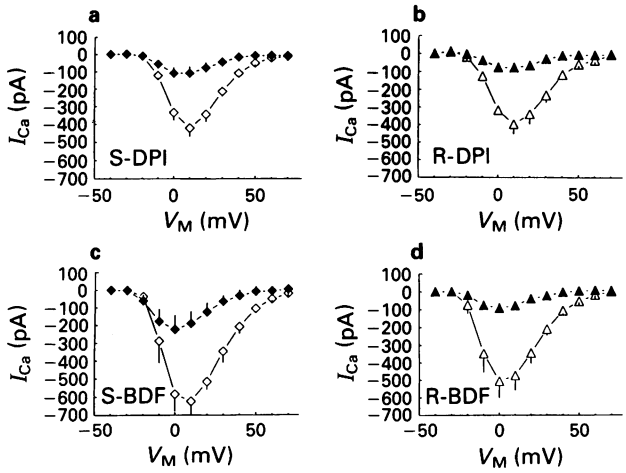

Figure 3 Effects of the enantiomers on the current-voltage relations of I_{Ca}. (a–d) I_{Ca} measured as described in Figure 2, before (open symbols) and 5 min after (closed symbols) addition of S-DPI 201-106 (a), R-DPI 201-106 (b), S-BDF 8784 (c) and R-BDF 8784 (d). The concentration of each agent was 3 μmol l^{-1}. Mean values from 4–6 cells; vertical lines show s.e.mean.

R-BDF 8784 (3 μmol l^{-1}) are shown in Figure 3a–d, respectively. Each enantiomer of both DPI and BDF symmetrically reduced the amplitude of I_{Ca} without shifting the *I-V* curve along the voltage axis in individual cells. The apparent shift by 10 mV in Figure 3c is due to calculating mean values.

Concentration-response curves for the depression of I_{Ca}

The degree of inhibition of I_{Ca} depended on the concentration of the investigated compound (Figure 4). In order to estimate quantitative differences (e.g. IC_{50} values), concentration-response curves were constructed on the assumption that each drug was able to depress I_{Ca} completely, the Hill coefficient was taken as -1. The IC_{50} values obtained under these conditions are summarized in the legend of Figure 4. The concentration-response curves were identical with the exception of the one for racemic BDF 8784, which lay further to the right along the concentration axis.

Steady-state inactivation of I_{Ca} after exposure to R-BDF 8784 and R-DPI and voltage -dependence of the block

In order to estimate the influence of the membrane potential on the block induced by R-DPI and R-BDF 8784, the steady-state availability of I_{Ca} was measured. From a holding potential of -100 mV, the membrane was depolarized to various potentials (range -60 mV to $+10$ mV) by conditioning prepulses of 2 s in duration. The remaining I_{Ca} was tested with a final clamp step of 300 ms in duration from -40 mV to 0 mV. Some of these experiments were performed at 37°C (5 controls, 1 with R-DPI and 3 with R-BDF). Since the resulting curves were not different from the ones measured at room temperature, the data were pooled for further analysis. The results are shown in Figure 5. In control runs before drug application, the potential for 50% inactivation of I_{Ca} ($V_{1/2}$) had a mean value of -23.9 ± 0.8 mV. In the presence of R-DPI (10 μmol l^{-1}), the inactivation curve was shifted to more negative membrane potentials by 12.6 ± 2.6 ($n = 6$). With R-BDF 8784 (10 μmol l^{-1}), the shift was of similar magnitude and direction, i.e. 12.0 ± 1.9 mV ($n = 4$).

From Figure 5 it is evident, that after depolarization of the membrane for 2 s to -60 mV in the presence of either R-DPI or R-BDF 8784, a small block of about 5–20% of the maximum Ca^{2+} current was still present. We have tested in a few experiments whether the block could be relieved if the membrane was held at more negative potentials for a longer period of time. With the conditioning potential set to

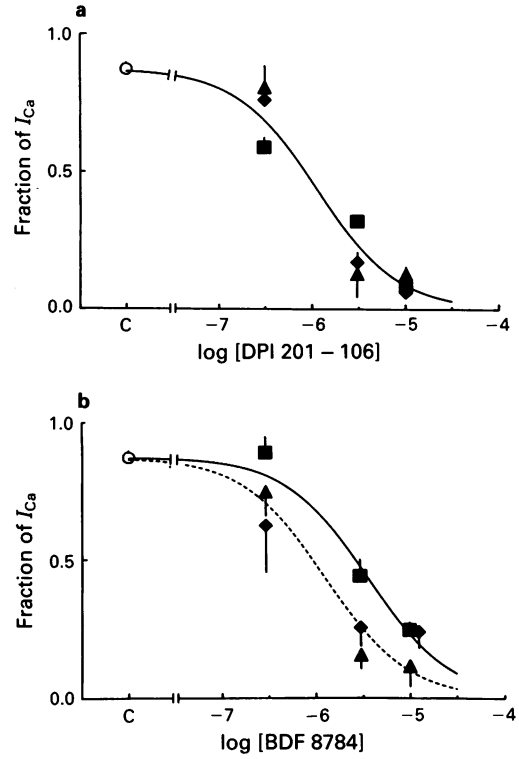

Figure 4 Concentration-response curves for the decrease in I_{Ca} by the DPI and BDF compounds. (a) Effects of racemic DPI 201-106 (■), R-DPI 201-106 (▲), and S-DPI 201-106 (◆). (b) Effects of racemic BDF 8784 (■), R-BDF 8784 (▲), and S-BDF 8784 (◆). I_{Ca} was measured at 7 min after the addition of each agent. The open symbol represents the time matched control value (○) which gives an estimate of the spontaneous decline of I_{Ca} in the control cells ('run-down'). The continuous concentration-response curves were fitted to the experimental data points by a nonlinear regression fit algorithm according to the method of Marquardt (GraphPad, ISI software). The following IC_{50} values (in μmol l^{-1}) were obtained for the racemate, the R- and the S-enantiomer: DPI 201-106, 1.1; 1,2; 1,1; BDF 8784, 3,6; 1.1; 1.3, respectively. IC_{50} values (in μmol l^{-1}) for the calculation of the curves in (a) and (b) were: 1.1 and 3.6 for continuous curves in (a) and (b) respectively and 1.3 for dashed curve in (b).

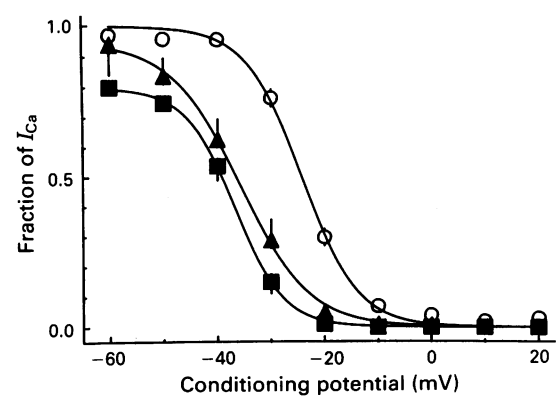

Figure 5 Steady-state inactivation of I_{Ca} (fraction of available I_{Ca}). The steady-state inactivation curve was measured with conditioning prepulses (duration 2 s) ranging from -60 mV to $+10$ mV. The holding potential was kept at -100 mV. The remaining I_{Ca} was tested with a final clamp step from -40 to 0 mV (duration 300 ms, 25 ms at -40 mV). The currents were normalized to the maximum amplitude obtained before the addition of drug. The control values 7–8 min after membrane rupture were pooled for both groups of cells (controls, ○). Measurements were then repeated 7 min after addition of R-DPI 201-106 (▲) and R-BDF 8784 (■), 10 μmol l^{-1}. The continuous curves were fitted to the experimental data points by use of a non-linear regression fit algorithm (GraphPad, ISI software). The mean potentials for $V_{1/2}$ were -23.9 ± 0.8 mV, controls, $n = 10$; -35.9 ± 2.0 mV, R-DPI, $n = 6$; -36.8 ± 1.4 mV, R-BDF 8784, $n = 4$.

-100 mV for 1 min, the block of I_{Ca} either with **R**-DPI or with **R**-BDF 8784 had disappeared (data not shown). Therefore, at a holding potential of -100 mV, I_{Ca} seemed fully available also in the presence of the agents, whereas I_{Ca} was strongly inhibited at a holding potential of -40 mV (compare Figure 4).

Discussion

The main findings of our study were that the racemate and the enantiomers of DPI and its analogue BDF 8784 inhibited I_{Ca} in guinea-pig cardiac myocytes in a non-stereoselective manner. The IC_{50}-value of 1.1 μmol l^{-1} for racemic DPI corresponds well with the value of 1.6 μmol l^{-1} reported by Holck & Osterrieder (1988), despite the fact that these authors increased concentrations cumulatively whereas we employed one concentration in each cell only. In our experiments, the racemic BDF 8784 seemed somewhat less potent than the enantiomers (Figure 4). However, this small difference in potency may well be an artifact due to the large scatter of results because of the poor solubility of the compounds in aqueous solution.

We have always observed a decrease in net current at the end of the clamp steps. At room temperature, I_{Ca} is known to inactivate more slowly (Isenberg *et al.*, 1987). Therefore, some residual I_{Ca} can be expected at the end of a clamp step of 300 ms duration due to incomplete inactivation, which is also reduced by the inhibitory effects of BDF 8784 and DPI 201-106. As an alternative explanation, the compounds may increase outward currents (potassium or background currents). For the time being, we cannot distinguish between these two possibilities.

The lack of stereoselectivity for the inhibitory effect of BDF 8784 on cardiac calcium currents was surprising since the enantiomers had different binding affinities for sites in the L-type calcium channels of skeletal muscle (Zech *et al.*, 1989). One possible explanation could be that there are differences between skeletal and heart muscle membranes in affinity of drugs to L-type calcium channels. No binding data for BDF 8784 enantiomers in heart muscle membranes are as yet available.

There is a substantial discrepancy between the K_i-values described for the binding of BDF 8784 and DPI on Ca^{2+}-channels (nanomolar range, Zech *et al.*, 1989) and the IC_{50} values given in this paper (micromolar range). Similar discrepancies between the concentration ranges of drug binding and drug effects have been found with the dihydropyridine Ca^{2+} channel blockers (Bean, 1984; Hamilton *et al.*, 1987). They are explained by the potential dependence of drug affinity for Ca^{2+} channels, i.e. the dihydropyridines bind with much higher affinity to inactivated channels in depolarized vesicular membranes used for binding assays than to resting channels in polarized cells. Differences between binding data for the enantiomers of BDF 8784 and our IC_{50} values could have an analogous explanation. The block of I_{Ca} by DPI and by BDF 8784 was clearly voltage-dependent and increased with membrane depolarization, which could reflect higher affinity of the binding sites when the channels are inactivated. Finally, the poor solubility of the compounds could contribute to the discrepancy between binding data reported and IC_{50} values measured in voltage clamp experiments, since in the binding experiments, membranes were incubated in pure DMSO to avoid aqueous solutions, whereas the Tyrode solution in the voltage clamp experiments contained only 1% DMSO.

Several agents previously thought to be selective modulators of sodium channels were found later also to influence Ca^{2+} channels (e.g. veratridine and batrachotoxin, Romey & Lazdunski, 1982). *Vice versa*, Ca^{2+} channel modulators like bepridil also modify Na^+ channels (Yatani *et al.*, 1986). Thus BDF 8784 and DPI are not unique in modifying both types of cation channels. The phenomenon may relate to the pronounced lipophilic properties of these drugs or it could indicate that Na^+ and Ca^{2+} channels have so many structural similarities (Hosey & Lazdunski, 1988) that they accommodate similar binding domains for these agents (Yatani *et al.*, 1988). However, since DPI affects Na^+ channels stereoselectively but Ca^{2+} channels non-stereoselectively, there must be differences in binding sites on both types of channel or in the access pathways to them.

The potential dependence of the block of Ca^{2+} channels was studied with the **R**-enantiomers only in order to avoid interference with current measurement from modified Na^+ channels. Both with **R**-DPI and **R**-BDF 8784, the block clearly depended on membrane potential. With conditioning pulses of 2 s duration, the apparent inactivation curve for I_{Ca} was shifted to more negative potentials with about 20% of the maximum current blocked by **R**-BDF 8784 at -60 mV. However, long lasting clamp pulses to -100 mV were sufficient for complete relief of the Ca^{2+} channels from the block.

In conclusion, although binding data from the literature suggest stereoselective actions at least for BDF 8784, we could not detect significant differences in the calcium channel blocking effects of the enantiomers of DPI and BDF 8784 and their racemic mixtures in guinea-pig cardiac myocytes.

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Endothelium-dependent and independent relaxation of the rat aorta by cyclic nucleotide phosphodiesterase inhibitors

[1]Narcisse Komas, [2]Claire Lugnier & Jean-Claude Stoclet

Université Louis Pasteur de Strasbourg, Laboratoire de Pharmacologie Cellulaire et Moléculaire, Faculté de Pharmacie, CNRS URA 600, BP 24 67401 Illkirch, France

1 The effects of selective inhibitors of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) phosphodiesterases (PDEs) were investigated on PDEs isolated from the rat aorta and on relaxation of noradrenaline (1 μM) precontracted rat aortic rings, with and without functional endothelium.

2 Four PDE forms were isolated by DEAE-sephacel chromatography from endothelium-denuded rat aorta: a calmodulin-activated PDE (PDE I) which hydrolyzed preferentially cyclic GMP, two cyclic AMP PDEs (PDE III and PDE IV) and one cyclic GMP-specific PDE (PDE V). The latter was selectively and potently inhibited by zaprinast. The two cyclic AMP PDEs were discriminated by specific inhibitors: one was inhibited by cyclic GMP (PDE III) and by new cardiotonic agents (milrinone, CI 930, LY 195115 and SK&F 94120); the other was inhibited by denbufylline and rolipram (PDE IV). None of these drugs significantly inhibited PDE I.

3 The PDE III inhibitors caused endothelium-independent relaxations of rat aortic rings with the following EC_{50} values (μM concentration producing 50% relaxation): LY 195115: 3.4, milrinone: 5.7, CI 930; 7.8, SK&F 94120: 14.7. Neither N^G-monomethyl-L-arginine (L-NMMA, 300 μM), an inhibitor of the L-arginine-NO pathway, nor L-arginine (1 mM) modified the effect of PDE III inhibitors. However, methylene blue (10 μM) an inhibitor of soluble guanylate cyclase abolished relaxation induced by PDE III inhibitors except in the case of compound CI 930.

4 The specific PDE IV and PDE V inhibitors both produced endothelium-dependent relaxations which were inhibited by L-NMMA and by methylene blue (10 μM). In the presence of L-NMMA, relaxation was restored by subsequent addition of L-arginine.

5 The relaxant effects of denbufylline and rolipram were studied in the presence of drugs stimulating either adenylate cyclase (forskolin and isoprenaline) or soluble guanylate cyclase (sodium nitroprusside, SNP), or inhibiting PDE III (milrinone). In endothelium-denuded rings, a relaxing effect of both denbufylline and rolipram was found in the presence of milrinone (EC_{50} values 1.7 and 12 μM, respectively) or SNP (EC_{50} values 12.3 and 124 μM, respectively), but not in the presence of forskolin or isoprenaline. However in the presence of functional endothelium, relaxations produced by PDE IV inhibitors were significantly potentiated by forskolin, isoprenaline, milrinone and SNP (respective EC_{50} values for denbufylline: 2, 2, 0.4 and 0.7 μM and for rolipram: 7, 13, 7 and 1.2 μM).

6 These results indicate that the relaxant effects of inhibitors of the cyclic AMP-specific PDE IV are markedly enhanced by cyclic GMP elevating agents and by the PDE III inhibitor milrinone. They support the hypothesis that cyclic GMP enhances cyclic AMP-mediated relaxation, possibly through the inhibition of the cyclic GMP-inhibited PDE III.

Keywords: Cyclic AMP; cyclic GMP; endothelium; PDE III inhibitors; PDE IV inhibitors; rat aortic rings; relaxation

Introduction

An increase in adenosine 3':5' cyclic-monophosphate (cyclic AMP) or guanosine 3':5'-cyclic monophosphate (cyclic GMP) level has been correlated to smooth muscle relaxations caused by different drugs in vascular smooth muscle (see Bär, 1974; Kukovetz *et al.*, 1981; Ignarro & Kadowitz, 1985). In most tissues, the concentrations and thus the actions of cyclic AMP and cyclic GMP are regulated by cyclic nucleotide phosphodiesterases (PDEs). Multiple PDE isoforms have been isolated from several tissues and they have been characterized by their substrate specificity, their modulation by calmodulin or by cyclic GMP, their pharmacological responses to drugs and their primary sequence. Recently Beavo & Reifsnyder (1990) proposed to classify these PDEs into five major families called PDE I, II, III, IV and V, this classification was used in the current paper.

We have previously isolated three PDEs from bovine aortic smooth muscle (Lugnier *et al.*, 1986): a calmodulin-sensitive enzyme belonging to type I PDEs, a cyclic AMP-specific PDE selectively inhibited by rolipram (ROI-PDE, type IV) and a PDE V selectively inhibited by zaprinast (cyclic GMP-PDE, type V). Recently, Lindgren *et al.* (1990) showed the existence of another PDE, a cyclic GMP-inhibited low K_m cyclic AMP-PDE (CGI-PDE, type III) in rat aortic tissue. This PDE is also present in the heart, where it is the target of new cardiotonic drugs of the milrinone type (Harrison *et al.*, 1986a,b; Reeves *et al.*, 1987; Komas *et al.*, 1989; Silver, 1989). Since these drugs cause important decreases in systemic arterial pressure and peripheral vascular resistance (Hayes *et al.*, 1985; Gristwood *et al.*, 1986a; Uretresky *et al.*, 1986; Murali *et al.*, 1987), it is of interest to investigate the respective role of PDE III and the other cyclic AMP or cyclic GMP-specific PDEs (PDE IV and PDE V) in vascular smooth muscle. The respective role of these enzymes is not clear in blood vessels.

In the current study, we have isolated the PDEs from the rat aorta, and we have investigated the relaxant effects on rat aortic rings of selective inhibitors of the PDEs found in this

[1] Present addresss: Laboratory of Cellular Metabolism, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

[2] Author for correspondence.

tissue. Since the PDEs of endothelial and smooth muscle cells from the same vessel may not be identical (Lugnier & Schini, 1990), the endothelium was removed from the aorta before isolation of the PDEs. In addition the influence of the endothelium derived relaxing factor (EDRF) discovered by Furchgott & Zawadzki (1980) was investigated in contraction experiments by use of aortic rings with and without functional endothelium and by inhibition of its production and effect. EDRF has been identified as NO (Palmer *et al.*, 1987) or an NO-conjugated thiol (Myers *et al.*, 1990) which stimulates guanylate cyclase. The relaxing effects of PDE inhibitors were thus studied in the absence and in the presence of N^G-monomethyl-L-arginine (L-NMMA, Rees *et al.*, 1989) an inhibitor of NO production from L-arginine (Palmer *et al.*, 1988), of L-arginine and of methylene blue, an inhibitor of guanylate cyclase (Martin *et al.*, 1985).

Methods

Isolation of aortic phosphodiesterases

Thoracic aortae from 20 female Wistar rats (350–400 g) were removed and cleaned of adventitial connective tissue. The vessels were then opened out lengthways to expose the luminal surface which was then repeatedly rubbed with blunt forceps to remove the endothelial cells. Tissue without endothelium (1.9 g) was then minced and homogenized with a glass pestle homogenizers in 6 vol. of buffer A (25 mM sodium phosphate pH 6.6, 0.25 M sucrose, 2 mM Mg acetate (MgAc), 1 mM dithiothreitol, 5 mM ethylene glycol *bis* (β-aminoethylether) N,N,N',N',tetraacetic acid (EGTA), 2000 u ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} trypsin inhibitor from soya bean). The homogenate was centrifuged at 105,000 *g* for 60 min. The supernatant was applied to an anion-exchange diethylaminoethyl (DEAE)-sephacel column and first eluted by buffer B (buffer A without EGTA) until no absorbance was detected in the eluate at 280 nm. The elution was performed at a flow rate of 30 ml h^{-1} with a linear gradient of NaCl (0–0.5 N) in buffer B. Fractions (3.5 ml/tube) were collected, assayed for PDE activity and dialyzed overnight against buffer C (25 mM sodium phosphate, pH 6.6, 2 mM MgAc, 1 mM EGTA). For long-term storage without loss of activity, bovine serum albumin (BSA, 1 mg ml^{-1}) was added to the desired fractions, which were then stored at −80°C.

Since DEAE-sephacel did not allow the separation of PDE V from PDE I (Figure 1a) as did DEAE-cellulose chromatography (Lugnier *et al.*, 1986), PDE V from DEAE-sephacel chromatography (fractions from 22 to 30) was repurified by high performance liquid chromatography (h.p.l.c.) (Figure 2) as reported previously (Komas *et al.*, 1991).

Phosphodiesterase assay

PDE activities were determined according to Wells *et al.* (1975), in the presence of calcium ($CaCl_2$, 10 μM) and an excess of calmodulin (18 nM), or in the absence of calmodulin and calcium but in the presence of 1 mM EGTA. Unless otherwise indicated, the substrate concentration ([^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP) was 1 μM. Both incubation time and enzyme concentration in the assay medium were adjusted so that no more than 15% of the substrate was hydrolyzed under the assay conditions. The concentrations of drugs that produced 50% inhibition of substrate hydrolysis (IC_{50}) were calculated by nonlinear regression from concentration-response curves including five or six concentrations of inhibitor. To prevent the influence of cross-contamination between PDE III and PDE IV, PDE inhibitors were tested in the presence of 100 μM rolipram (for PDE III) or 100 μM cyclic GMP (for PDE IV). In these conditions the activity of the contaminating form was inhibited by more than 97%. PDE inhibitors were dissolved in dimethylsulphoxide (DMSO) at a final concentration of 1%. At this concentration, DMSO had no significant effects on PDE activity.

Preparations of rat aortic rings

Female Wistar rats (350–400 g) were killed by cervical dislocation. Thoracic aortae were removed carefully to protect the endothelial cells, cleaned of adventitial connective tissue and cut into 2 to 2.5 mm wide transverse rings. The rings were mounted under 2 g of resting tension in organ baths containing 20 ml of modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.25, KH_2PO_4 1.14, $MgSO_4$ 1.19, glucose 10, $NaHCO_3$, 25, at 37°C and gassed with 95% O_2 and 5% CO_2. Tissues were allowed to equilibrate for 2 h with periodical washing before experiments were begun. Tension was measured with an isometric force transducer (Celaster, Lusignan, France).

The endothelium was removed from some aortic rings by gently rubbing the intimal surface with a wooden stick. Removal of functional endothelium was verified by the lack of any relaxation to acetylcholine (1 μM) in rings precontracted with noradrenaline (1 μM). Aortic preparations with functional endothelium exhibited at least 50% relaxation under the same conditions.

Measurement of aortic relaxation

Most experiments were done on sets of four rings from the same aorta: two rings with and two rings without endothelium. Tissues were contracted with 1 μM noradrenaline. When the contractile response reached a steady state, PDE inhibitors were added cumulatively. Relaxation produced by each concentration of PDE inhibitor was measured after 5 min incubation and value was expressed as a percentage of the initial noradrenaline-induced tone. The concentration of a PDE inhibitor giving 50% of relaxation (EC_{50}) was estimated by non-linear regression of each log concentration-effect curve.

The role of nitric oxide, NO, released from the endothelium was studied with N^G-monomethyl-L-arginine (L-NMMA). This compound was added at the concentration of 300 μM to one pair of rings 10 min before addition of 1 μM noradrenaline. Then responses were obtained to either increasing doses of PDE inhibitors as described above or to an EC_{50} dose of PDE inhibitors. Either L-arginine (1 mM) or solvent (water) was then added to each bath 10 min after the addition of the last concentration of PDE inhibitor. Methylene blue (10 μM) was added to each bath at the end of the experiment.

For the study of PDE IV inhibitors (rolipram and denbufylline) in combination with isoprenaline, forskolin, milrinone and sodium nitroprusside (SNP), contraction was elicited by $PGF_{2\alpha}$ (2 μM) in the presence of prazosin (0.1 μM) added 15 min prior to $PGF_{2\alpha}$. When contraction had stabilized after 20 min, an appropriate concentration of isoprenaline (0.1 μM), forskolin (3 μM), milrinone (30 nM) and SNP (1 nM) was then added to decrease the tension to 90% of the initial contraction. Ten min later, a concentration-response curve to rolipram or denbufylline was obtained. The net relaxation to rolipram or denbufylline was determined 5 min after administration of the drug and was expressed as a percentage of the initial tension. In preliminary experiments, denbufylline and rolipram caused similar relaxation whether the aortic rings were contracted with $PGF_{2\alpha}$ or noradrenaline.

Statistical analysis

Statistical comparisons were performed according to analysis of variance (ANOVA) followed by Newman-Keuls' test, or Student's *t* test. Results are expressed as the mean ± s.e.mean. In figures, vertical bars indicate ± s.e.mean and are not included when the size of the symbol exceeds the value of the s.e.mean. Statistical significance was assumed when $*P < 0.05$; $** P < 0.01$ or $*** P < 0.005$.

Drugs

[8-^{3}H]-cyclic AMP (30–50 Ci mmol^{-1}) and [8-^{3}H]-cyclic GMP (5–15 Ci mmol^{-1}) were obtained from New England Nuclear. Leupeptin and trypsin inhibitor from soya bean were purchased from Sigma. Aprotinin (Iniprol) was obtained from Choay (Paris). Noradrenaline bitartrate (Sigma) was dissolved in Milli Q water (Millipore) containing HCl 34 mM and Na_2SO_3 7.9 mM. A 14 mM stock solution of $PGF_{2\alpha}$ (Upjohn) was used and dilutions made daily in Millipore-treated water. Prazosin (Sigma) was prepared daily in Milli Q water. Forskolin (Sigma) and sodium nitroprusside (Merck) were diluted in absolute ethanol. Isoprenaline (Sigma) was diluted in Milli Q water. Milrinone (Sterling Wintrop), denbufylline (BRL 30892, Beecham-Wülfing), rolipram (Schering), CI 930 [3(2H)-pyridazinone, 4,5-dihydro-6-[4-(1h-imidazol-1-yl) phenyl]-5-methyl-monohydrochloride] (Warner-Lambert), LY 195115 (1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3 pyridazinyl)-2*H*-indole-2-one) (Lilly Research Laboratories) and SK&F 94120 (5-(4-acetamidophenyl)pyrazin-2(1H)-one) (Smith Kline & French Research) were dissolved in DMSO 100%. Zaprinast (M&B 22948, May Baker) was dissolved in NaOH 0.1 N giving a stock solution of 10 mM.

For experiments on aortic relaxation, the final concentrations of DMSO, NaOH and ethanol in the organ bath never exceeded 0.5%, 0.0001 N and 0.05% respectively.

Results

Effects of different phosphodiesterase inhibitors on aortic PDEs

DEAE-sephacel chromatography resolved two major peaks (Figure 1a) the first was activated by calmodulin and the second insensitive to calmodulin (Figure 1a and b). This latter fraction could be, in the presence of 100 μM rolipram or cyclic GMP, resolved into two PDEs: a cyclic GMP-inhibited PDE (PDE III, fractions from 46 to 50) and a rolipram-sensitive PDE (PDE IV, fractions from 52 to 59). The first peak eluted by DEAE-sephacel was repurified by h.p.l.c. (Figure 2) and resolved into a calmodulin-independent cyclic GMP-specific PDE (PDEV, fractions from 25 to 27) and a calmodulin-dependent cyclic GMP-PDE (PDE I, fractions from 28 to 31).

The non-catecholamine, non-glycoside cardiotonic agents (LY 195115, SK&F 94120, CI 930 and milrinone) which are currently being developed for the treatment of congestive heart failure, and other reference PDE inhibitors (rolipram, denbufylline and zaprinast) were tested on both PDE III, PDE IV and PDE V. The IC_{50} values obtained for these drugs are shown in Table 1. PDE III activity was potently inhibited by cardiotonic agents (IC_{50} around 1 μM). The order of potency was CI 930 > LY 195115 ≥ milrinone > SK&F 94120. Rolipram, denbufylline and zaprinast were ineffective on PDE III. In contrast, PDE IV activity was, as expected,

Table 1 Inhibition of PDE III, PDE IV and PDE V isolated from rat aorta by reference drugs (IC_{50}, μM)

Drugs tested	*PDE III*	*PDE IV*	*PDE V*
LY195115	0.7 ± 0.2	NS	74 ± 11
CI 930	0.2 ± 0.1	NS	68 ± 17
Milrinone	1.0 ± 0.3	NS	84 ± 16
SK&F 94120	1.5 ± 0.4	NS	NS
Denbufylline	NS	0.6 ± 0.2	11 ± 0.6
Rolipram	—	1.0 ± 0.2	407 ± 117
Zaprinast	NS	NS	0.8 ± 0.3

Results are the mean of at least three determinations obtained for three different enzymatic preparations. PDE III + 100 μM rolipram represented 61 ± 2% of the PDE activity and PDE IV + 100 μM cyclic GMP represented 61 ± 1% of the PDE activity of the respective preparations, using 1 μM cyclic AMP as substrate. PDE V was assayed at 1 μM cyclic GMP as substrate. NS = not significant (IC_{50} values ≫ 200 μM).

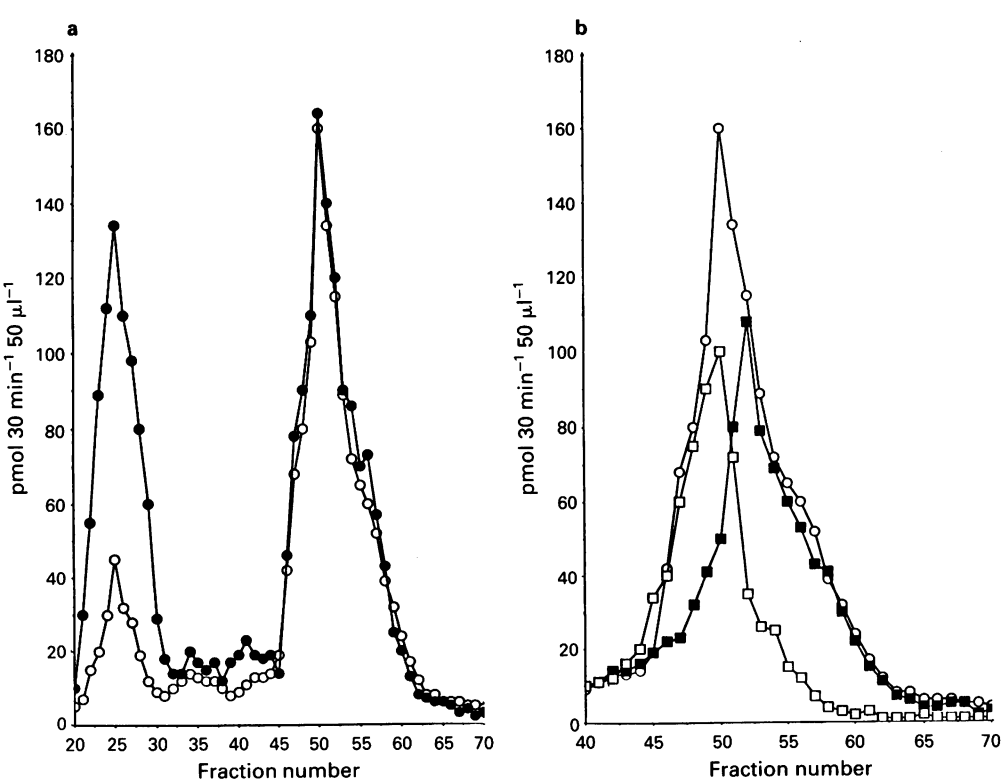

Figure 1 Chromatographic resolution of phosphodiesterase (PDE) activity from rat aorta eluted from a DEAE-sephacel column. (a) Activity was assayed with 1 μM cyclic AMP in the presence of EGTA (○) and 1 μM cyclic AMP in the presence of calmodulin/calcium (●). (b) Activity was assayed with 1 μM cyclic AMP in the presence of EGTA (○); 1 μM cyclic AMP + 5 μM cyclic GMP (■) and 1 μM cyclic AMP + 100 μM rolipram (□).

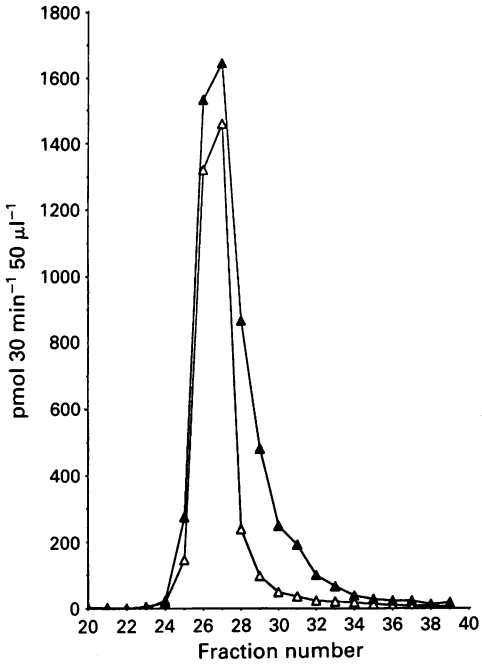

Figure 2 Representative elution profile of PDE V obtained after rechromatography by h.p.l.c. of the endothelium-denuded aorta first fraction (22–30) isolated by DEAE-sephacel. PDE activity was determined using 1 µM cyclic GMP + EGTA (△) and 1 µM cyclic GMP + calmodulin/Ca^{2+} (▲).

much more sensitive to rolipram and denbufylline than to cardiotonic drugs and zaprinast. This latter compound had no inhibitory effect on either PDE III or PDE IV.

These compounds were also studied on PDE V, with all tested cardiotonic drugs having IC_{50} values around 70 µM (except SK&F 94120; IC_{50} > 300 µM). Denbufylline was more potent on PDE V (IC_{50} = 11 µM) than rolipram (IC_{50} > 300 µM) while zaprinast selectively and potently inhibited PDE V (IC_{50} = 0.8 µM). None of drugs in Table 1 (up to 300 µM) inhibited PDE I significantly except zaprinast which displayed an IC_{50} of 20 ± 5 and 30 ± 8 µM in the presence or absence of calmodulin respectively, cyclic GMP (1 µM) being used as substrate.

Relaxation of rat aortic rings by selective PDE III inhibitors

Selective PDE III inhibitors relaxed noradrenaline precontracted rat aortic rings in a concentration-dependent manner (Figure 3), with the following order of potency: LY 195115 ≥ milrinone ≥ CI 930 > SK&F 94120. Relaxations were endothelium-independent, as there were no significant differences between the concentration-response curves and the EC_{50} values obtained in aortic rings with or without functional endothelium (Table 2). To ascertain that relaxation was not due to release of EDRF, L-NMMA was used to block EDRF production. Figure 4 shows that pretreatment of aortic rings with L-NMMA did not modify the relaxation-responses to

Table 2 Relaxing potency of PDE III inhibitors on noradrenaline-precontracted (1 µM) rat aortic rings (EC_{50}, µM)

Inhibitors	*With functional endothelium*	*Without functional endothelium*
LY 195115	3.4 ± 0.9 (n = 6)	3.1 ± 0.9 (n = 6)
Milrinone	5.7 ± 1.0 (n = 6)	9.0 ± 3.0 (n = 6)
CI 930	7.8 ± 3.0 (n = 8)	5.1 ± 2.0 (n = 8)
SK&F 94120	14.7 ± 4.0 (n = 6)	14.2 ± 5.0 (n = 6)

The results are given as mean ± s.e.mean. Numbers in parentheses indicate the number of rings used.

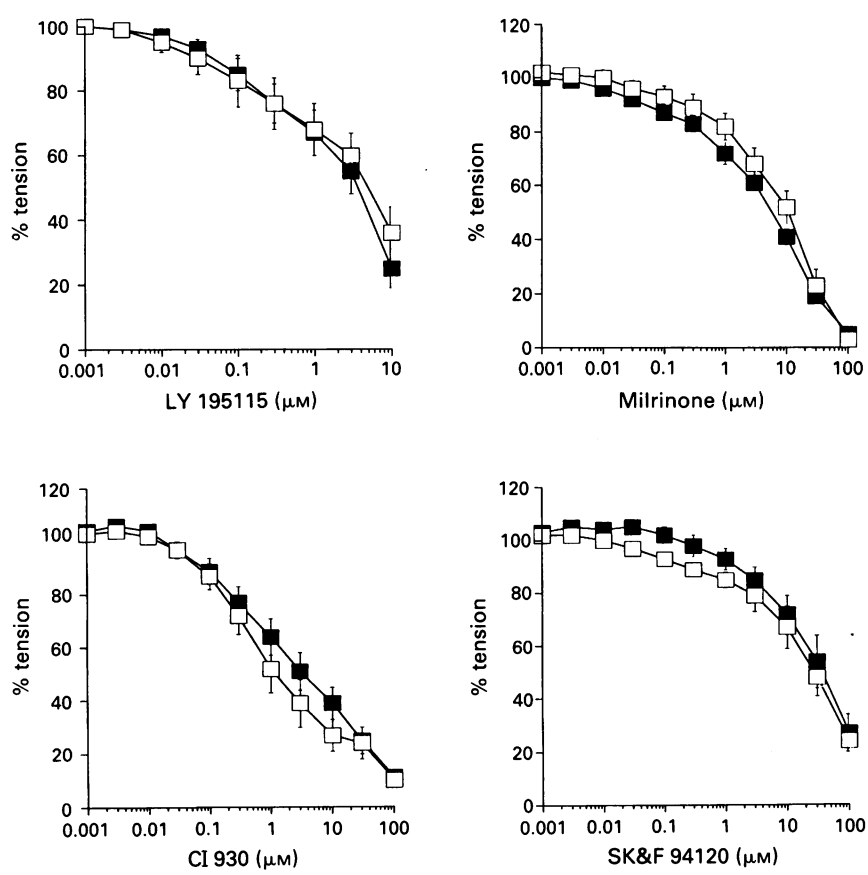

Figure 3 Relaxation of noradrenaline-precontracted rat aortic rings by PDE III inhibitors in the presence (■) or absence (□) of functional endothelium. (n = 8 rings).

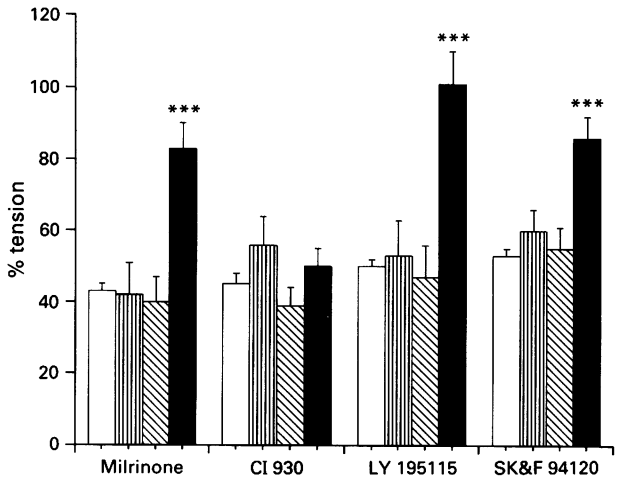

Figure 4 Relaxation of rat aorta with functional endothelium by PDE III inhibitors, in the absence (□) and in the presence of 300 μM N^G-monomethyl-L-arginine (▥), 1 mM L-arginine (▨) or 10 μM methylene blue (■). Results are expressed as a percentage of tension induced by noradrenaline alone (1 μM) and are mean of $n = 8$ to 14 experiments (vertical bars indicate s.e.mean). PDE III inhibitors were used at their EC_{50} values: LY 195115 3 μM, milrinone 7 μM, CI 930 6 μM and SK&F 94120 14 μM. *** $P < 0.005$ compared with the control. Significant differences were determined by ANOVA followed by Newman-Keuls' test.

PDE III inhibitors. Further addition of L-arginine had no effect. However methylene blue (10 μM) abolished relaxation induced by PDE III inhibitors except in the case of compound CI 930.

Effects of PDE V inhibitors on relaxation of rat aortic rings

In rings without functional endothelium, zaprinast, a selective inhibitor of PDE V, induced little relaxation (maximum 30% at 100 μM, the highest contraction used to avoid solubilization problems, Figure 5). In contrast, in rings with functional endothelium, zaprinast produced a concentration-dependent relaxation which was significantly different (Student's t test) from

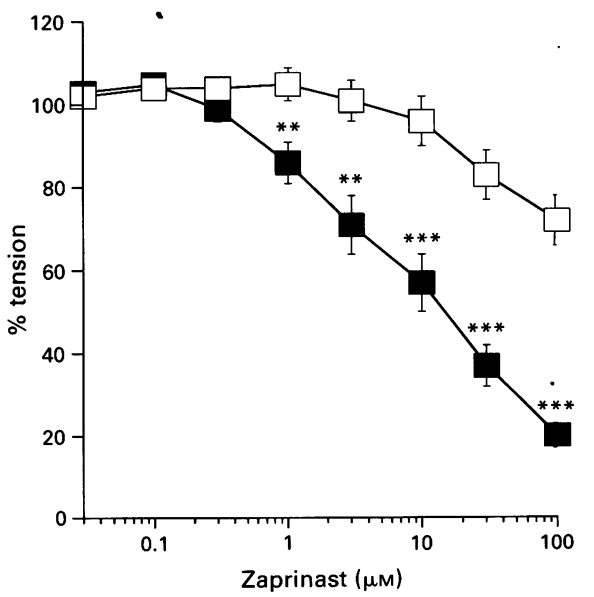

Figure 5 Relaxant effect of zaprinast on rat aortic rings without and with functional endothelium. Concentration-response curves in the presence (■) or absence (□) of endothelium. (EC_{50} value in the presence of endothelium 32 ± 9 μM, $n = 6$). Significant differences were determined by ANOVA (** $P < 0.01$, *** $P < 0.005$ compared without functional endothelium) followed by Newman-Keuls' test.

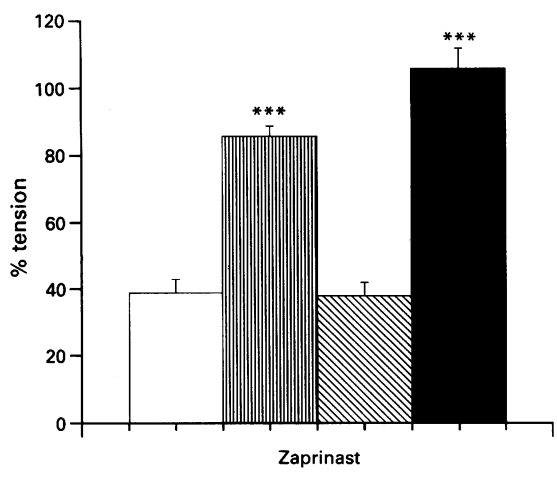

Figure 6 Relaxation of rat aorta with functional endothelium by zaprinast in the absence (□) and in the presence of 300 μM N^G-monomethyl-L- arginine (▥), 1 mM L-arginine (▨) or 10 μM methylene blue (■). Results are expressed as a percentage of tension induced by noradrenaline alone (1 μM) and are mean of 6 to 12 experiments (vertical bars indicate s.e.mean). Zaprinast was used at 30 μM. *** $P < 0.005$ compared with the control. Significant differences were determined by ANOVA followed by Newman-Keuls' test.

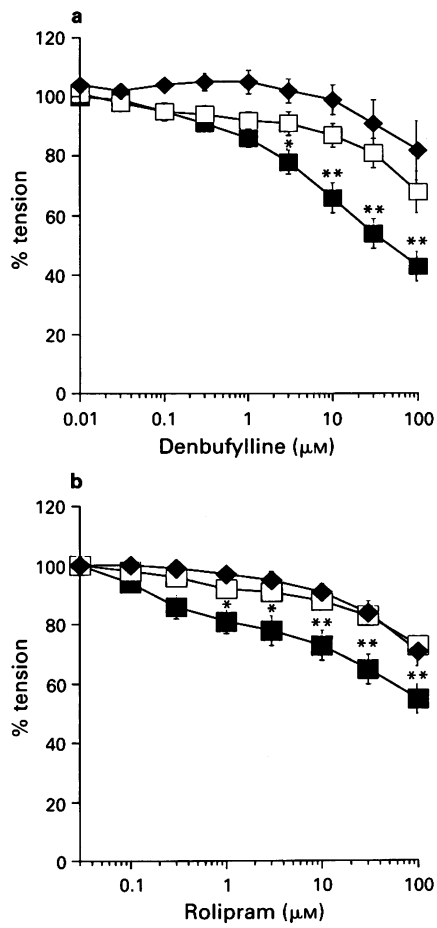

Figure 7 Relaxant effects of PDE IV inhibitors (denbufylline and rolipram) in rat aortic rings without and with functional endothelium. Concentration-response curves in the absence of functional endothelium (□) and with functional endothelium in the absence (■) or presence (◆) of N^G-monomethyl-L-arginine (L-NMMA) ($n = 8$). Significant differences were determined by ANOVA (* $P < 0.05$, ** $P < 0.01$, compared without functional endothelium) followed by Newman-Keuls' test. No significant difference was found between the concentration-response curves obtained in the absence of functional endothelium or in the presence of functional endothelium with L-NMMA.

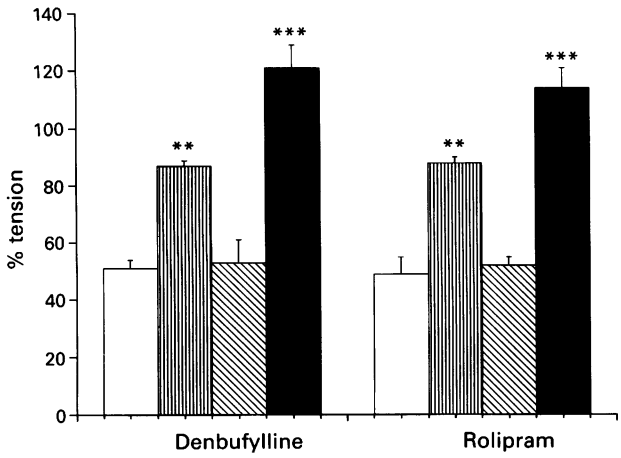

Figure 8 Relaxation of rat aorta with functional endothelium by PDE IV inhibitors in the absence (□) and in the presence of 300 μM N^G-monomethyl-L-arginine (▥), 1 mM L-arginine (▨) or 10 μM methylene blue (■). Results are expressed as a percentage of tension induced by noradrenaline alone (1 μM) and are mean of 10 to 14 experiments (vertical bars indicate s.e.mean). PDE IV inhibitors were used at their EC_{50} values, 30 μM for denbufylline and 100 μM for rolipram. ** $P < 0.01$, *** $P < 0.005$ compared with the control. Significant differences were determined by ANOVA followed by Newman-Keuls' test.

responses elicited in the absence of functional endothelium. The endothelium-dependent relaxation of rat aortic rings induced by zaprinast was significantly inhibited after 10 min pre-exposure to L-NMMA and the effect of zaprinast was restored by subsequent addition of 1 mM L-arginine (Figure 6). The inhibitory effects of L-NMMA were not due to endothelium injury because, after washing out L-NMMA, the relaxant ability of zaprinast was restored. Methylene blue abolished the endothelium-dependent relaxation induced by the PDE V inhibitor, zaprinast.

Effects of PDE IV inhibitors on relaxation of rat aortic rings

As shown in Figure 7 PDE IV inhibitors, rolipram and denbufylline, did not produce any significant relaxation, except at very high concentrations, in rat aortic rings without functional endothelium. In the presence of functional endothelium, these compounds caused a concentration-dependent relaxation (Figure 7). As in the case of zaprinast, the endothelium-dependent relaxation effects induced by these drugs were inhibited by L-NMMA (Figures 7 and 8). Further addition of L-arginine restored relaxation. Methylene blue abolished the endothelium-dependent relaxation induced by the PDE IV inhibitors (Figure 8).

Effects of PDE IV inhibitors on vascular relaxations in combination with other cyclic nucleotide elevating agents

The influence of pretreatment by compounds which are able to increase levels of either cyclic AMP (isoprenaline, a β-adrenoceptor agonist; forskolin, an adenylate cyclase activator, and milrinone, a PDE III inhibitor) or cyclic GMP (SNP, a soluble guanylate cyclase activator) were investigated on relaxation responses elicited by PDE IV inhibitors. A relatively low concentration of each compound (which relaxed precontracted rings by about 10%) was used to look for potentiation of the effects of rolipram and denbufylline.

Figure 9 and Table 3 show the effects of these drugs on rat aortic relaxations induced by PDE IV inhibitors. In the absence of functional endothelium, isoprenaline and forskolin (Figures 9a and 9b) did not significantly modify the relaxant effects elicited by rolipram or denbufylline, whereas SNP and

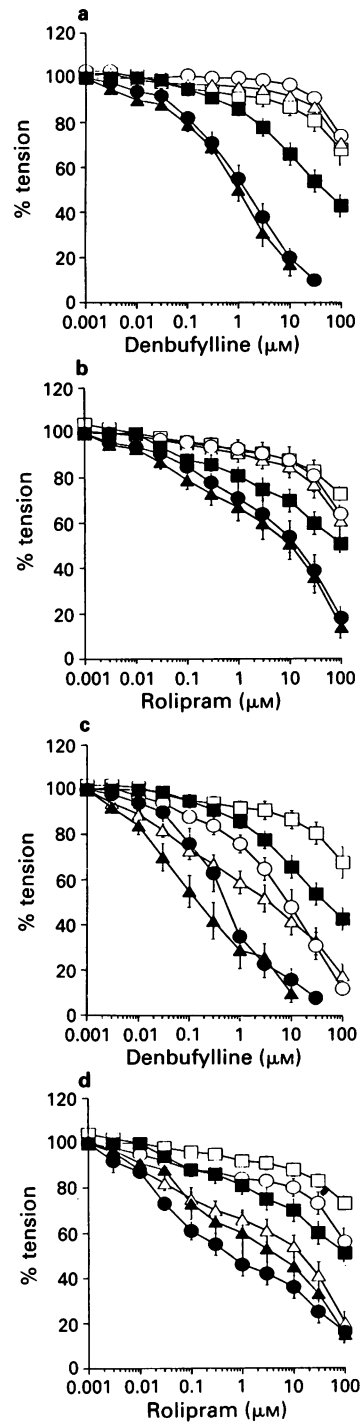

Figure 9 Concentration-relaxation curves of PDE IV inhibitors (denbufylline and rolipram) on prostaglandin $F_{2\alpha}$-precontracted rat aorta without (open symbols) and with (closed symbols) functional endothelium, control (□) and (■). (a and b) In the presence of 0.1 μM isoprenaline (△) and (▲), or 3 μM forskolin (○) and (●). (c and d) Sodium nitroprusside (SNP) 1 nM (○) and (●) or 30 nM milrinone (△) and (▲). Without functional endothelium, curves obtained for denbufylline and rolipram in the presence of milrinone and SNP were significantly different (ANOVA) from those elicited by denbufylline or rolipram alone ($n = 10$ rings). With functional endothelium, relaxant responses to denbufylline and rolipram in the presence of all four compounds were significantly different from those obtained with denbufylline or rolipram alone ($n = 10$ rings). See legend of Table 3 for experimental details.

milrinone (Figures 9c and 9d) enhanced the relaxant effect of these compounds (EC_{50} values 1.7 ± 0.6 and 12.2 ± 2.6 μM for denbufylline, 12.3 ± 4 and 124 ± 26 μM for rolipram, respectively). SNP weakly but significantly potentiated the

relaxant effect of rolipram and it strongly potentiated the relaxant effect induced by denbufylline. Milrinone caused a similar potentiation of relaxant responses induced by both rolipram and denbufylline.

In the presence of functional endothelium, denbufylline (Figures 9a and 9c) and rolipram (Figures 9b and 9d) caused concentration-dependent relaxations (EC_{50} values 30 ± 9 and $152 \pm 18\,\mu M$, respectively) by themselves in these conditions. The relaxant effects of rolipram and denbufylline were potently potentiated by all drugs used (isoprenaline and forskolin, Figures 9a and b; milrinone and SNP, Figures 9c and 9d). This effect was always more marked with denbufylline than with rolipram. Table 3 shows that in the presence of forskolin and isoprenaline, the EC_{50} values of rolipram and denbufylline were decreased by 15 fold and that in the presence of milrinone these values were even more strongly decreased (25 fold for rolipram and 75 fold for denbufylline). In the presence of SNP, the EC_{50} values of rolipram and denbufylline were in accordance with the IC_{50} values of these compounds obtained on rat aortic PDE IV.

Thus two classes of compounds could be distinguished in these experiments (i) compounds which are able to increase cyclic AMP levels by activating adenylate cyclase (isoprenaline and forskolin) enhanced the effect of denbufylline and rolipram only when functional endothelium was present, (ii) compounds like milrinone and SNP which respectively are able to inhibit PDE III or to increase cyclic GMP level, potentiated relaxation induced by denbufylline and rolipram in the absence as well as in the presence of functional endothelium.

Discussion

This study shows that PDE activity from rat aortic smooth muscle can be chromatographically resolved into different forms which hydrolyze either cyclic GMP or cyclic AMP. As in cardiac tissues, two calmodulin-insensitive cyclic AMP-PDE forms were found in rat aorta: PDE III and PDE IV. The selective and potent effects of cardiotonic drugs on vascular smooth muscle PDE III suggest that this PDE belongs to the same PDE family as the cardiac PDE III (Harrison *et al.*, 1986a; Komas *et al.*, 1989). The IC_{50} values of positive inotropic agents on PDE III were similar to those obtained for purified cardiac PDE III (Harrison *et al.*, 1986b). In contrast PDE III was insensitive to rolipram, denbufylline and zaprinast under all conditions used.

It is well established that, in cardiac tissues, PDE III inhibitors produce their positive inotropic effects by inhibition of cardiac PDE III (Weishaar *et al.*, 1987; Kariya & Dage, 1988; Ito *et al.*, 1988; Muller *et al.*, 1990a) and certain of these compounds significantly increase cyclic AMP content in these tissues (Gristwood *et al.*, 1986b). The different PDE III inhibitors used in the present study were all able to relax precontracted rat isolated aorta (in the presence or the absence of functional endothelium) with varying potencies. This finding is in accordance with the results obtained by Kauffman *et al.* (1987), who have shown that cardiotonic dihydropyridazinones were potent vasodilator agents. Since in this study, neither addition of L-NMMA nor the removal of endothelium affected the relaxation produced by PDE III inhibitors and since no PDE III has been found in endothelial cells (Lugnier & Schini, 1990), it appears unlikely that functional endothelium plays an important role in the relaxant actions of these drugs. The present results show that relaxation of rat aorta was produced by PDE III inhibitors at concentrations that were indeed active in selectively inhibiting the enzyme isolated from the same tissue. Our results also confirm the observation of Kauffman *et al.* (1987) that the soluble guanylate cyclase inhibitor, methylene blue was able to inhibit relaxation produced by some PDE III inhibitors, although this was not the case with CI 930. The present findings lend further support to the previous suggestion that PDE III inhibition caused endothelium-independent relaxation through an increase in both cyclic AMP and cyclic GMP levels (Kauffman *et al.*, 1987). The effect of methylene blue would suggest that inhibition of cyclic GMP hydrolysis could contribute to relaxation. This is conceivable because cyclic GMP, although generally considered as competitive inhibitor of PDE III, can be hydrolyzed by the enzyme with low velocity (V_{max} for cyclic GMP $= 0.6\,\mu M\,min^{-1}\,mg^{-1}$; V_{max} for cyclic AMP $= 6\,\mu M\,min^{-1}\,mg^{-1}$; Beavo, 1988). However, such interpretation needs further confirmation since our results show that neither the presence of functional endothelium, the basal EDRF production of which activates guanylate cyclase and increases the cyclic GMP level in rat aorta (Martin *et al.*, 1986a), nor the inhibition of the L-arginine–NO pathway by L-NMMA, had any influence on aortic relaxation induced by PDE III inhibitors.

Whatever the mechanisms involved, PDE III inhibitors are potent relaxant agents in the aorta, in spite of their relative inability to produce positive inotropy in rat cardiac tissue compared to their cardiac effect in other species (Alousi *et al.*, 1983; Kobylarz *et al.*, 1985; Weishaar *et al.*, 1987). In agreement with the results of Martin *et al.* (1986a,b), we have found that the selective PDE V inhibitor, zaprinast, induced a relaxation that was endothelium-dependent. An effect of this drug on the endothelium seems unlikely, since no PDE V has been found in endothelial cells (Lugnier & Schini, 1990). The likelihood that the endothelium-dependent relaxation induced by zaprinast in rat aortic rings was mediated by EDRF was evident from the effects of the L-NMMA. This compound selectively abolished the endothelium-dependent relaxation

Table 3 Influence of cyclic nucleotide elevating agents on the potency (EC_{50}, μM) of rolipram and denbufylline to relax prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted rat aortic rings, with or without functional endothelium

	Denbufylline		*Rolipram*	
	with endothelium	*without endothelium*	*with endothelium*	*without endothelium*
Control	30.2 ± 9.0	NS	152 ± 18	NS
Isoprenaline $0.1\,\mu M$	1.7 ± 0.6	NS	13 ± 5	NS
Forskolin $3\,\mu M$	1.4 ± 0.6	NS	6.7 ± 3.0	NS
Sodium nitroprusside 1 nM	0.7 ± 0.3	12.3 ± 4.0	1.2 ± 0.5	124 ± 26
Milrinone 30 nM	0.4 ± 0.02	1.7 ± 0.3	6.7 ± 3.0	12.2 ± 2.6

Results are expressed as mean of 6 experiments ± s.e.mean. Control values were obtained in the absence of any cyclic nucleotide elevating agents. In order to obtain the same level of contraction prior to the addition of denbufylline or rolipram, the aortic rings were stimulated either by $1\,\mu M$ $PGF_{2\alpha}$ (control rings) or by $2\,\mu M$ $PGF_{2\alpha}$ (rings exposed to cyclic nucleotide elevating agent at a concentration reducing contraction by 10%). NS = Not significant (EC_{50} values $\gg 200\,\mu M$).

induced by zaprinast in rat aortic rings, and in the presence of L-arginine, this inhibition by L-NMMA was completely restored (Figure 6). Recently, Weishaar et al. (1990) showed that zaprinast significantly potentiated the in vitro vascular relaxant response to atrial natriuretic factor (ANF). Altogether these observations suggest that the relaxant effects of zaprinast are due to the inhibition of the hydrolysis of cyclic GMP formed in response to EDRF released from endothelial cells (this study) or following stimulation of particulate guanylate cyclase by ANF (Weishaar et al., 1990).

It is well established that in cardiac tissue from different species, PDE IV inhibitors do not cause an inotropic effect by themselves (Gristwood et al., 1986a,b; England, 1989). However, Muller et al. (1990b) reported that positive inotropic effects of rolipram and denbufylline could only be obtained in the presence of either an adenylate cyclase activator (forskolin 15–100 μM) or a PDE III inhibitor (SK&F 94120, 2–10 μM). This was also observed in the presence of high concentration of another PDE III inhibitor milrinone (100 μM) by Shahid & Nicholson (1990). In rat aorta without functional endothelium, the PDE IV inhibitors (rolipram and denbufylline) were by themselves weak relaxant agents, acting at high concentrations only. However, these agents induced an endothelium-dependent relaxation which was abolished by L-NMMA and by methylene blue. This observation shows that the relaxing effect of PDE IV inhibitors was dependent on EDRF production and subsequent activation of cytosolic guanylate cyclase. There are different possibilities with respect to the mechanisms involved; either PDE IV inhibitors acted through an increase in EDRF production, or activation of cytosolic guanylate by basal EDRF was responsible for the enhancement of the relaxant effect of PDE IV inhibitors in smooth muscle cells, or both. The first mechanism cannot be entirely excluded, because PDE IV has been found in endothelial cells (Lugnier & Schini, 1990). However, it is not likely because cyclic AMP elevating drugs have been reported to decrease rather than to increase the initial release of EDRF by cultured bovine aortic endothelial cells (Lückhoff et al., 1990). Other authors found that neither cyclic GMP nor cyclic AMP regulate the EDRF release from bovine aortic endothelial cells (Kuhn et al., 1991). On the contrary the following evidence is in favour of the second mechanism: (1) in endothelium-denuded aortic rings, the guanylate cyclase activating agent SNP, markedly enhanced the relaxing effect of PDE IV inhibitors; (2) PDE IV from rat aortic smooth muscle was inhibited by rolipram and denbufylline at concentrations (in the micromolar range) which are consistent with the relaxing effects (also in the micromolar range) of these drugs in the presence of functional endothelium and SNP; (3) it has recently been reported that nitrovasodilators and nitroprusside have synergistic effects with isoprenaline in rat aortic smooth muscle (Maurice & Haslam, 1990; Maurice et al., 1991); (4) a low concentration of the PDE III inhibitor milrinone (30 nM) also enhanced the effects of rolipram and denbufylline, although other agents interfering with cyclic AMP metabolism (isoprenaline and forskolin) were unable to do so in endothelium-denuded rings (at least at concentrations producing by themselves a threshold inhibitory effect). The similarity between these effects of milrinone and SNP, which was rather unexpected, could be explained if potentiation of the relaxant effect of PDE IV inhibitors resulted from the inhibition of the cyclic GMP-inhibited PDE III either directly by milrinone or as a consequence of the accumulation of endogenous cyclic GMP under the influence of SNP. The hypothesis of an involvement of PDE III is not unlikely, since the affinities of cyclic GMP for PDE III ($K_i = 0.1\,\mu$M; Beavo, 1988) and for cyclic GMP-dependent protein kinase ($K_a = 0.1\,\mu$M; Lincoln et al., 1988) are similar. This hypothesis is not only consistent with the present findings, but also with the results of Maurice & Haslam (1990) showing that SNP, at a concentration which increases cyclic GMP level, also enhances isoprenaline-induced increases in cyclic AMP content in rat aorta. Indeed, the hydrolysis of cyclic AMP by PDE III isolated from rat aorta was inhibited by micromolar range of cyclic GMP (Figure 1b).

In conclusion, the current study shows that rat aortic media-adventitia layer contains four PDE forms respectively presenting the properties of PDE I, PDE III, PDE IV and PDE V. The results obtained with selective inhibitors show that both inhibitors of PDE IV (a cyclic AMP-specific enzyme) and of PDE V (a cyclic GMP-specific enzyme) produced endothelium-dependent relaxation of the rat aorta, whereas inhibitors of PDE III (a cyclic GMP-inhibited cyclic AMP phosphodiesterase) induced endothelium-independent relaxation. Furthermore the results indicate that the influence of endothelium was due to EDRF production from L-arginine and subsequent activation of guanylate cyclase, and they show synergistic actions of PDE IV inhibitors with milrinone (a PDE III inhibitor) and with SNP (a guanylate cyclase activator). Altogether these findings and previous results (Maurice & Haslam, 1990; Maurice et al., 1991) suggest that in the rat aorta, cyclic GMP exerts a potentiating effect on cyclic AMP-mediated relaxing action. Inhibition of cyclic AMP hydrolysis by PDE III could be involved in this effect of cyclic GMP.

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Supersensitivity to tetrodotoxin and lignocaine of sea anemone toxin II-treated sodium channel in guinea-pig ventricular muscle

Matomo Nishio, Tsuyoshi Ohmura, Shigeru Kigoshi & [1]Ikunobu Muramatsu

Department of Pharmacology, Fukui Medical School, Matsuoka, Fukui 910–11, Japan

1 Sea anemone toxin II (ATX II, 20–30 nM) doubled the action potential duration in guinea-pig papillary muscles without affecting the maximum rate of rise of the action potential ($\dot{V}_{max}$) and the resting potential.

2 Tetrodotoxin and lignocaine shortened the prolonged action potential in the ATX II-treated papillary muscles in concentrations (30 nM – 3 μM) at which these drugs did not suppress the $\dot{V}_{max}$.

3 Whole-cell voltage-clamp experiments with single ventricular cells showed that ATX II produced a slowly decaying inward sodium current following a transient sodium current upon depolarization.

4 The ATX II-induced slowly decaying current was reduced by tetrodotoxin or lignocaine in concentrations (300 nM–1 μM for tetrodotoxin, 3–10 μM for lignocaine) at which these drugs failed to affect the $\dot{V}_{max}$ in cells not treated with ATX II.

5 These results suggest that sodium channel modification by ATX II not only changes its kinetics but also increases the susceptibility of the channel to block by tetrodotoxin and lignocaine.

Keywords: Na channel; supersensitivity; sea anemone toxin II; tetrodotoxin; lignocaine; guinea-pig ventricular muscle

Introduction

Several neurotoxins and local anaesthetics bind with high affinity and specificity to different sites of voltage-sensitive sodium channels and modify their properties. At present, six different receptor sites for toxins and one site for local anaesthetics are recognized (Narahashi, 1974; Hondeghem & Katzung, 1977; Catterall, 1977; 1980; 1984; 1988; Catterall & Beress, 1978; Coraboeuf *et al.*, 1979; Jaimovitch *et al.*, 1982; Barchi, 1988; Trimmer & Agnew, 1989).

Sea anemone toxin II (ATX II) is a polypeptide toxin of 4935 molecular weight isolated from *Anemonia sulcata* (Alsen, 1983). This toxin, like other polypeptide toxins (α-scorpion toxins, anthopleurin A), binds to receptor site 3 among the six distinct sites mentioned above, resulting in a slowing or block of the inactivation process of sodium channels (Romey *et al.*, 1976; Isenberg & Ravens, 1984; Wu & Narahashi, 1988). ATX II has also been reported to enhance the binding and action of lipid-soluble toxins such as batrachotoxin acting at receptor site 2 (Catterall, 1984; Sheldon *et al.*, 1986). More recently, the sensitivity to tetrodotoxin (a site 1 toxin) and lignocaine (a local anaesthetic) of axonal sodium channels has been demonstrated to be enhanced by another site 3 toxin, anthopleurin A (Muramatsu *et al.*, 1990). The evidence suggests that site 3 toxins can interact with other toxins which bind to different receptor sites. The purpose of the present study is to examine the possible interaction of ATX II with tetrodotoxin or lignocaine in the cardiac sodium channels.

Methods

Recording of action potential in guinea-pig papillary muscle

Male guinea-pigs weighing 250–350 g were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and the hearts were quickly isolated. The papillary muscles were dissected from the ventricles. The preparation was mounted in a tissue bath and perfused continuously with normal external solution of the following composition (mM); NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, NaH_2PO_4 0.21 and glucose 5.6. The bathing solution was gassed with 95% O_2 and 5% CO_2 at 30°C. Square wave pulses of 1 ms duration were applied every 10 s. The membrane potential and the maximum rate of depolarization ($\dot{V}_{max}$) of action potential were recorded by a conventional microelectrode technique (Muramatsu *et al.*, 1987), digitized using a PCM recording system (RP-880, NF Circuit Design Block, Japan) and stored on videocassette (NV 730, National, Japan) for later analysis. The membrane potential was simultaneously recorded on a strip chart recorder (RJG-4122, Nihon Kohden, Japan).

Preparation of single ventricular cells

Guinea-pig heart was dissected and mounted on the Langendorf apparatus to perfuse the coronary artery with oxygenated and pre-warmed (37°C) normal external solution of the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, glucose 10 and HEPES 5. The pH of the external solution was adjusted to 7.3 with NaOH. After the heart had been cleared of blood, the perfusate was changed to Ca-free external solution containing 0.4 mg ml^{-1} collagenase (Type 1, Sigma, St. Louis, U.S.A.) and the solution was recirculated by use of a peristaltic pump for 25 min. Thereafter, the collagenase was washed by 100 ml of high K-low Cl solution (storage solution). The composition of the storage solution was (mM): taurine 10, oxalic acid 14, glutamic acid 80, KCl 25, KH_2PO_4 10, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid (HEPES) 10, glucose 11, ethylene glycol *bis* (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 0.5, pH was adjusted to 7.2 by adding KOH (Isenberg & Klöckner 1982). The preparation was stored in this solution at 4°C for the experiments.

Whole-cell clamp experiments

A piece of tissue was dissected from the ventricle mentioned above and the cells were dispersed in the experimental chamber (0.5 ml in volume) filled with the storage solution. After the cells had settled on the floor of the chamber, the perfusion with normal external solution was started at a rate of 2–3 ml min^{-1}. The temperature of the perfusate in the experimental chamber was maintained at 33°C. Patch electrodes were made from glass capillaries of 1.5 mm o.d. with a double step microelectrode puller (PP-83, Narishige, Japan). After the tip had been polished by heat, the electrode was filled with internal solution of following composition (mM): potassium aspartate 100, KCl 20, $MgCl_2$ 2.5, HEPES 5, EGTA 5, adenosine-5'-triphosphate 5 and phosphocreatine 5.

[1] Author for correspondence.

The pH was adjusted to 7.3 with KOH. Outer diameter of the electrode tip was 2–5 μm and the electrode resistance ranged between 1 and 3 MΩ. Patch clamp amplifier (S-3666, Nihon Kohden) was used to record the whole-cell membrane currents. After the electrode tip was touched to the cell surface, a negative pressure of 20–40 cmH_2O was applied to the interior of the electrode. Within a few seconds, a giga-ohm seal was established between the electrode tip and the cell surface. Then the membrane patch at the tip of the electrode was ruptured by applying further suction of -50 to -100 cmH_2O. Rupture was indicated by a large increase in the capacitive current. The current signals and membrane potentials were displayed on an oscilloscope (VC-10, Nihon Kohden) and stored by the same recording system as that used in the action potential experiments.

Drugs used were sea anemone toxin II (Ferring, Kiel, Germany), tetrodotoxin (Sankyo, Tokyo, Japan) and lignocaine (Sigma).

Experimental values are given as mean ± s.e.mean.

Results

Effects of tetrodotoxin and lignocaine on membrane potential in the presence or absence of sea anemone toxin (ATX II)

Guinea-pig papillary muscle had a resting potential of -84 ± 1 mV in amplitude and produced action potentials of 359 ± 15 ms in duration at 90% repolarization (APD_{90}; $n = 12$). ATX II (30 nM) gradually prolonged the action potential (Figure 1b left). The effect reached its maximum within 30 min and was observed for more than 30 min. ATX II at concentrations of 20–30 nM doubled the action potential duration (approximately 700 ms at APD_{90}). At such concentrations, ATX II failed to affect the maximum rate of rise of action potential ($\dot{V}_{max}$) and the resting potential. At higher concentrations (100 nM and higher), ATX II prolonged the action potential remarkably (more than 1 s) but variably (data not shown); therefore, constant prolongation could not be elicited by electrical stimulation at intervals of 10 s. ATX II at 20–30 nM was used in the subsequent experiments.

The prolonged action potential under treatment with ATX II was shortened by the addition of 100 nM tetrodotoxin (Figure 1b middle). However, in muscles not treated with ATX II, tetrodotoxin at 100 nM had no effect on the action potential duration, and a higher concentration of tetrodotoxin (10 μM) was required to suppress the action potential duration (Figure 1a). $\dot{V}_{max}$ was also suppressed by high concentrations of tetrodotoxin (such as 10 μM) both in the ATX II-treated and -untreated muscles.

Figure 2 shows the effects of lignocaine on the ATX II-untreated and -treated papillary muscles. Lignocaine at 100 nM shortened the action potentials prolonged by ATX II (Figure 2b middle). In the muscles not treated with ATX II, however, application of 100 nM lignocaine had no effect on the action potential duration (Figure 2a middle). Lignocaine at 10 μM also shortened the action potentials of ATX II-treated muscles more potently than those of ATX II-untreated muscles. (Figure 2a right and 2b right). $\dot{V}_{max}$ was suppressed

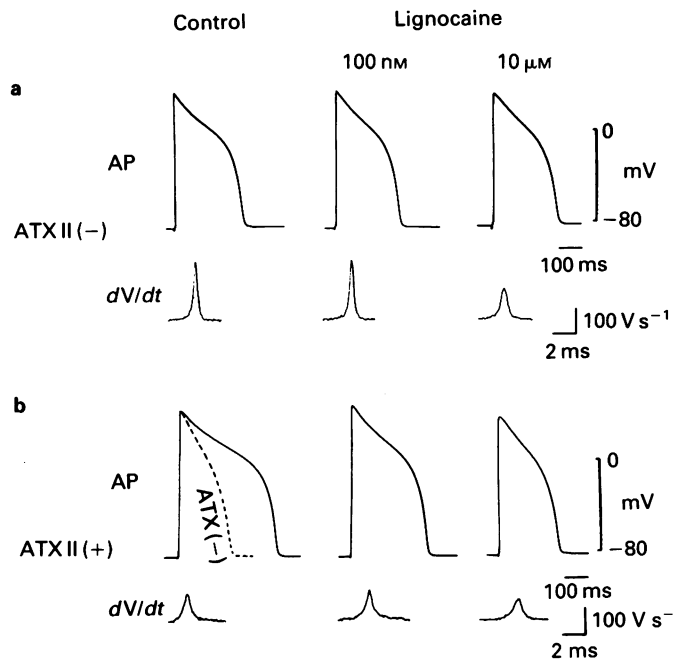

Figure 2 Effects of lignocaine on action potential and dV/dt in the absence (a) or presence (b) of 30 nM sea anemone toxin (ATX II). Experimental conditions are the same as those in Figure 1.

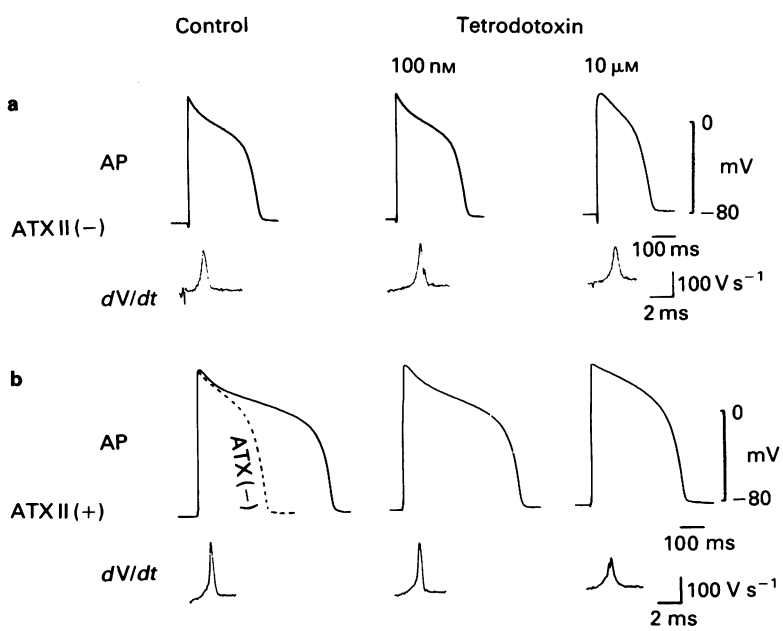

Figure 1 Effects of tetrodotoxin on action potential and rate of rise of action potential (dV/dt) in guinea-pig isolated papillary muscles in the absence (a) or presence (b) of 30 nM sea anemone toxin II (ATX II). Records (a) and (b) were obtained from two different cells. The muscles were stimulated every 10 s. Microelectrode experiments at 30°C.

by lignocaine at a high concentration (10 μM) and the effect was not affected by ATX II treatment (Figure 2a right and 2b right).

Concentration-inhibition curves for tetrodotoxin and lignocaine on the action potential duration and $\dot{V}_{max}$ in the ATX II-treated papillary muscles are shown in Figure 3. Relative decreases in prolongation of APD_{90} and in $\dot{V}_{max}$ were plotted against the concentrations of tetrodotoxin or lignocaine. As shown in the insets of Figure 3, the difference in the APD_{90} before and after application of each drug in the ATX II-treated muscles was measured and its ratio to the prolonged portion of APD_{90} by ATX II was expressed as a percentage (a% in the inset of Figure 3). Inhibition of $\dot{V}_{max}$ by treatment with tetrodotoxin or lignocaine was expressed as a percentage of the control $\dot{V}_{max}$ which was measured in the presence of ATX II before treatment with each drug (b% in the inset of Figure 3). Tetrodotoxin and lignocaine shortened the action potential duration in the ATX II-treated papillary muscles in concentrations at which these drugs did not suppress the $\dot{V}_{max}$ (30 nM–1 μM). In contrast to the curves for $\dot{V}_{max}$, the concentration-inhibition curves for action potential duration have a plateau phase ranging from 30 to 300 nM tetrodotoxin or from 100 nM to 1 μM lignocaine, suggesting a biphasic inhibition.

Effects of tetrodotoxin and lignocaine on sea anemone toxin (ATX II)-modified ionic currents

In the presence of 2 mM $CoCl_2$, sodium inward currents of the single ventricular cells were produced by depolarizing steps to 0 mV from the resting potential (−80 mV) at intervals of 10 s. Figure 4a shows representative recordings before and after ATX II application. Before treatment with ATX II, only a transient inward sodium current flowed. Within 2 min after perfusion with 30 nM ATX II, the transient sodium current was followed by a slowly decaying inward current which was not present in the control (Figure 4a). The slow component developed its maximum within 5 min after the application of ATX II and was observed constantly for more than 30 min.

The slow component developed under treatment with ATX II was inhibited either by tetrodotoxin or lignocaine in concentrations at which these drugs failed to affect the $\dot{V}_{max}$ in cells not treated with ATX II. Figure 4b and c show the representative results where 300 nM tetrodotoxin or 3 μM lignocaine was added after treatment with 30 nM ATX II for 10 min. Both the transient and slow components were markedly reduced or not observed after application of tetrodotoxin at high concentrations (10 μM and higher, data not shown).

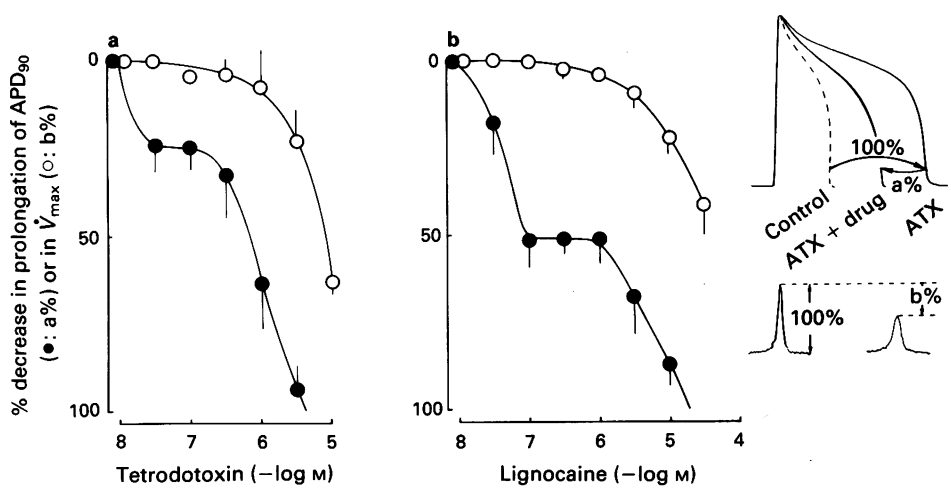

Figure 3 Concentration-inhibition curves for tetrodotoxin (a) and lignocaine (b) of action potential duration and maximal rate of rise of the action potential ($\dot{V}_{max}$) in the sea anemone toxin (ATX II)-treated papillary muscle. Percentage decreases in prolongation of action potential duration at 90% repolarization (APD_{90}, ●) and in $\dot{V}_{max}$ (○) were plotted against the concentration of tetrodotoxin (a) or lignocaine (b). Concentration of ATX II at which it doubled action potential duration (20–30 nM) was used. Insets show how the percentage decrease in prolongation of action potential duration (a%) and in $\dot{V}_{max}$ (b%) were measured. Mean (n = 3–4) with s.e. (shown by vertical lines).

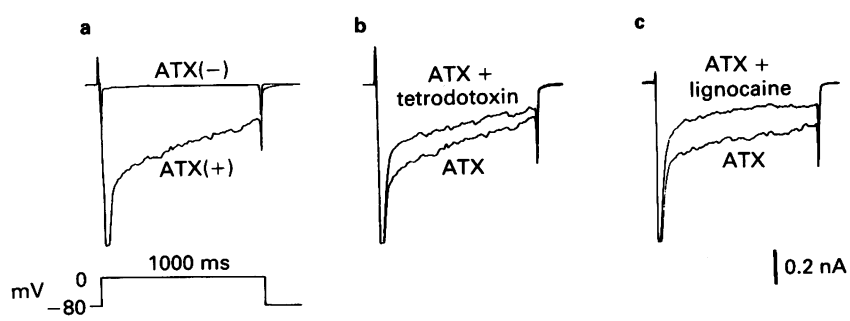

Figure 4 Effects of tetrodotoxin and lignocaine on the inward current of sea anemone toxin (ATX II)-treated guinea-pig single ventricular cells isolated with collagenase. Whole-cell voltage-clamp recordings at 33°C. Membrane was held at −80 mV (resting potential) and test pulses to 0 mV for 1 s were delivered every 10 s. Perfusate contained 2 mM $CoCl_2$. (a) The cell was treated with ATX II for 10 min. (b) Sodium inward currents before and after application of 300 nM tetrodotoxin in the presence of ATX II are superimposed. (c) Sodium inward currents before and after application of 3 μM lignocaine in the presence of ATX II are superimposed. Concentration of ATX II at which ATX II doubled action potential duration (20–30 nM) was used. The holding current at −80 mV was zero.

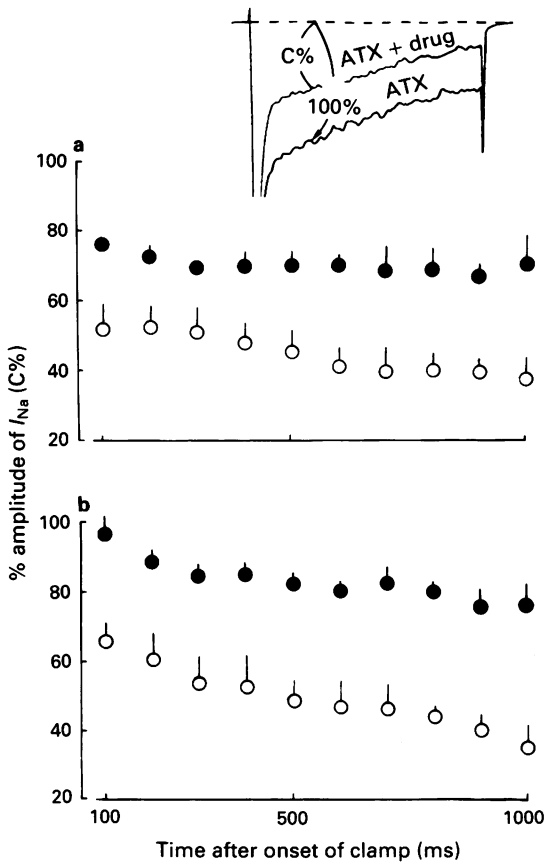

Figure 5 Effects of tetrodotoxin and lignocaine on sea anemone toxin (ATX II)-modified sodium current at different points of time during 1 s test pulses in ATX II-treated single ventricular cells. Inset shows how the percentage amplitude of inward sodium current (c%) was calculated. The current amplitude was measured from the recordings of the experiments in Figure 4. Mean (n = 3–4) with s.e. shown by vertical lines. (a) Tetrodotoxin: (●) 300 nM; (○) 1 μM. (b) Lignocaine: (●) 3 μM; (○) 10 μM.

The amplitudes of the slowly decaying current before and after application of tetrodotoxin or lignocaine were compared as shown in the inset of Figure 5 and the ratios (c% in Figure 5) were plotted against the time after onset of clamp pulse. Tetrodotoxin suppressed ATX II-modified inward current to the same extent at all the time points measured during the depolarizing test pulse, whereas the inhibition by lignocaine progressively increased during the depolarization.

Discussion

ATX II (20–30 nM) prolonged the action potential duration of guinea-pig papillary muscle without affecting the $\dot{V}_{max}$ and resting potential. Under whole-cell voltage-clamp conditions with single guinea-pig ventricular cells, ATX II at such concentrations produced a slowly decaying inward current, which was absent in cells not treated with ATX II. These results are consistent with the previous reports that ATX II prolonged action potentials by inhibiting the sodium channel inactivation in crayfish giant axon (Romey *et al.*, 1976; Low *et al.*, 1979), myelinated nerve fibres (Bergman *et al.*, 1976; Romey *et al.*, 1976) and guinea-pig ventricular cells (Honerjäger, 1982; Isenberg & Ravens, 1984).

The prolonged action potential was shortened by the addition of either tetrodotoxin or lignocaine in concentrations at which they failed to affect action potential duration and $\dot{V}_{max}$ in muscles not treated with ATX II. These phenomena were consistent with the results obtained from voltage-clamp experiments, in which slowly decaying inward current developed during treatment with ATX II was inhibited by extremely low concentrations of tetrodotoxin (300 nM) or lignocaine (3 μM) i.e. at such low concentrations, tetrodotoxin or lignocaine selectively inhibited the prolonged part of the action potential and the slowly decaying inward sodium current. It has been recognized that there are two sodium channel subtypes (Barchi, 1988; Trimmer & Agnew, 1989): one population of channels is blocked by nanomolar concentrations of tetrodotoxin (e.g. neural sodium channels), whereas the other is resistant or not very sensitive to tetrodotoxin (e.g. mammalian cardiac sodium channels). Recently, single channel measurements in cultured muscle have separated two populations of sodium channels having distinct biophysical properties that might correspond to these tetrodotoxin-sensitive and tetrodotoxin-resistant channel subtypes (Weiss & Horn, 1986).

The sensitivity of sodium channels to tetrodotoxin can also be altered according to the state of innervation. In skeletal muscle, sodium channels in neonatal muscle are resistant to tetrodotoxin, but high affinity binding develops after innervation (Pappone, 1980; Frelin *et al.*, 1983). Denervation of mature skeletal muscle induces the appearance of tetrodotoxin-resistant sodium channels (Pappone, 1980; Rogart,1985). Sodium channels expressed in *Xenopus* oocytes from mRNA isolated from cardiac cells show only high sensitivity (Sutton *et al.*, 1988).

Taking these findings and our present results together, two possible explanations can be considered for changes in sensitivity of cardiac sodium channels to tetrodotoxin and lignocaine after treatment with ATX II: (1) ATX II induces a new class of sodium channels with high affinity for tetrodotoxin and lignocaine; (2) ATX II modifies the existing sodium channels resulting in a change of sensitivity to tetrodotoxin and lignocaine. Some marine toxins have been reported to induce or create new classes of channels (Dubois *et al.*, 1983; Kobayashi *et al.*, 1987; Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988). Selective inhibition of the prolonged part of the action potential and of the slowly decaying inward current by tetrodotoxin and lignocaine may favour the former possibility. However, $\dot{V}_{max}$ and the resting potential were not affected by ATX II. Voltage-dependency of the slowly decaying inward current is much the same as that of the normal sodium inward current (Isenberg & Ravens, 1984). Therefore, it seems that ATX II does not induce new channels with activation kinetics different from that of the existing sodium channels. Rather, it is more likely that ATX II inhibits the inactivation process of existing sodium channels and that only the modified sodium channels become more sensitive to tetrodotoxin and lignocaine. Biphasic curves of concentration-inhibition relationships for tetrodotoxin and lignocaine may reflect the complexity of the modification of sodium channels by ATX II.

Recently, Muramatsu *et al.* (1990) reported that tetrodotoxin-sensitive sodium channels of crayfish giant axon become more sensitive to tetrodotoxin and lignocaine after treatment with anthopleurin A, another site 3 toxin. This evidence also supports the second possibility that susceptibility of sodium channels to tetrodotoxin or lignocaine is enhanced after modification with site 3 toxins such as ATX II and anthopleurin A, regardless of whether they are neuronal (tetrodotoxin-sensitive) or cardiac (tetrodotoxin-low sensitive) sodium channels. Site 3 toxins have been reported to enhance the binding and actions of lipid-soluble toxins acting at the receptor site 2 in neuroblastoma cells (Catterall, 1980) and cardiac myocytes (Sheldon *et al.*, 1986).

Lignocaine and other local anaesthetics are known to block sodium channels differently according to the state of the channels; i.e. resting, open and inactivated states (Hille, 1977; Hondeghem & Katzung, 1977; Matsubara *et al.*, 1987). Open channel block by lignocaine may be more likely to occur under conditions where the inactivation of sodium channels is inhibited by ATX II. As shown in Figures 4 and 5, a progressive increase in the lignocaine-induced inhibition of slowly decaying inward current during the depolarizing pulse may

represent such an additional block (open channel block) in contrast to the case of tetrodotoxin. However, detailed analysis is required in further experiments using single channel recordings.

Neurotoxins and local anaesthetics have been demonstrated to bind to several different sites in the sodium channel and to produce different electrophysiological effects. The present study further suggests the existence of close and complex interactions between the binding sites.

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Non-adrenergic, non-cholinergic neural activation stabilizes smooth-muscle tone independently of eicosanoid factors in guinea-pig isolated airways

[1]A. Lindén, *A. Ullman, C-G. Löfdahl & B-E. Skoogh

Departments of Pulmonary Medicine and *Clinical Pharmacology, University of Göteborg, Sweden

1 We examined the effect of variations in resting smooth-muscle tone induced by eicosanoid inhibitors on the direction and magnitude of the non-adrenergic, non-cholinergic (NANC) nervous response in guinea-pig isolated airways.

2 NANC responses (in the presence of 1 μM atropine, 10 μM guanethidine) to electrical field stimulation (1200 mA; 0.5 ms; 240 s; 3 Hz) were studied in guinea-pig isolated airway preparations ($n = 4$–7) taken from the proximal trachea, the distal trachea and the main bronchus. Tissues were treated with either the cyclo-oxygenase-blocker, indomethacin (10 μM), or the leukotriene receptor antagonist, FPL 55712 (11.5 μM), to modulate the resting tone.

3 Depending on the level of resting tone prior to electrical field stimulation, NANC activation induced either a contraction or a relaxation converging towards a similar level of tone, a 'tonus equilibrium'. This tonus equilibrium displayed an increasing level towards the periphery (3 ± 3 (s.e.mean) % for the proximal trachea, 28 ± 3% for the distal trachea and 54 ± 4% for the main bronchus, in % of maximum active tension). After NANC activation, the tonus returned to a level similar to the resting tone.

4 We conclude that changes in resting smooth-muscle tone not only affect the magnitude but also the direction of the NANC response. It is suggested that the NANC nervous system is a stabilizing factor in the regulation of airway smooth-muscle tone, independent of eicosanoid factors. An increasing contractile component and a decreasing relaxant component of the NANC system towards the peripheral airways is indicated.

Keywords: FPL 55712; indomethacin; main bronchus; NANC; resting tone; segmental distribution; trachea

Introduction

Both a contractile and a relaxant non-adrenergic, non-cholinergic (NANC) response have been demonstrated in the airways of guinea-pig and man *in vitro* (Coburn *et al.*, 1973; Richardson *et al.*, 1976; Davis *et al.*, 1982; Szolcsanyi *et al.*, 1982; Andersson *et al.*, 1983; Lundberg *et al.*, 1983a; Taylor *et al.*, 1984) and there are data which indicate a neural origin for both NANC components (Davis *et al.*, 1982; Szolcsanyi *et al.*, 1982; Andersson *et al.*, 1983; Lundberg *et al.*, 1983a).

In a recent study on guinea-pig isolated airways, we demonstrated that indomethacin decreased the active component of the tension in the resting preparation, the resting smooth-muscle tone, and increased the contractile as well as decreased the relaxant NANC response (Lindén *et al.*, 1991a). In the distal trachea, relaxations were even changed into contractions. One explanation of this could be that both the magnitude and the direction of the NANC response are related to the resting smooth-muscle tone. If so, the NANC system may be a stabilizing factor in the regulation of airway smooth-muscle tone.

The purpose of the present study was to examine whether both the magnitude and the direction of the NANC response are related to the resting smooth-muscle tone in guinea-pig airways. Different levels of resting tone were obtained by modulating the spontaneous airway tone with eicosanoid inhibitors (Lindén *et al.*, 1991b) and then activating the NANC system at the different levels of resting tone thus obtained. In this way, the importance of eicosanoid factors during NANC activation was also evaluated.

[1]Author for correspondence at: University Lung Clinic, Box 17301, S-402 64 Göteborg, Sweden.

Methods

Preparation

Twenty-four, specific pathogen-free, Dunkin-Hartley guinea-pigs (350–500 g) were killed by cervical dislocation and exsanguinated. The thoracic contents were removed and placed in a 200 ml oxygenated (94% O_2; 6% CO_2) dissection bath filled with Krebs-Ringer solution (KR composition, mM: NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6) at room temperature. The extrapulmonary airways were dissected free and airway rings were cut transversally. Each of these airway rings contained four to five cartilaginous rings. Two airway rings from the proximal (cervical) trachea, the distal (intrathoracic) trachea and the main bronchi from each animal were used. The airway rings were opened longitudinally along the anterior, cartilaginous part and mounted on steel hooks as isolated strips. These 'frontally-opened airway rings' were mounted in custom-made, temperature-controlled (37°C) and oxygenated 8 ml organ baths. These baths were continuously flushed with fresh KR (0.4 ml min^{-1}). Histological evaluation of the mounted airway rings showed that epithelial and smooth muscle cells were intact (Lindén *et al.*, 1991a).

Tension recordings

Isometric tension was continuously recorded on a Grass Polygraph (7D) via Grass force transducers (FT03). We used a recently developed method (Lindén *et al.*, 1991b), separating the active component, the tonus, from the passive component of the recorded total tension.

In order to compensate for the time-decay of the passive tension in the preparations, we used a linear mathematical model (Lindén *et al.*, 1991b) in which the passive tension (g) equals the formula $1.2 - [0.004 \times t]$. t represents time (min after initial adjustment of the applied, passive tension).

There was no significant difference in the level or decay of passive tension in the trachea and the main bronchus (Lindén *et al.*, 1991b) nor was there any difference in the decay of passive tension between control and indomethacin- or FPL 55712-treated preparations (Lindén *et al.*, 1991b).

The active component of the total (active plus passive) tension (g) within each preparation was calculated by subtracting the passive tension. This was done immediately before, at the end of and 25 min after electrical field stimulation of the NANC system, as well as during the maximum 5-hydroxytryptamine (5-HT)-induced contraction. Within each preparation, the levels of active tension were related to the maximum 5-HT-induced active tension (% max). Within each airway segment, the tonus equilibrium was calculated as the mean of the active tension levels at the end of the electrical field stimulation, in all three treatment groups.

Electrical field stimulation

Electrical field stimulation (EFS) from constant current generators was applied via rectangular platinum electrodes (5 × 30 mm) mounted parallel to the airway preparations (10 mm apart). We used square-wave biphasic impulses of 0.5 ms duration (Lindén *et al.*, 1991a) and a current of 1200 mA, which were found to activate the contractile and the relaxant component of the NANC system to a similar degree (data not shown). The total duration of stimulation for NANC activation was 240 s in order to evoke contractile and relaxant responses of large magnitude (Lindén *et al.*, 1991a). At a frequency of 3 Hz, these simulation parameters produced submaximal, tetrodotoxin-sensitive contractile and relaxant NANC responses (Lindén *et al.*, 1991a).

Experimental design

The passive component of the total tension in the resting preparation was applied as follows: 25 min of equilibration with the adjustment of the total (active plus passive) tension to 1.4 g was followed by a control contraction (12 Hz; 20 s) to avoid subsequent sliding on the steel hooks connected to the preparations. A wash-out followed. The active component of the total tension was then eliminated with 0.3 mM theophylline which produces a maximum relaxation in guinea-pig airways (Jeppson *et al.*, 1982; Lindén *et al.*, 1991b). In the presence of this drug the remaining, passive tension was adjusted to 1.2 g and the theophylline was then washed out. In preliminary experiments, we ascertained that 1.2 g was an optimum applied, passive tension for contractile responses to EFS (12 Hz; 20 s) in both the trachea and the main bronchus (data not shown).

After adjusting the applied tension, atropine (1 μM) and guanethidine (10 μM) were added to the KR in all treatment groups in order to obtain cholinergic blockade (Andersson *et al.*, 1981; Lundberg *et al.*, 1983; Taylor *et al.*, 1984) and adrenergic depletion (Kamikawa & Shimo, 1976; Grundström *et al.*, 1981; Brock *et al.*, 1988). In the resting preparations, three different levels of active tension (resting tone) were established by the pharmacological manipulation of the spontaneous airway tone, by use either of the cyclo-oxygenase inhibitor, indomethacin (10 μM), or the leukotriene receptor antagonist FPL 55712 (11.5 μM) or the vehicle (Lindén *et al.*, 1991b). We have previously demonstrated that the spontaneous airway tone was stable for at least 2 h (Lindén *et al.*, 1991b), which exceeded the total duration of the present experiments. Preparations were incubated with the eicosanoid inhibitors for an equilibration period of 70 min and the NANC system was then activated by EFS (3 Hz, 240 s). After the EFS, a wash-out was performed. The preparations were then flushed with fresh KR containing the above-mentioned drugs for 25 min. At the end of the experiment 10 μM 5-HT was added which produced a maximum serotonin-induced contraction. A maximum 5-HT-induced contraction reached about 80% of a maximum carbachol-induced (0.1 mM) contraction in both the trachea and the main bronchus (data not shown).

Data analysis

The results were expressed as the mean (± s.e.mean). The statistical evaluation of data was performed by use of Student's *t* test (two-tail) for unpaired data with 95% confidence intervals (Table 2) and by means of a parametric correlation coefficient determined at 95% confidence intervals (Colton, 1974); *n* equals the number of guinea-pigs (independent observations).

Drugs

Atropine (Sigma), guanethidine (Ciba-Geigy), 5-HT (Sigma) and theophylline (Draco) were dissolved in KR-solution. Indomethacin (Merck) and FPL 55712 (7-[3-(4-acetyl-3-hydrox-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid, Fisons) were dissolved in ethanol (70%), producing a final ethanol concentration of $<0.35\,ml^{-1}$ in the organ baths (vehicle). All the drug solutions were made up immediately before use.

Results

Characteristics of NANC responses

Depending on the airway segment and the level of active tension prior to stimulation, either contractile or relaxant NANC responses with one dominant phase were observed, as demonstrated by some original tracings in Figure 1. This pattern was observed in all preparations.

Ability of the NANC system to stabilize airway smooth-muscle tone

In terms of relative active tension (% max), FPL 55712 decreased the resting tone and indomethacin abolished it, thereby producing different levels of active tension prior to stimulation in the three treatment groups of each airway segment (Figure 2). The levels of maximum active tension were of a similar range in all treatment groups (Table 1).

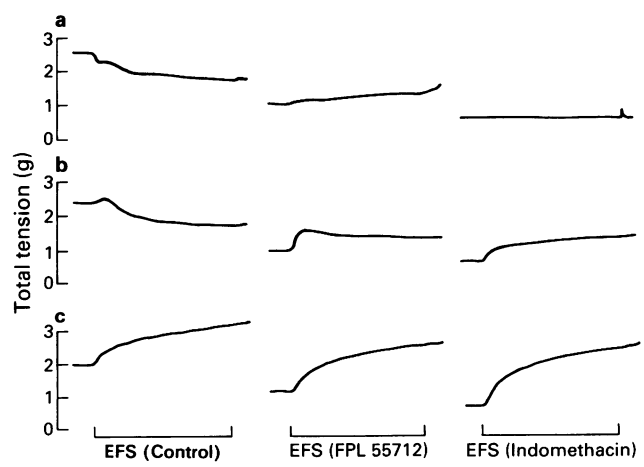

Figure 1 Original tracings of NANC responses of guinea-pig isolated airways: (a) proximal trachea, (b) distal trachea and (c) main bronchus after treatment with vehicle, the leukotriene receptor antagonist, FPL 55712 (11.5 μM) or the cyclo-oxygenase inhibitor, indomethacin (10 μM). NANC responses were evoked by electrical field stimulation (EFS; 1200 mA; 0.5 ms; 240 s; 3 Hz).

Table 1 Maximum 5-hydroxytryptamine-induced (10 μM) active* tension in guinea-pig isolated airways with and without eicosanoid inhibitors

	Tension (g)		
	Proximal trachea (n = 6)	*Distal trachea* (n = 6)	*Main bronchus* (n = 4–7)
Control (vehicle)	2.52 (0.35)	1.86 (0.20)	2.06 (0.11)
FPL 55712 (11.5 μM)	2.01 (0.20)	1.31 (0.12)	1.86 (0.18)
Indomethacin (10 μM)	1.95 (0.23)	1.60 (0.18)	2.26 (0.20)

* After excluding the passive component of the recorded isometric tension, see Methods.
S.e.means are shown in parentheses.

Depending on the level of active tension prior to stimulation in the distal trachea, NANC activation by EFS induced either contraction or relaxation, converging towards a similar level of active tension, a 'tonus equilibrium' (EFS in Figure 2). Before NANC activation by EFS, there was a pronounced difference in the level of active tension between the control and the indomethacin-treated group. At the end of EFS, this difference was significantly reduced. The 95% confidence interval for the NANC-induced reduction of the difference between the control and the indomethacin group is presented in a separate table (Table 2). Prior to EFS, there was also a pronounced difference in the level of active tension between the control and the FPL 55712-treated group which was significantly reduced at the end of EFS (Table 2).

NANC responses converging towards a tonus equilibrium were also observed in the other two airway segments, although a very small contractile response was observed in the proximal trachea and no relaxant response was observed in the main bronchus. These NANC responses significantly decreased the difference in active tension noted prior to EFS (Table 2) except in the case of the difference between the control and the FPL 55712 group in the main bronchus (Table 2). A 'converging trend' could, however, also be identified in that case. After NANC activation, the tension returned towards a level similar to the active tension prior to stimulation in the majority of treatment groups (Figure 2). The converging pattern induced by NANC activation was also observed in all segments when the tension was expressed in terms of total tension (active plus passive component, data not shown).

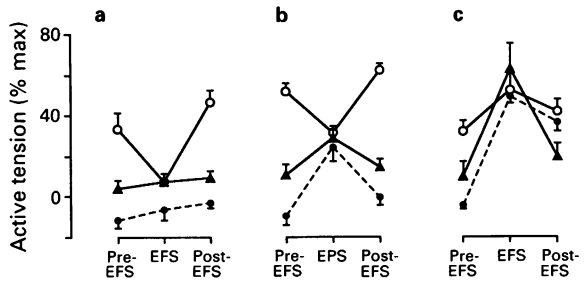

Figure 2 NANC responses at different levels of resting tone in guinea-pig isolated airways: (a) proximal trachea, (b) distal trachea and (c) main bronchus. Different levels of active tension prior to NANC activation (resting tone) were established by pretreatment with vehicle (○——○), the leukotriene receptor antagonist FPL 55712 (11.5 μM, ▲——▲) or the cyclo-oxygenase inhibitor indomethacin (10 μM, ●---●). NANC activation was evoked by electrical field stimulation (1200 mA; 0.5 ms; 240 s; 3 Hz). The active tension is presented as mean percentage (with bars indicating the s.e.mean) of maximum 5-hydroxytryptamine-induced (10 μM) active tension (% max) prior to (Pre-EFS), during (EFS) and after (Post-EFS) electrical field stimulation. n = 4–7 guinea-pigs. Depending on the level of resting tone, NANC activation induced either a contraction or a relaxation, converging towards a similar level of active tension, a 'tonus equilibrium'.

The contractile NANC response was most pronounced in preparations of the main bronchus following indomethacin or FPL 55712 treatment, where the active tension was abolished or decreased prior to stimulation. The pattern in the distal trachea was similar. The relaxant response was pronounced in the control preparations of the proximal and the distal trachea, where the active tension prior to stimulation was higher than in the indomethacin and FPL 55712 groups.

The tonus equilibrium, towards which responses converged during NANC activation, was at a higher level in the more peripheral airways (3 ± 3% and 28 ± 3% and 54 ± 4%, as a percentage of maximum active tension for the proximal trachea, the distal trachea and the main bronchus respectively; n = 15–18). The correlation between the level of the tonus equilibrium and the airway segment was high (r = 0.84) and significant (r = 0.74–0.91 in terms of the 95% confidence interval, n = 15–18).

Discussion

In this study, we confirmed the previously reported effect of eicosanoid inhibitors on spontaneous airway tone in guinea-pig airways (Lindén *et al.*, 1991b). We obtained evidence that these changes in resting smooth muscle tone affect not only the magnitude but also the direction of the response to NANC stimulation. In the distal trachea, a high level of resting tone led to a relaxation, whereas a low level of resting tone led to a contraction during NANC activation. The NANC responses converged, with and without different eicosanoid inhibitors. Thus the NANC responses adjusted the tone towards a similar level, a tonus equilibrium. These findings suggest that the NANC nervous system is a stabilizing factor in the regulation of airway smooth muscle tone, independent of eicosanoid factors.

A converging adjustment of tone induced by NANC activation is a new aspect of the role played by the NANC system in the airways. It has previously been reported that there is a relationship between the magnitude of the relaxant NANC response and the level of total tension (active plus passive component) prior to stimulation in guinea-pig isolated

Table 2 Statistical analysis of the converging effect on airway smooth-muscle tone induced by NANC activation

	NANC-induced reduction of difference in active tension (95% confidence intervals*, in % of maximum active tension)		
	Proximal trachea (n = 6)	*Distal trachea* (n = 6)	*Main bronchus* (n = 4–7)
Control minus Indo	8–52%	30–79%	9–56%
Control minus FPL 55712	5–52%	24–54%	−43–66%

* Student's t distribution (two-tailed).
Indo = indomethacin.

airways (Souhrada *et al.*, 1980; Karlsson *et al.*, 1984; Thompson *et al.*, 1988) and similar results have been obtained in human isolated airways (Davis *et al.*, 1982; de Jongste *et al.*, 1987). Kamikawa & Shimo (1989) demonstrated that the magnitude of the contractile NANC response depends on the level of passive tension prior to stimulation in guinea-pig isolated bronchi. We add to this previous data the finding that the level of active tension prior to stimulation, the resting tone, not only determines the magnitude of the NANC response but also appears to determine whether NANC neural activation leads to a relaxation or a contraction. Furthermore, this connection between the resting tone and the NANC response produces a converging adjustment of tone during NANC activation. Apart from the possible, physiological importance of these findings, they also highlight the need for caution when examining contractile and relaxant NANC responses against a varying resting tone.

The present study, as well as previous studies, suggest that the relaxant NANC component dominates in the proximal guinea-pig trachea (Coburn *et al.*, 1973; Kamikawa & Shimo, 1976). However, when the resting tone was reduced in the distal trachea, we also observed a moderate contractile NANC component: this confirms the results of two recent studies (Belvisi *et al.*, 1990; Lindén *et al.*, 1991a). In the guinea-pig main bronchus, on the other hand, the contractile NANC component appeared to dominate: this agrees with most previous studies (Andersson *et al.*, 1983; Lundberg *et al.*, 1983b; Frossard *et al.*, 1987; Kamikawa & Shimo, 1989). In contrast, Grundström *et al.* (1981) found no response to electrical field stimulation in the atropinized main bronchus, whereas Souhrada *et al.* (1980) registered relaxant NANC response in this segment. These discrepancies might be attributable to differences in resting tone prior to stimulation. It is, therefore, possible that a relaxant NANC response could be produced in the guinea-pig main bronchus, as in human bronchi (Richardson *et al.*, 1976; Davis *et al.*, 1982), if the resting tone is increased prior to NANC activation.

The data in this study are expressed in terms of relative active tension in order to detect segmental differences in NANC responses related to maximum contractions. We found this transformation acceptable since the levels of maximum 5-HT-induced active tension were of a similar range in all treatment groups. Furthermore, the converging pattern was also observed when studying the total tension (active plus passive component) expressed in grams.

The level of the tonus equilibrium increased towards the more peripheral airway segments. This suggests segmental differences in the balance between the contractile and the relaxant NANC component in the guinea-pig and agrees with two recent studies of the NANC system (Belvisi *et al.*, 1990; Lindén *et al.*, 1991a). It is possible that these segmental differences reflect differences in the forces applied on the extra- and intrathoracic airways as well as on the extra- and intrapulmonary airways by the surrounding tissue (Mead *et al.*, 1970). The increasing level of the tonus equilibrium towards the periphery also emphasizes the fact that the trachea from guinea-pigs is not necessarily a relevant *in vitro* model for physiological or pathological conditions in the bronchi.

It could be argued that an indomethacin-induced increase in the contractile NANC response follows the loss of relaxant prostanoids released by electrical field stimulation. Another possibility is an indomethacin-induced increase in the release of contractile leukotrienes due to electrical field stimulation, since it has been reported that indomethacin shunts arachidonic acid metabolism towards the synthesis of leukotrienes (Kreutner *et al.*, 1990). In the present study, however, leukotriene receptor antagonism and cyclo-oxygenase inhibition produced contractile NANC responses of a similar magnitude in the main bronchus. This indicates that the modulation of the NANC responses induced by indomethacin and FPL 55712 is secondary to the reduced resting tone that is present before stimulation. Furthermore, the NANC responses converged towards a similar level of tone both during cyclo-oxygenase inhibition and during leukotriene antagonism. This finding suggests that, although endogenous eicosanoids are potent modulators of airway smooth muscle tone *in vitro* (Ullman *et al.*, 1990; Dahlén *et al.*, 1983; Lindén *et al.*, 1991b), these agents have a minor effect on tone during NANC activation.

The mechanism controlling the direction of the NANC response is unclear. The present data suggest, however, that this mechanism is non-adrenergic, non-cholinergic and independent of eicosanoids. It is possible that the mechanism connecting the level of resting tone and the direction of the NANC response is neural but it might also involve the actin-myosin system, within the smooth muscle cell.

Nishikawa *et al.* (1983) demonstrated that myosin light chain kinase and protein kinase C may phosphorylate different sites on the myosin molecule. In a subsequent study (Nishikawa *et al.*, 1984), the same group found an inverse relationship between the phosphorylation induced by the two kinases. There was also evidence for an inhibitory effect on the actin-activated MgATPase when myosin was phosphorylated by myosin light chain kinase and protein kinase C compared to phosphorylation of myosin light kinase alone. Based on these findings, Nishikawa *et al.* (1984) suggested that, whereas phosphorylation induced by myosin light chain kinase favours contraction, phosphorylation induced by protein kinase C may favour relaxation. If true, this means that the smooth muscle cell may be protected against severe contraction via activation of protein kinase C.

It was recently reported that one transmitter candidate for the relaxant NANC response, vasoactive intestinal polypeptide (Matzusaki *et al.*, 1980; Palmer *et al.*, 1986; Ellis *et al.*, 1989; Li *et al.*, 1991) is absent in the lung tissue of asthmatics but not in the tissue of normal subjects (Ollerenshaw *et al.*, 1989). In another study, asthmatics also demonstrated an increase in substance P immunoreactive nerve fibres (Ollerenshaw *et al.*, 1990), which probably mediate the major part of the contractile NANC response (Lundberg *et al.*, 1983a,b). It might therefore be important to consider the finding that NANC neural activation stabilizes airway smooth-muscle tone when evaluating the pathophysiological role of the NANC system in obstructive airways disease. It is possible that an imbalance between the contractile and the relaxant components of the NANC response contributes to pathological airway obstruction.

In conclusion, both the direction and the magnitude of the NANC response in guinea-pig isolated airways appeared to be related to the resting tone prior to NANC activation such that this neural activation stabilizes airway smooth muscle tone. The NANC responses converged towards a tonus equilibrium that displayed segmental differences. Endogenous eicosanoids had a minor effect on airway smooth muscle tone during NANC activation. These findings suggest that the NANC nervous system is a stabilizing factor in the regulation of airway smooth muscle tone, independent of eicosanoid factors.

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Thermogenic and lipolytic effect of yohimbine in the dog

Jean Galitzky, *Michel Vermorel, Max Lafontan, Paul Montastruc & [1]Michel Berlan

Laboratoire de Pharmacologie Médicale et Clinque, Faculté de Médecine, INSERM U 317, 37 allées J. Guesde, 31400 Toulouse, France; and *Centre de recherches zootechniques et vétérinaires, INRA, Theix, 63122, Ceyrat, France

1 Lipid mobilization during a hypocaloric diet may be enhanced by a pharmacological approach using α_2-adrenoceptor antagonists since these drugs are known to increase sympathetic tone and stimulate lipolysis. Studies were undertaken in the dog in order to evaluate the effects of oral yohimbine administration (α_2-adrenoceptor antagonist) on heat production, metabolic, endocrinological and cardiovascular parameters.

2 Acute oral yohimbine (0.25 or 0.40 mg kg^{-1}) provoked an increase in plasma non-esterfied fatty acids. The drug increased sympathetic nervous system activity as indicated by the increased level of plasma noradrenaline. These effects persisted during the entire experimental period (4 h). The increase in plasma noradrenaline level was two fold higher with the higher dose of yohimbine (0.4 mg kg^{-1}). The plasma adrenaline level was increased only with the higher dose.

3 Yohimbine transiently increased plasma insulin and the effect was dose-dependent.

4 Yohimbine (0.25 mg kg^{-1}) enhanced heart rate and arterial blood pressure.

5 The effect of yohimbine on oxygen consumption, carbon dioxide and heat production was determined by indirect calorimetry. The drug (0.25 mg kg^{-1}) increased O_2 consumption and CO_2 and heat production 30 min after its administration and the effect persisted over the experimental period. The respiratory quotient, rather low in the fasting animals, remained unchanged.

6 The present work indicates that thermogenesis and lipid mobilization are enhanced during fasting in the dog by α_2-adrenoceptor blockade. Yohimbine also induced a transient increase in plasma insulin level and increased heart rate and blood pressure. The lipid mobilization plus the action on thermogenesis observed after yohimbine draw attention to the putative interest of α_2-antagonists in the pharmacological treatment of obesity during restricted calorie intake.

Keywords: Yohimbine; lipomobilization; catecholamines; thermogenesis; fasting dog

Introduction

Starvation or energy restriction induce compensatory changes in metabolic rate as reflected by the reduction in total energy expenditure. Reduction of the size of the digestive tract, reduction of the protein turnover and of ionique active transport (mainly Na^+/K^+) contribute to compensatory mechanisms (Ma & Foster, 1985; Farrel & Williams, 1989; Rumpler *et al.*, 1989). Lipid mobilization is accelerated during the early period of energy restriction or fasting in man and animals. The decrease in plasma insulin levels is a major factor involved in the induction of lipid mobilization (Cahill *et al.*, 1966; Livavivathana *et al.*, 1978; Jensen *et al.*, 1987). In man (James *et al.*, 1981; Scheen *et al.*, 1981; Dulloo & Miller, 1987) and in the rat (Young & Landsberg, 1977), starvation is followed by a lowering of the level of adrenergic activity. Thus, a probable decrease in sympathetic nervous system activity could be partly responsible for the reduction of energy expenditure and the weight-loss resistance usually observed during obesity therapy based on caloric restriction (Landsberg & Young, 1978) suggesting that enhancement of sympathetic activity could offer new perspectives (Dulloo, 1988).

A pharmacological approach aiming at an improvement in lipid mobilization and an increase in thermogenesis could be of potential interest in the treatment of obesity. The direct stimulation of β-adrenoceptors with β-agonists increases the metabolic rate and lipolysis. However, the use of classical (isoprenaline) or new (RO 16-8714) β-adrenoceptor agonists should be avoided since these agents possess powerful cardiovascular effects (Henny *et al.*, 1987) and some of them are rapidly eliminated after administration. The new atypical β-adrenoceptor agonist (BRL 26830A) which increases lipolysis and thermogenesis in animals may represent a more attractive approach since minor cardiovascular effects were observed after acute or chronic administration in man (Connacher *et al.*, 1988).

The use of α_2-adrenoceptor antagonists may also be of interest for both lipid mobilization and thermogenesis increment. These compounds have been shown to increase the sympathetic tone either through an action on the central nervous system and/or the blockade of the presynaptic α_2-adrenoceptors located on the sympathetic nerve endings. In consequence, these drugs raise plasma catecholamine and non-esterified fatty acid (NEFA) levels. The effect on NEFA levels is due both to a direct blockade of antilipolytic α_2-adrenoceptors located on the adipocytes and an indirect action through the stimulation of fat cell β-adrenoceptors by noradrenaline. These two mechanisms could exert additive effects on lipid mobilization (Zahorska-Markiewicz *et al.*, 1986; Galitzky *et al.*, 1988; Berlan *et al.*, 1991).

The present study was undertaken to evaluate lipomobilization together with sympathetic nervous system activation and thermogenesis. This comparative approach was carried out using the dog as a model. The properties of dog fat cell adrenoceptors have previously been defined and are very similar to those reported in human adipocytes (Milavec-Krisman & Wagner, 1978; Berlan *et al.*, 1982; Taouis *et al.*, 1987).

Methods

Endocrino-metabolic and cardiovascular measurements

Six normal female mongrel dogs (9–19 kg) were used for the studies. Several days before each experiment, the dogs were trained to stand still for 3 to 4 h on a Pavlov table, and accustomed to immobilization and blood sampling. At 09 h 00 min., after 18–20 h fasting, the dogs were placed on the Pavlov table. The systolic and diastolic blood pressures were recorded by means of a catheter introduced into the abdominal aorta

[1] Author for correspondence.

Table 1 Effect of oral yohimbine administration (0.25 or 0.40 mg kg^{-1}) on plasma glucose, insulin, noradrenaline and adrenaline levels and on cardiovascular parameters (heart rate, systolic and diastolic blood pressure) in 6 conscious dogs

Time (min)	0		60		240	
Dosage (mg kg^{-1})	0.25	0.40	0.25	0.40	0.25	0.40
Glucose (mmol l^{-1})	5.2 ± 0.1	5.2 ± 0.1	4.5* ± 0.2	4.5* ± 0.1	4.7 ± 0.1	4.5 ± 0.4
IRI (μu ml^{-1})	19.6 ± 2.8	22.9 ± 6.7	41.0* ± 7.3	64.9* ± 23	29.1 ± 8.5	25.0 ± 7.8
Noradrenaline (pg ml^{-1})	242 ± 35	404 ± 92	1031* ± 189	2006* ± 505	979* ± 194	1403* ± 335
Adrenaline (pg ml^{-1})	180 ± 31	146 ± 22	294 ± 52	384* ± 116	269 ± 93	253 ± 47
Heart rate (beats min^{-1})	72 ± 7	—	101* ± 12	—	89 ± 17	—
SBP (mmHg)	161 ± 12	—	198* ± 17	—	185 ± 7	—
DBP (mmHg)	98 ± 8	—	127* ± 9	—	101 ± 7	—

IRI: immuno-reactive insulin; SBP: systolic blood pressure; DBP: diastolic blood pressure.
Values are mean ± s.e.mean.
* $P < 0.05$ when compared to corresponding values measured at time 0 min.

via the left fermoral artery under local anaesthesia (xylocaine 5%) and connected to a Gould P23ID transducer on one channel of a Honeywell recorder. Mean blood pressure was measured by electrical integration of systolic and diastolic blood pressure. Heart rate was obtained with a heart period meter triggered by blood pressure. Another catheter was placed in the jugular vein for blood sampling. The dog remained quiet until the beginning of the experiment. One blood sample was taken before drug administration. Then, yohimbine (pellets from Houde Laboratory, Paris) was administered orally. The dog received placebo or various doses of yohimbine (0.25 or 0.40 mg kg^{-1} orally). The different administrations were separated by at least one week. Other blood samples were taken 15, 30, 60, 120, 180, 240 min after yohimbine administration.

The blood samples were collected in heparinized tubes and immediately centrifuged; plasma was collected and frozen at −20°C until used. Blood glucose was determined with a glucose-oxidase technique (Biotrol kit), and insulin (IRI) by radio-immunoassay (commercial radiommunoassay kit, Institut Pasteur, Paris) with human insulin used as a standard. Non-esterified fatty acids (NEFA) were determined by an enzymatic procedure by the Wako method (commercial kit, Biolyon, Lyon) with oleic acid (1 mmol l^{-1}) in 0.2% Triton X-100 used as a standard.

For catecholamine determination, blood was collected on lithium heparin with 10 mM sodium metabisulphite and centrifuged at 5000 g at 0°C; the plasma was stored at −80°C. Catecholamines were selectively isolated from the plasma by adsorption on activated alumina, then eluted with 0.1 M perchloric acid. Dihydroxybenzylamine was used as internal standard to monitor recovery from the extraction step. Catecholamines were assayed by high performance liquid chromatography (h.p.l.c.) using electrochemical (amperometric) detection (Waters h.p.l.c. system) (Valet *et al.*, 1988).

Heat production

Heat production of dogs with or without yohimbine treatment was determined by indirect calorimetry as previously described by Vermorel *et al.* (1973). Several days before the measurements the dogs were trained to stay in a wired cage similar to the respiration chamber. On the measurement days fasting dogs (18 h) were housed in a 300 l air conditioned (20°C) open-circuit respiration chamber.

Gas exchanges were determined each minute with dual-channel paramagnetic O_2 (20–21%) and infrared CO_2 (0–1%) analysers. A computer programme was designed to account for the dead space of the chamber. Consequently, the variations in O_2 consumption and CO_2 production of dogs were recorded after a 4 and 3 min delay, respectively.

One hour after the dog had been introduced into the chamber, it received 0.5 mg yohimbine (or placebo) orally. Gas exchanges and heat production were monitored from 30 min before until 240 min after yohimbine (or placebo) administration.

Data analysis

Values presented are means ± s.e.mean. Student's paired t test was used for comparisons between matched pairs; differences were considered significant when P was less than 0.05. The Mann Whitney U test was used for analysis of unpaired data.

Results

Metabolic and endocrinological effects of yohimbine

Results are depicted in Figure 1 and Table 1. The placebo did not modify the plasma NEFA concentration. The higher dose of yohimbine induced an increase in plasma NEFA levels and a significant effect was already apparent 30 min after its

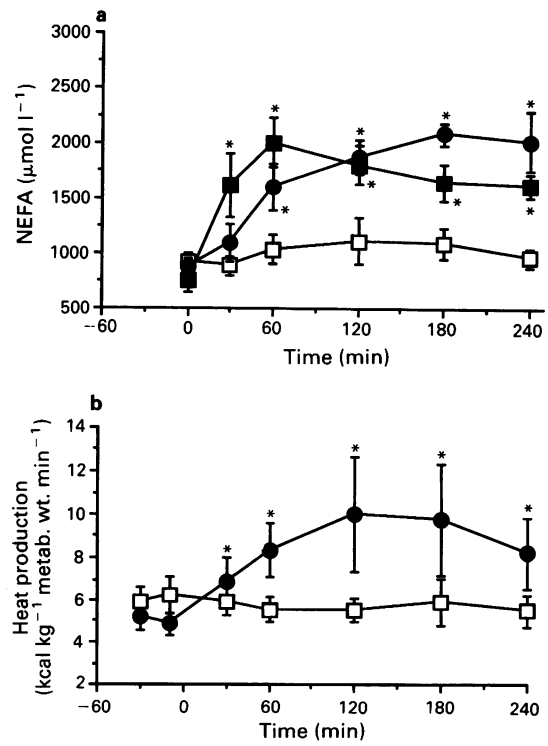

Figure 1 Effect of oral placebo or yohimbine administration (0.25 or 0.40 mg kg^{-1}) on non-esterified fatty acids plasma levels (NEFA) (a) and heat production (b) in 4 conscious dogs: yohimbine 0.25 mg kg^{-1} (●); 0.40 mg kg^{-1} (■); placebo (□). For heat production, each experimental point represents the average values over 10 min of heat production (derived from oxygen consumption measured every min). Values are mean with s.e.mean shown by vertical bars. *$P < 0.05$ when compared to corresponding values measured at time 0 min.

administration, the maximal increment being observed 30 min later. Plasma levels of NEFA remained significantly increased for at least 240 min. The lower dose of yohimbine (0.25 mg kg^{-1}) was only effective on the level of NEFA 60 min after its administration and the maximal effect was similar to that observed with 0.4 mg kg^{-1} (with regard to the highest levels reached and the duration of the action of the drug) (Figure 1).

Yohimbine also stimulated insulin secretion. The effect was transient since at the end of the experimental period, insulin values did not differ from those measured before yohimbine administration. The increase measured at time 60 min was greater with the higher dose (+183%) than with the lower dose (+89%). Yohimbine also promoted a small but significant (60 and 120 min after its administration) decrease in plasma glucose level. The effect, not observed with placebo, was similar whatever the yohimbine dose used.

A large increase in the plasma noradrenaline level was observed 60 and 240 min after yohimbine administration. The two doses were effective but the increase was two fold higher with 0.40 mg kg^{-1} (at time 60 min) and in both cases, the effect persisted until the end of the experimental period. At 240 min, the plasma levels of noradrenaline were similar. The higher dose of yohimbine also increased plasma adrenaline only 60 min after its administration but the lower dose was without significant effect. Placebo administration did not modify the parameters studied (Table 2).

Effect of yohimbine on respiratory exchanges and heat production

Measurement of respiratory exchange and heat production was investigated only with 0.25 mg kg^{-1} yohimbine since this dose significantly increased plasma noradrenaline and NEFA levels with a minor effect on insulin release.

Placebo administration was without any effect on O_2 consumption, CO_2 and energy production. Yohimbine significantly increased the respiratory exchange and heat production as compared with the pre-administration values and with the placebo (Figure 1). Maximal responses were achieved between 120 and 240 min and reached 102 and 81% when compared to placebo values. Since the increase in O_2 consumption or CO_2 production was of the same order, there was no significant effect of yohimbine on the respiratory quotient which was approximately 0.75–0.77 before and after yohimbine or placebo administration.

Effect of yohimbine on blood pressure and heart rate

Yohimbine administration induced a progressive and significant rise in blood pressure. The maximal effect occurred 60 min after its administration and then progressively decreased (Table 1). The heart rate also increased; maximal values were measured 60 min after yohimbine administration and then, heart rate progressively decreased. The values measured 240 min after yohimbine did not differ significantly from pre-administration values. Placebo administration did not modify the parameters studied (Table 2).

Table 2 Effect of oral placebo administration on plasma glucose, insulin, noradrenaline and adrenaline levels and on cardiovascular parameters (heart rate, systolic and diastolic blood pressure) in 4 conscious dogs

Time (min)	0	60	240
Glucose (mmol l^{-1})	5.5 ± 0.3	5.7 ± 0.2	5.5 ± 0.1
IRI (μu ml^{-1})	15.5 ± 4.1	12.2 ± 3.2	16.3 ± 3.8
Noradrenaline (pg ml^{-1})	320 ± 80	370 ± 73	322 ± 72
Adrenaline (pg ml^{-1})	134 ± 37	133 ± 46	155 ± 16
Heart rate (beats min^{-1})	78 ± 9	71 ± 10	73 ± 10
SBP (mmHg)	165 ± 10	155 ± 9	157 ± 4
DBP (mmHg)	87 ± 8	78 ± 5	85 ± 3

IRI: immuno-reactive insulin; SBP: systolic blood pressure; DBP: diastolic blood pressure.
Values are mean ± s.e.mean.

Discussion

This study demonstrates that yohimbine, administered to the dog by an oral route at dosages commonly tested in man (Galitzky *et al.*, 1988; Berlan *et al.*, 1991), increases lipid mobilization, the activity of the adrenergic system and heat production over a period of at least 4 h. The lipolytic response promoted by yohimbine is linked to stimulation of the adipose tissue β-adrenoceptors since propranolol administration completely blocked the response (Taouis *et al.*, 1988). The present results confirm previous observations in the dog in which higher doses of yohimbine were administered by the intravenous route (Taouis *et al.*, 1988). Interestingly, the lower dose used in this study (0.25 mg kg^{-1}) promoted a lipomobilization of the same magnitude as that previously measured with a two fold higher dose given by the i.v. route, whereas plasma noradrenaline and insulin increments were about two fold less (Taouis *et al.*, 1988). Since, in the present study, the plasma NEFA level increase was of the same magnitude with the two different doses of oral yohimbine (Figure 1), while insulin secretion and noradrenaline (Table 1) release were dose-dependent, it is suggested that the β-lipolytic pathway is fully operative with 0.25 mg yohimbine.

Thermogenesis was also increased by yohimbine. The smaller dose used in the study promoted an increase (about 100%) of respiratory exchanges and heat production (Figure 1). This effect was observed over the experimental period and the basal level was not recovered within 4 h. This thermogenic action of yohimbine is attributable to the activation of the sympathetic nervous system (reflected by the increasing plasma noradrenaline levels). The thermogenic capacities of catecholamines and other sympathomimetic drugs have been demonstrated previously. Adrenaline and noradrenaline increase the metabolic rate in man (Jung *et al.*, 1979; Katzeff & Danforth, 1981). Ephedrine (a sympathomimetic drug) also stimulates thermogenesis at a dose of 0.25 mg kg^{-1} (Astrup *et al.*, 1985). A methylxanthine like caffeine is also effective in thermogenesis since this drug increases the resting metabolic rate in young men by 16% (Acheson *et al.*, 1980).

In the present study, yohimbine, even at the lower dose, significantly increased both heart rate and blood pressure. This action could represent a major limitation for the potential use of yohimbine as an antiobesity drug. However, the stimulation of heat production largely exceeded the stimulant effect on heart rate and blood pressure. The thermogenic effect of yohimbine in dog (+100%) was also large when compared to the effect of other thermogenic drugs used in man (Jung *et al.*, 1979; Acheson *et al.*, 1980; Katzeff & Danforth, 1981). Indeed, the other parameters were also largely increased, particularly NEFA and noradrenaline plasma levels. The challenge of the utilization of this compound is now to determine whether the stimulation of thermogenesis and lipolysis could operate at lower doses of yohimbine which would be effective on thermogenesis without affecting cardiovascular parameters. It has been shown that in non-obese and obese human subjects, similar doses of oral yohimbine (0.20 mg kg^{-1}) induced lipid mobilization and sympathetic activation but were without effect on blood pressure, heart rate and plasma insulin or glucose levels (Galitzky *et al.*, 1988; Berlan *et al.*, 1991). It will be essential to check whether such a therapeutic dose is thermogenic in man.

The origin of the thermogenic action of yohimbine remains to be established. Since there is good evidence for an effect via

the activation of the sympathetic nervous system, it could be proposed that the β-adrenoceptor stimulation promotes a thermogenic effect by stimulation of the energetic metabolism in brown fat or in the muscles. In rodents, it has been demonstrated that ephedrine increases thermogenesis in brown adipocytes via β-adrenoceptor stimulation (Scheidegger *et al.*, 1984).

However, the presence of brown adipose tissue in adult dogs has never been established or investigated although this species has frequently been studied in the veterinary field (Joshua, 1970; De Bruijne, 1979) so a possible effect of noradrenaline on this tissue cannot be ruled out. In man, probably possessing lower amounts of brown adipose tissue, its involvement in thermogenesis has not been clearly demonstrated. The effect of ephedrine as a thermogenic agent arises from a major action on the skeletal muscle (Astrup *et al.*, 1985).

The effect of yohimbine on substrate utilization involved in thermogenesis activation could be deduced from the respiratory quotient. The respiratory quotient was not modified after yohimbine administration. Thus, the drug increased both lipid and carbohydrate oxidation. As proposed by Henny *et al.* (1987), lipolysis and triglyceride resynthesis processes in adipose tissue can also partly contribute to the thermogenesis through so-called futile cycles.

Drugs blocking α_2-adrenoceptors provoke insulin secretion through blockade of the α_2-adrenoceptors of the pancreatic β-cells. In the present study, yohimbine increased plasma insulin. Moreover, this effect on insulin secretion was less intense with the lower dose used while its effects on NEFA were not dose-related (Figure 1). This result confirms a previous investigation showing that the effect on insulin is transient while the effect on the plasma NEFA level is more sustained (Taouis *et al.*, 1988). It has been shown that in the dog, the insulin secretion induced by yohimbine is attributable to adrenergic activation since this effect is absent after β-blockade (Taouis *et al.*, 1988). It could be postulated that in fasting conditions (high plasma level of NEFA and low insulin and glucose levels), the increment in plasma NEFA levels predominates and that in these conditions, the insulinotropic action of noradrenaline will have a 'firing point' higher than that of its lipolytic action.

In man, it has been shown that yohimbine induces some similar pharmacodynamic effects to those observed in the dog. Moreover, in healthy male or female volunteers as well as in obese females a similar oral dosage of yohimbine did not induce either insulin or cardiovascular modifications (Galitzky *et al.*, 1988; Berlan *et al.*, 1991) whereas it did induce an increase in plasma NEFA and noradrenaline levels.

Further experiments should be undertaken to assess the possible usefulness of yohimbine (or, other new α_2-antagonists) in obesity therapy. The data from Peterson *et al.* (1988) showed that an increase in body fat stores is associated with depression of sympathetic nervous system activity in man. In addition, Klein *et al.* (1989) showed that long fasting periods even after 84 h, failed to increase or only moderately increased plasma noradrenaline levels. Taken together, these results indicate that drugs like yohimbine, able to increase sympathetic activity, lipid mobilization and thermogenesis during fasting or dietary therapy, should be of interest in the reduction of weight in obese subjects.

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The guinea-pig ileum preparation as a model for 5-HT_{1A} receptors: anomalous effects with RS-30199-193

Catherine Small, Christine M. Brown, William S. Redfern & [1]Michael Spedding

Syntex Research Centre, Research Park, Riccarton, Edinburgh EH14 4AP

1 Agents that have high and selective affinity for the 5-HT_{1A} site such as 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and N,N-dipropyl-5-carboxamidotryptamine (DP5CT) inhibited the responses to field stimulation in guinea-pig ileum preparations; the inhibitory effects were antagonized by methiothepin and spiperone, consistent with effects at the 5-HT_{1A} site.

2 The inhibitory effects of DP5CT were pronounced in Tyrode solution containing low Ca^{2+} (0.9 mM), but were much less apparent in Tyrode solution containing 1.8 or 5.4 mM Ca^{2+}.

3 Responses to DP5CT were abolished by pretreatment with phorbol dibutyrate (3 μM), whereas the responses to UK14304 were only slightly inhibited.

4 Buspirone and ipsapirone (1 μM) inhibited the responses to field stimulation, and the effects were resistant to idazoxan, but inhibited by 8-OH-DPAT or spiperone.

5 RS-30199-193 (5-chloro-2-methyl-1,2,3,4,8,9,10,10a-octahydronaphth-[1,8-cd]-azepine hydrochloride) an azepine with high affinity for the 5-HT_{1A} site in rat cerebral cortex in binding experiments, augmented contractions, but did not antagonize the responses to DP5CT or to 8-OH-DPAT.

6 The hybrid compound of RS-30199-193 with buspirone, RS-64459-193 (5-chloro-2-[4-(8-azaspiro[4,5] decane-7,9-dione)-but-1-yl]-1,2,3,4,8,9,10,10a-octahydronaphth[1,8-cd]-3-azepine hydrochloride) maintained high affinity for the 5-HT_{1A} binding site in rat brain and both inhibited the response to field stimulation and antagonized the responses to 8-OH-DPAT and DP5CT. Thus the buspirone side chain when added to RS-30199-193 appears either to induce affinity for a distinct subset of receptors in the guinea-pig ileum or is required for functional effectiveness at the 5-HT_{1A} receptor.

Keywords: 5-HT_{1A} receptors; buspirone; 8-hydroxy-2-(di-n-propylamino)tetralin; DP5CT; partial agonists; 5-hydroxytryptamine; RS-30199-193

Introduction

The multiplicity of distinct binding sites for 5-hydroxytryptamine (5-HT) has led to the definition of distinct receptor sites and subdivision into 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors has gained acceptance (Bradley *et al.*, 1986), as has the definition of four subtypes of 5-HT_1 receptors (A, B, C, D). Several new therapeutic entities such as buspirone and ipsapirone have affinity for the 5-HT_{1A} site.

Although there are many studies on binding sites for 5-HT_{1A} ligands (Peroutka & Snyder, 1979; Middlemiss & Fozard, 1983; Hamon *et al.*, 1984; Hoyer *et al.*, 1985; Peroutka, 1986) there are relatively few functional assays to quantify agonist or antagonist effects. These assays include inhibition (Bockaert *et al.*, 1987) or stimulation (Markstein *et al.*, 1986) of adenylate cyclase activity in hippocampal neurones, or inhibition of electrical spike activity in the same tissue (Rowan & Anwyl, 1987). We have therefore extended the functional assay proposed by Fozard & Kilbinger (1985) and Mir *et al.* (1988) which measures inhibition of responses to field stimulation in the guinea-pig ileum preparation.

5-HT_{1A} receptors in the guinea-pig ileum myenteric plexus preparation have been shown to inhibit acetylcholine release following field stimulation and the 5-HT_{1A}agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; Arvidsson *et al.*, 1981) inhibits the contractile responses to field stimulation (Fozard & Kilbinger, 1985). We have modified this assay by using the more potent and selective agonist, N,N-dipropyl-5-carboxamidotryptamine (DP5CT; Hagenbach *et al.*, 1986) to investigate the roles of agonists and antagonists at 5-HT_{1A} receptors. We show that reproducible responses to DP5CT may be obtained in media containing low Ca^{2+} and that responses to DP5CT readily cross-desensitize with responses to other 5-HT_{1A} receptor agonists. Preliminary accounts of part of this work have already been published (Brown *et al.*, 1989).

[1] Author for correspondence.

Methods

Guinea-pig ileum preparation

Ileum preparations, taken from female Dunkin-Hartley guinea-pigs in the weight range of 250–500 g, were set up in 30 ml isolated organ baths filled with low Ca^{2+} Tyrode solution (composition, mM: NaCl 137, KCl 2.7, $CaCl_2$ 0.9, $MgCl_2$ 1.1, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.5; pH 7.4; 37°C; gassed with oxygen) and stimulated via two parallel stainless steel electrodes, one of which passed through the lumen, at 0.1 Hz (1 ms pulse duration, supramaximal voltage). Cumulative concentration-response curves were obtained to 8-OH-DPAT (threshold 10 nM) and DP5CT (threshold 0.1 nM, maximal 30 nM) only once in any tissue to avoid desensitization; the tissues were preincubated with antagonists for 40 min. In some experiments antagonism of the responses to DP5CT was expressed as apparent pA_2 values by the method of Arunlakshana & Schild (1959). Resting tension was adjusted to 1 g and responses were recorded isometrically on a Hugo-Sachs or LectroMed chart recorder. Experiments using phorbol dibutyrate were performed in a fume hood. Phorbol dibutyrate (3 μM) was incubated with the preparations for 30 min, during which time the preparations developed marked spontaneous activity, and then washed out for 20 min.

Binding studies

A washed, total membrane fraction of rat or pig brain was used in the majority of binding studies. In some preparations (Table 1) tissue (heart and lung) from Dunkin Hartley guinea-pigs was used. Tissue was homogenized in 25 vol ice cold Tris HCl buffer (50 mM Tris HCl pH 7.4) with a polytron PT10 tissue disrupter. The homogenate was centrifuged at 48,000 g_{av} in a refrigerated centrifuge at 4°C for 15 min. After the third wash the pellet was incubated in 25 vol buffer at 37°C for

Table 1 Binding assays used to define type of receptor

Receptor	*Tissue*	*Ligand*	*Incubation*	*Non-specific drug*	*Standard*
5-HT_{1A}	Rat cerebral cortex	[^{3}H]-8-OH-DPAT 1.0 nM	15 min 37°C	Buspirone 3×10^{-6} M	Buspirone
5-HT_{1A}	Rat cerebral cortex	[^{3}H]-5-HT 2.0 nM	10 min 37°C	Spiperone 10^{-6} M	Buspirone
5-HT_{1A}	Rat cerebral cortex	[^{3}H]-WB4101 2.0 nM	40 min 37°C	5-HT 10^{-5} M	Buspirone
5-HT_{1B}	Rat striatum	[^{125}I]-cyanopindolol 30 pM	40 min 37°C	5-HT 10^{-5} M	RU 24969
5-HT_{1C}	Pig choroidal plexus	[^{3}H]-mesulergine 1.0 nM	15 min 37°C	5-HT 10^{-5} M	Mesulergine
5-HT_{1D}	Baboon cortex	[^{3}H]-5-HT 2.0 nM	15 min 37°C	5-HT 10^{-5} M	Rauwolscine
5-HT_2	Rat frontal cortex	[^{3}H]-ketanserin 1.0 nM	10 min 37°C	Methysergide 2×10^{-6} M	Ritanserin
α_1	Rat cerebral cortex	[^{3}H]-prazosin 0.5 nM	30 min 25°C	Phentolamine 10^{-5} M	Prazosin
α_2	Rat cerebral cortex	[^{3}H]-yohimbine 2.0 nM	30 min 25°C	Phentolamine 10^{-5} M	Yohimbine
β_1	Guinea-pig lung	[^{3}H]-dihydroalprenolol 1.0 nM	30 min 25°C	Isoprenaline 10^{-4} M	Propranolol
β_2	Guinea-pig left ventricle	[^{125}I]-cyanopindolol 0.9 pM	30 min 25°C	Isoprenaline 10^{-4} M	Propranolol
D_2	Rat striatum	[^{3}H]-spiperone 0.1 nM	30 min 37°C	(+)-Butaclamol 10^{-6} M	Haloperidol
CEB	Rat cerebral cortex	[^{3}H]-nitrendipine 0.2 nM	2 h 25°C	Nifedipine 10^{-6} M	Nifedipine

10 min before being recentrifuged. The final pellet was resuspended in 3 ml buffer and stored under liquid nitrogen until required.

The membrane fraction was incubated to equilibrium with radiolabelled drug in the presence of 8–15 concentrations of the test compound or a highly selective drug to define non-specific binding using the conditions shown in Table 1. Separation of bound from free ligand was achieved with a Brandel M24 cell harvester at a constant vacuum pressure of 22 mmHg. Bound radioactivity was determined by liquid scintillation spectrometry. The inhibition of specific binding of the radioligands by competing ligands was analysed graphically to estimate IC_{50} (concentration of competitor displacing 50% of specifically-bound radioligand), by use of a non-linear least square programme run on a BBC microcomputer. The IC_{50} was converted to an affinity constant (K_i) by use of the equation of Cheng & Prusoff (1973).

RS-30199-193 (5-chloro-2-methyl-1,2,3,4,8,9,10,10a octahydronaphth-[1,8-cd]-azepine)hydrochloride, RS-64459-193 (5-chloro-2-[4-(8-azaspiro[4,5]decane-7,9-dione)-but-1-yl]-1,2,3,4,8,9,10,10a-octahydronaphth[1,8-CD]3-azepine hydrochloride), UK14304 (5-bromo-6-[2-imidazolin-2-yl-amino]-quinoxaline hydrochloride), DP5CT, 8-OH-DPAT, buspirone, idazoxan and ipsapirone were synthesized by Dr R. Clark, Institute or Organic Chemistry, Syntex, Palo Alto, U.S.A. ^{3}H-labelled ligands were obtained from Du Pont, U.K. or Amersham, U.K., all other drugs were purchased from Sigma. All drugs were dissolved in distilled water, with the exception of phorbol dibutyrate which was dissolved in ethanol. Drugs were stored on ice and solutions were prepared daily.

Values refer to mean ± s.e.mean; Student's t test was used for comparisons.

Results

Responses to 8-OH-DPAT and DP5CT

Guinea-pig ileum preparations responded to field stimulation (0.1 Hz, 1 ms pulse duration, supramaximal voltage) with stable contractions which were abolished by atropine (0.1 μM) and by tetrodotoxin (0.3 μM). DP5CT (1 nM–100 nM) and 8-OH-DPAT (10 nM–10 μM) inhibited the contractile responses in Tyrode solution containing 0.9 mM Ca^{2+}. The concentration-response curve to DP5CT was monophasic, with a maximum inhibition of 40 ± 4% (n = 50; Figure 1). In contrast, the response to 8-OH-DPAT was biphasic with low concentrations (<0.1 μM) causing 30% inhibition, but higher concentrations (>10 μM) caused full inhibition of the responses to field stimulation (Figure 2). Both phases of the concentration-response curves to 8-OH-DPAT were resistant to idazoxan (1 μM), ketanserin (0.1 μM) and prazosin (0.1 μM; data not shown). The responses to DP5CT and the responses to low concentrations of 8-OH-DPAT were antagonized by 0.1 μM spiperone but the responses to high concentrations of 8-OH-DPAT were resistant to spiperone (Figures 1–2). Responses to DP5CT were antagonized by methiothepin (Figure 1). In experiments in which a full range of methio-

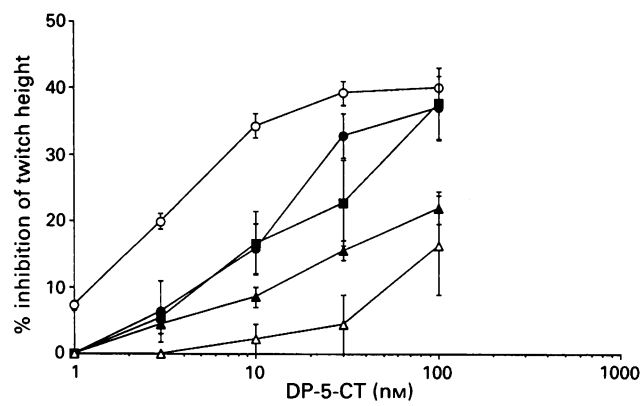

Figure 1 Concentration-response curves to N,N-dipropyl-5-carboxamidotryptamine (DP5CT, ○, control, n = 50) in the presence of spiperone (■, 0.1 μM, n = 5), buspirone (▲, 1 μM, n = 3), ipsapirone (●, 1 μM, n = 3), and methiothepin (△, 1 μM, n = 6). Responses are measured as the inhibition of the contractile responses of guinea-pig ileum to field stimulation in the presence of 0.9 μM Ca^{2+}.

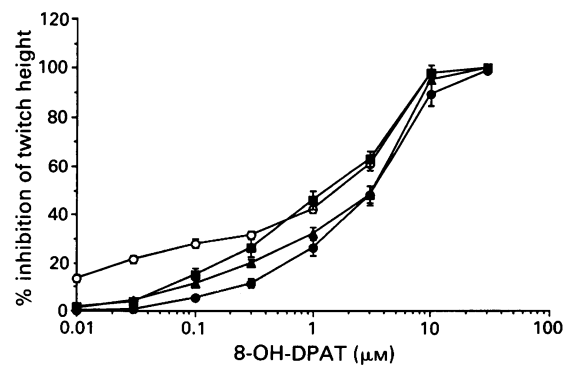

Figure 2 Concentration-response curves to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, ○, control, n = 4) in the presence of spiperone (■, 0.1 μM, n = 13), buspirone (●, 1 μM, n = 23), ipsapirone (▲, 1 μM, n = 13). Responses are measured as the inhibition of the contractile responses of guinea-pig ileum to field stimulation in the presence of 0.9 mM Ca^{2+}.

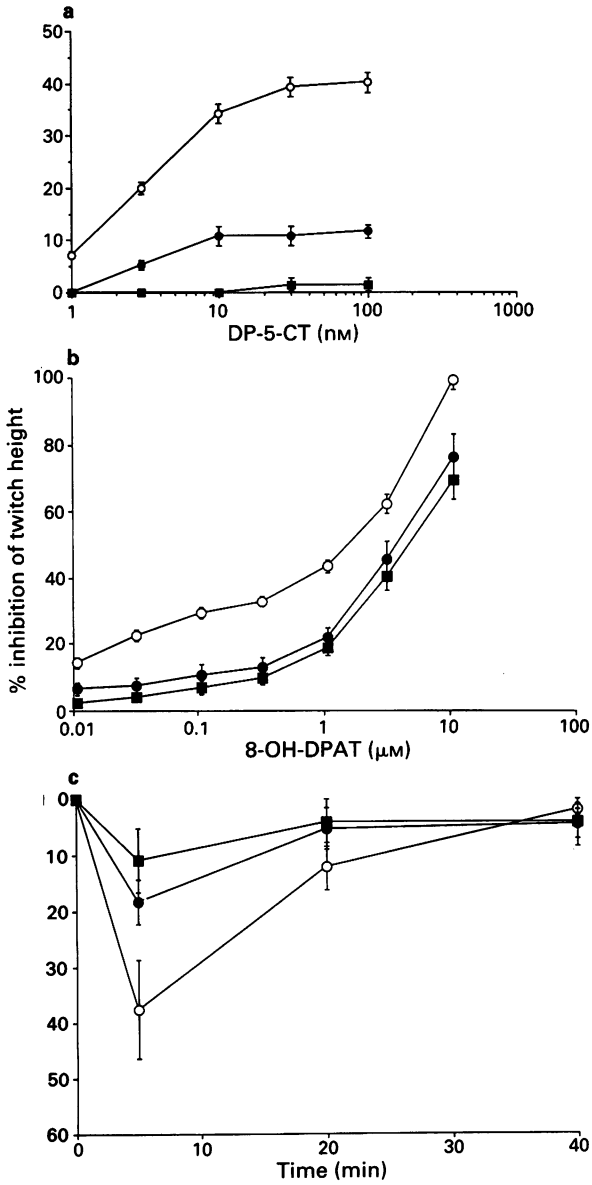

Figure 3 (a) Concentration-response curves to N,N-dipropyl-5- carboxamidotryptamine (DP5CT) in the presence of 0.9 mM Ca^{2+} (○, $n = 50$), 1.8 mM Ca^{2+} (●, $n = 4$) or 5.4 mM Ca^{2+} (■, $n = 4$). Responses are measured as the inhibition of the contractile responses of guinea-pig ileum to field stimulation. (b) Concentration-response curves to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) in the presence of 0.9 mM Ca^{2+} (○, $n = 45$), 1.8 mM Ca^{2+} (●, $n = 4$) or 5.4 μM Ca^{2+} (■, $n = 4$). (c) Inhibitory effects of buspirone (1 μM) on the contractile responses of the guinea-pig ileum to field stimulation in the presence of 0.9 (○), 1.8 (●) or 5.4 (■) mM Ca^{2+} ($n = 4$).

thepin concentrations (0.03, 0.1, 0.3, 1 μM) were used, the concentration-response curves to DP5CT were shifted to the right, with reduction of the maximum response only occurring at 1 μM; in these experiments methiothepin had a pA_2 value of 7.3 (slope 0.9) indicating a site of action at 5-HT$_1$ receptors (Bradley *et al.*, 1986). In contrast, responses to DP5CT were resistant to ketanserin (0.1 μM; not shown). Responses to DP5CT were not modified in preparations taken from guinea-pigs pretreated with *para*chlorophenylalanine (PCPA; 150 mg kg^{-1}, i.p., for 2 days, $n = 4$).

Changes in calcium concentration

Increasing the Ca^{2+} concentration from 0.9 M to 1.8 or 5.4 mM caused increases in the responses to field stimulation (18.9 + 3.3%, 1.8 mM; 30.4 + 6.1%, 5.4 mM, $n = 9$). The responses to DP5CT and the responses to low concentrations of 8-OH-DPAT were inhibited by increasing the Ca^{2+} concentration of the medium to 1.8 and 5.4 mM (Figure 3a,b) indicating that high sensitivity to 5-HT$_{1A}$ agonists was reduced with high Ca^{2+} concentrations. The inhibitory responses to buspirone were also reduced in higher Ca^{2+} concentrations (Figure 3c). In all other experiments in this paper, the Ca^{2+} concentrations were maintained at 0.9 mM.

Effects of buspirone and ipsapirone on responses to 8-OH-DPAT and DP5CT

Buspirone (1 μM, Figures 3c and 4) and ipsapirone inhibited the responses to field stimulation (19 ± 1%, $n = 14$ and 16 ± 1%, $n = 6$ respectively), although the inhibition faded back to baseline over a 40 min incubation period.

The responses to buspirone and/or ipsapirone were resistant to the α_2-adrenoceptor antagonist, idazoxan (1 μM), were reduced by pretreatment with spiperone (0.1 μM) and were abolished by pretreatment with a threshold concentration of 8-OH-DPAT (10 nM; Table 2). Pretreatment of tissues with buspirone or ipsapirone (1 μM for 40 min) allowed responses to field stimulation to return to control levels, but under these conditions responses to subsequent addition of DP5CT and

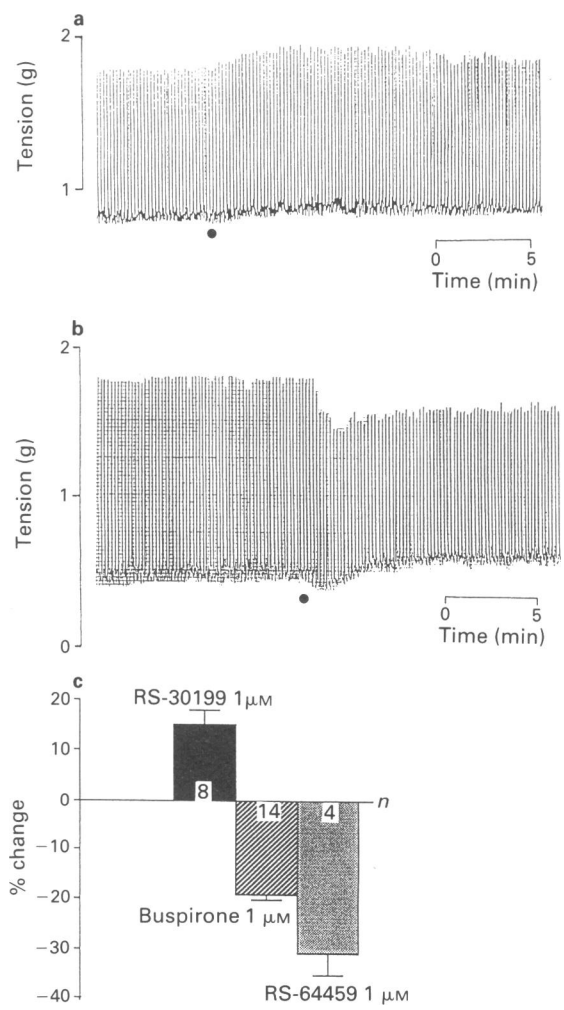

Figure 4 Effects of (a) RS-30199-193 (1 μM), (b) buspirone (1 μM) and RS-64459-193 on responses to field stimulation of the guinea-pig ileum. The histogram (c) shows the percentage change in twitch height for buspirone ($n = 14$), RS-30199-193 ($n = 8$) and RS-64459-193 ($n = 4$). Vertical bars represent s.e.mean.

Table 2 Inhibition (−) or augmentation (+) of contractile responses of guinea-pig ileum to field stimulation

Compounds	*% change*	*s.e.mean*	n
RS-30199-193 1 μM	+15.0	2.0	8
RS-30199-193 1 μM + 8-OH-DPAT 10 nM	+18.0	2.0	3
RS-30199-193 1 μM + PCPA	+38.6	5.8	3
Buspirone 1 μM	−20.0	1.0	14
Buspirone 1 μM + idazoxan 0.1 μM	−20.0	1.5	6
Buspirone 1 μM + 8-OH-DPAT 10 nM	0	0.5	3
Buspirone 1 μM + spiperone 0.1 μM	−12.0	1.5	14
Buspirone 1 μM + spiperone 1 μM	−2.0	0.5	14
Ipsapirone 1 μM	−16.0	1.5	6
Ipsapirone 1 μM + idazoxan 0.1 μM	−10.0	1.0	6
Ipsapirone 1 μM + 8-OH-DPAT 10 μM	−1.0	1.0	5
RS-64459-193 1 μM	−30.7	4.5	4

The inhibition (in the presence of buspirone, ipsapirone or RS-64459-193) or augmentation (RS-30199-193) is calculated as % change of the initial twitch response. Antagonists were pre-incubated for 40 min.

8-OH-DPAT were markedly antagonized (Figures 1 and 2). These results indicate that agonists at 5-HT_{1A} receptors may cross-desensitize, or antagonize, each other. Responses to the α_2-adrenoceptor agonist, UK14304 were resistant to DP5CT (100 nM, dose-ratio <2, $n = 4$).

Effects of phorbol esters

Incubation of the preparations with phorbol dibutyrate (3 μM for 30 min) caused an increase in spontaneous contractions (20–50% of the responses to field stimulation) which was reversed on wash-out. Twenty minutes after wash-out of phorbol dibutyrate, responses to DP5CT were abolished, whereas responses to the α_2-adrenoceptor agonist UK-14304 were only slightly attenuated (Figure 5).

Effects of RS-30199-193

RS-30199-193 (Figure 6) is an agent with affinity for 5-HT_{1A} sites in rat cerebral cortex similar to that of buspirone (pK_i RS 30199-193 7.9, pK_i buspirone 8.0; Brown *et al.*, 1989); the compound is selective for 5-HT_{1A} binding sites over other 5-HT subtypes. It has moderate affinity for α_2-adrenoceptor binding sites but only low affinity for other subtypes tested (Table 3). However, this agent, in contrast to buspirone,

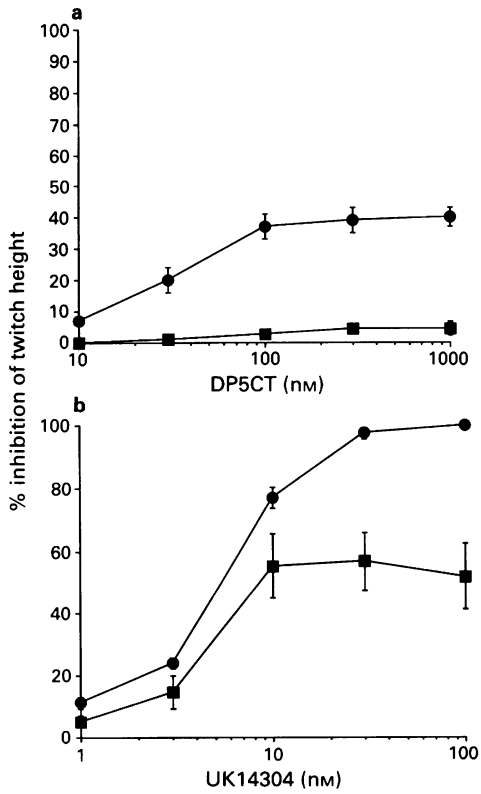

Figure 5 Inhibitory effects of (a) N,N-dipropyl-5-carboxamidotryptamine (DP5CT) and (b) (UK14304) on responses to field stimulation of guinea-pig ileum under control conditions (●) or after treatment of the preparations with phorbol dibutyrate (3 μM) for 30 min followed by a 20 min wash-out period (■). Vertical bars represent s.e.mean of at least 4 experiments.

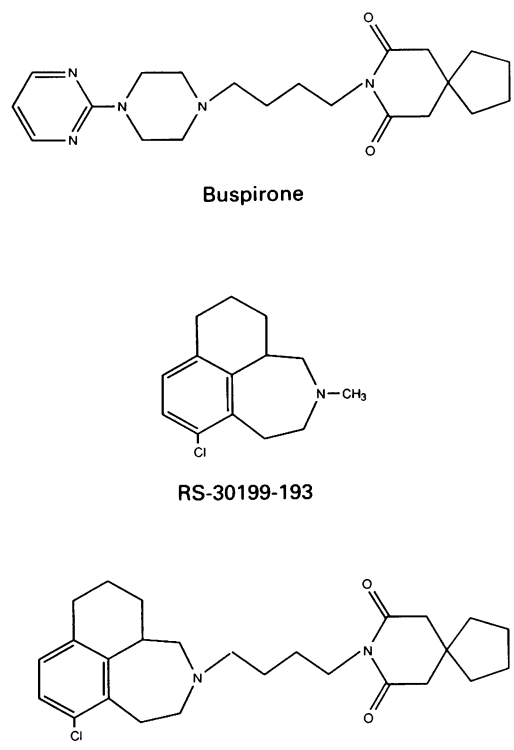

Figure 6 Structures of buspirone, 5-chloro-2-methyl-1,2,3,4,8,9,10, 10a-octahydronaphth-[1,8-cd]-azepine HCl (RS-30199-193) and (5-chloro-2-[4-(8-azaspiro [4,5]decane-7,9-dione)-but-1-yl]-1,2,3,4,8,9,10, 10a-octahydronaphth[1,8- cd]-3-azepine HCl (RS-64459-193).

Table 3 Affinity values of RS-30199-193

Receptor	*Ligand*	*Tissue*	pK_i (mean ± s.e.mean)
5-HT$_{1A}$	[^{3}H]-8-OH-DPAT	Rat cortex	7.91 ± 0.08
5-HT$_{1A}$	[^{3}H]-5-HT	Rat cortex	7.80 ± 0.1
5-HT$_{1A}$	[^{3}H]-WB4101	Rat cortex	7.98 ± 0.1
5-HT$_{1B}$	[^{125}I]-cyanopindolol	Rat striatum	<5
5-HT$_{1C}$	[^{3}H]-mesulergine	Pig choroid plexus	<5
5-HT$_{1D}$	[^{3}H]-5-HT	Baboon cortex	<5
5-HT$_2$	[^{3}H]-ketanserin	Rat frontal cortex	5.9 ± 0.1
α_1	[^{3}H]-prazosin	Rat cortex	6.2 ± 0.1
α_2	[^{3}H]-yohimbine	Rat cortex	6.94 ± 0.08
β_1	[^{3}H]-dihydroalprenolol	Guinea-pig heart	6.2 ± 0.2
β_2	[^{3}H]-dihydroalprenolol	Guinea-pig lung	5.7 ± 0.2
D_2	[^{3}H]-spiperone	Rat striatum	6.0 ± 0.3
CEB	[^{3}H]-nitrendipine	Rat striatum	<5

Binding assays were carried out as described in the methods section, using the appropriate ligands and assay conditions described in Table 1.

increased contractile responses to field stimulation and caused a threshold increase in tone (Figure 4). In addition, RS-30199-193 (1 μM) did not antagonize the responses to DP5CT (Figure 7). The increased contractility caused by RS-30199-193 was unaffected by pretreatment with a threshold concentration of 8-OH-DPAT (10 nM) and was slightly increased in preparations taken from animals pretreated with PCPA (Table 2). In four further experiments, the increased response to field stimulation was unaffected after desensitizing the preparations to 5-HT (1 μM). The increase in twitch height was unaffected by the presence of ICS-205-930 (0.3 μM 17.9 ± 1.0% augmentation, $n = 3$, compared with 21.7 ± 3.0% in control experiments, $n = 15$, NS). Higher concentrations of ICS-205-930 (1 μM) decreased the responses to field stimulation by more than 50% in the low Ca^{2+} medium used in the present experiments. Thus the increase in responses to field stimulation produced by RS-30199-193 does not seem to be mediated by 5-HT$_3$ or 5-HT$_4$ receptors.

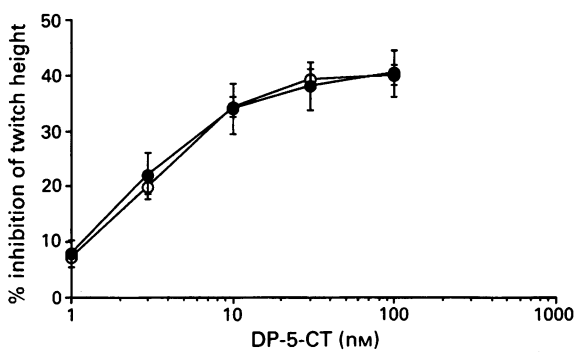

Figure 7 Comparison of the inhibitory effects of N,N-dipropyl-5-carboxamidotryptamine (DP5CT) on the responses of guinea-pig ileum to field stimulation in the presence (●, $n = 7$) or absence (○, $n = 50$) of (5-chloro-2-methyl-1,2,3,4,8,9,10,10a-octahydronaphth[1,8-cd]-azepine HCl (RS-30199, 1 μM). Vertical bars represent s.e.mean.

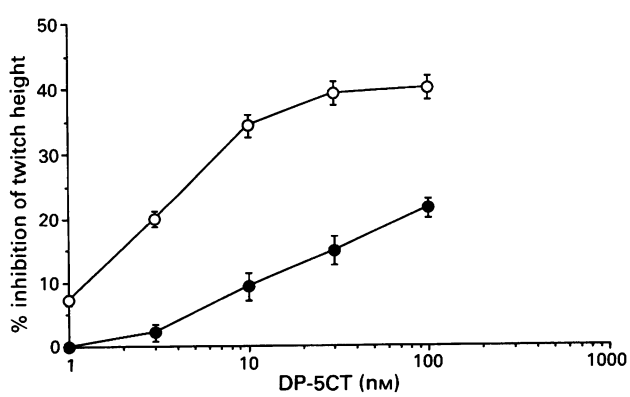

Figure 8 Comparison of the inhibitory effects of N,N-dipropyl-5-carboxamidotryptamine (DP5CT) on the responses of guinea-pig ileum to field stimulation in the presence (●, $n = 7$) or absence (○, $n = 50$) of (5-chloro-2-[4-(8-azaspiro[4,5]decane-7,9-dione)-but-1-yl]-1,2,3,4,8,9,10,10a-octahydronaphth[1,8-cd]-3-azepine HCl (RS-64459-193, 1 μM). Vertical bars represent s.e.mean.

Effects of RS-64459-193

RS-64459-193 (Figure 6) maintained high affinity for the 5-HT$_{1A}$ binding site in the rat brain (pK_i 8.10 ± 0.09 against [^{3}H]-8-OH-DPAT, pK_i 8.06 ± 0.1 against [^{3}H]-5-HT) but unlike RS-30199-193 behaved similarly to buspirone in the ileum in that responses to field stimulation were inhibited (30.7 ± 4.5%, $n = 4$; Figure 5; Table 2) and the compound antagonized subsequent responses to DP5CT (Figure 8); responses to UK 14304 were not antagonized (dose ratio <2, $n = 4$).

Discussion

Many subgroups of 5-HT receptors have been claimed to exist frequently only on the basis of radioligand binding assays; nevertheless, there is general agreement that the receptors may be classified into 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$ subgroups (Bradley *et al.*, 1986). Several subtypes of the 5-HT$_1$ site have been described and considerable interest in the 5-HT$_{1A}$ site has arisen on the basis of the anxiolytic properties of buspirone, which behaves as a weak agonist at 5-HT$_{1A}$ receptors in behavioural tests (Tricklebank *et al.*, 1984; Clague & Spedding, 1987). However, there are few functional *in vitro* correlates of affinity at this site. A frequently used test, inhibition of forskolin-induced adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation in guinea-pig hippocampal cells, has only a <25% maximal response, with correspondingly poor signal-to-noise ratio (e.g. Bockaert *et al.*, 1987) and in some systems stimulation of cyclase is seen (Markstein *et al.* 1986). We were therefore interested by the reports of Kilbinger & Fozard (1985) and Mir *et al.* (1988) showing that the inhibitory effects of 8-OH-DPAT on acetylcholine release in guinea-pig ileum preparations were mediated by 5-HT$_{1A}$ receptors.

8-OH-DPAT appears to cause inhibition in the ileum by two effects: a response mediated by 5-HT_{1A} receptors (<0.1 μM), which is responsible for up to 30% inhibition and an effect at higher concentrations which is resistant to 5-HT receptor and α-adrenoceptor antagonists (see also Kalkman *et al.*, 1986). DP5CT has 10 fold higher affinity for 5-HT_{1A} sites in radioligand binding experiments than does 8-OH-DPAT (Markstein *et al.*, 1986; Mir *et al.*, 1986) and we found this agent to be more potent than 8-OH-DPAT. Thus DP5CT would appear to be the most selective agonist available for this test, confirming the data of Hagenbach *et al.* (1986).

Therefore, there is good evidence that the inhibitory effects of DP5CT and of low concentrations of 8-OH-DPAT in this preparation are mediated via a direct interaction with receptors which may be classified as 5-HT_{1A}. Thus the inhibitory responses occur in the concentration-ranges at which these ligands are selective for 5-HT_{1A} receptors and have negligible affinity for other sites (Markstein *et al.*, 1986; Mir *et al.*, 1986). The effects of DP5CT were competitively antagonized by methiothepin with a pA_2 of 7.3. Spiperone, the antagonist which was first used to define 5-HT_{1A} sites (Peroutka & Snyder, 1979; Middlemiss & Fozard, 1983) was an effective antagonist of the effects of both DP5CT and 8-OH-DPAT. Furthermore, both buspirone and ipsapirone which are relatively selective, but less potent, ligands for the 5-HT_{1A} site caused spiperone-sensitive inhibition of the responses to field stimulation and these agents behaved as partial agonists and inhibited responses to 8-OH-DPAT. Responses to the agonsts faded with time (e.g. Figure 3c) and may be more akin to desensitization than partial agonism.

Buspirone and ipsapirone have been reported to be partial agonists in behavioural tests (Tricklebank *et al.*, 1984; Clague & Spedding, 1987) which are predictive of affinity for 5-HT_1 receptors (Goodwin & Green, 1985). However, in the present study, prolonged incubation of tissues with these drugs resulted in a waning of the inhibitory effects and subsequent antagonism of the effects of 8-OH-DPAT and DP5CT. Prolonged incubation with 8-OH-DPAT prevented the inhibitory effects of buspirone and ipsapirone. There appears to be a phenomenon of cross-desensitization, or antagonism, between these partial agonists, which has also been described in canine basilar artery (Taylor *et al.*, 1986).

The inverse Ca^{2+}-dependence of the responses to DP5CT and 8-OH-DPAT is typical of a presynaptic phenomenon, in that presynaptic responses to α_2-adrenoceptor agonists are reduced by increasing the Ca^{2+} concentration (Drew, 1978). As the total inhibition by DP5CT in this study was much less than that of α_2-adrenoceptor agonists, which fully inhibit the responses to field stimulation, a lowered Ca^{2+} level is especially important for the assay. The low maximal effects may relate to the number of receptor sites, the coupling to effector systems or it may be that none of the agents investigated was a full agonist. Whatever the reason, it is critical for the reproducibility of the assay that the Ca^{2+} concentration is maintained below 1.3 mM.

Phorbol esters have been shown to cause phenomena similar to those seen with receptor desensitization (Sibley *et al.*, 1985; Spedding, 1987) and these effects have been attributed to protein kinase C activation. In the present study, phorbol dibutyrate, a phorbol ester which rapidly penetrates into cells, abolished the responses to DP5CT while having less effect on the responses to UK14304 implying that protein kinase C is involved in the desensitization (or waning of the agonist effect in responses to the continued presence of the agonist).

With DP5CT, 8-OH-DPAT, buspirone and ipsapirone, inhibition of the responses to field stimulation could be related to affinity for the 5-HT_{1A} site. However, the structurally unrelated substance, RS-30199-193, showed similar affinity to buspirone in radioligand binding experiments for the 5-HT_{1A} site in rat cerebral cortex (Brown *et al.*, 1988) yet was devoid of partial agonist effects in the ileum and did not antagonize the inhibitory effects of DP5CT. This agent may therefore interact with a subset of 5-HT_{1A} sites (Wouters & Raedemaker, 1988) or interact with the sites in a novel way. In this respect, RS-30199-193 has been shown to be anxiogenic and not sedative in animal models whereas buspirone is anxiolytic at low doses and anxiogenic at high doses (Redfern *et al.*, 1989; Moulton & Morinan, 1990).

The cause of the increase in basal tone following RS-30199-193 is unknown, but would appear to be unrelated to the effects at 5-HT receptors in that it was resistant to all the antagonists tested. The increase was presumably not consequent on release of 5-HT because it was slightly increased in preparations taken from animals treated with PCPA.

When the buspirone side chain was added to RS-30199-193, the ability of the hybrid compound, RS-64459-193, to inhibit the responses to field stimulation and antagonize the responses to 8-OH-DPAT and DP5CT was similar to that of buspirone. Yet, despite this difference in the functional effects in the guinea-pig ileum, RS-64459-193 had similar affinity for the 5-HT_{1A} sites in the rat cortex. Thus the buspirone side chain when added to RS-30199-193 appears either to induce affinity for a distinct subset of receptors in the guinea-pig ileum or is required for functional effectiveness at the 5-HT_{1A} receptor.

In conclusion, this study demonstrates that transmurally-stimulated guinea-pig ileum preparation may be used to assess the effects of drugs acting at 5-HT_{1A} receptors and, in particular, to study partial agonist effects, but that the receptor in the ileum may not be identical to the binding site in rat cerebral cortex (see also Wouters & Raedemaker, 1988).

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Effects of histamine and activators of the cyclic AMP system on protein synthesis in and release of high molecular weight glycoproteins from isolated gastric non-parietal cells

[1] H.-K. Heim, A. Oestmann & K.-Fr. Sewing

Institut für Allgemeine Pharmakologie, Medizinische Hochschule Hannover, 3000 Hannover 61, Germany

1 Glycoprotein and protein synthesis in and release from pig isolated, enriched gastric mucous cells were measured by the incorporation of N-acetyl-[^{14}C]-D-glucosamine and [^{3}H]-L-leucine, respectively, into cellular and released acid precipitable material.

2 Histamine and activators of the adenosine 3':5'-cyclic monophosphate (cyclic AMP) system maximally stimulated total protein and glycoprotein synthesis in and release from the cells at concentrations of histamine (10 μM), forskolin (10–100 μM), 3-isobutyl-1-methylxanthine (100 μM), and dibutyryl cyclic AMP (1–3 mM), respectively. In the presence of 3-isobutyl-1-methylxanthine (30 μM) histamine stimulation was enhanced.

3 As shown by gel chromatography, stimulation by histamine (100 μM), forskolin (10 μM), 3-isobutyl-1-methylxanthine (100 μM) and dibutyryl cyclic AMP (1 mM) resulted in a release of high molecular weight ($\approx 2 \times 10^6$ daltons) glycoproteins from the cells. The histamine H_2-receptor antagonist, ranitidine (100 μM), blocked the effect of histamine.

4 We conclude that cyclic AMP-dependent processes are involved in the regulation of protein and glycoprotein synthesis in and the release of high molecular weight (mucous) glycoproteins from pig gastric non-parietal cells and that histamine may be a physiological activator of this system.

Keywords: Histamine; forskolin; cyclic AMP; protein; glycoprotein; gastric mucin; phosphodiesterase inhibitor

Introduction

Synthesis and secretion of high molecular weight (mucous) glycoproteins represents one of the major physiological functions of gastric mucous cells and is indispensable for the formation of the mucus layer in the stomach (Neutra & Forstner, 1987). Recent results argue for a role of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in the regulation of this process, since forskolin, a receptor-independent activator of the adenylate cyclase (Seamon & Daly, 1986), enhanced the release of high molecular weight glycoproteins from explants of rabbit gastric mucosa (Seidler & Sewing, 1989). However, indirect mechanisms may contribute to the effects observed in an organ culture preparation.

To confirm a function for cyclic AMP directly at the cellular level, we examined the influence of known activators of the cyclic AMP 'second messenger' system on protein and glycoprotein synthesis in and release from enriched porcine gastric mucous cells, as measured (Heim *et al.*, 1989) by the incorporation of N-acetyl-[^{14}C]-D-glucosamine ([^{14}C]-GlcNAc) and [^{3}H]-L-leucine, respectively, into trichloroacetic acid/phosphotungstic acid precipitable cellular and released macromolecules. As stimulants with different points of action in the cyclic AMP system we used, in addition to forskolin, histamine, shown by Gespach *et al.* (1982, 1983) and Sewing *et al.* (1985) to enhance cyclic AMP levels of gastric non-parietal cells via H_2-receptor activation, 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic AMP phosphodiesterases (Beavo, 1988), and dibutyryl cyclic AMP (db cyclic AMP), an activator of cyclic AMP-dependent protein kinases (Simon *et al.*, 1973).

Methods

Cell isolation

The techniques used have been described in detail previously (Heim *et al.*, 1989). Briefly, the following buffers were used: buffer A consisted of (mM): NaCl 74, $NaHCO_3$ 20, KCl 5, NaH_2PO_4 0.5, Na_2HPO_4 1, HEPES 50, glucose 11 and indomethacin 0.02. Buffer B had the same composition but contained additionally $CaCl_2$ 1, $MgCl_2$ 1.5 and bovine serum albumin 2 mg ml^{-1}. Corpus mucosa of slaughterhouse pigs was cut into squares of about 0.25 cm^2 and incubated for 30 min at 37°C in 150 ml of buffer A containing 188 mg of pronase E. The supernatant was discarded and the remaining tissue pieces were minced with scissors and incubated for 45 min in 100 ml of buffer B, containing 3600 units of collagenase type I and 11,600 units of collagenase type V. The suspension was filtered twice through a nylon mesh (pore size 200 μm) and the remainder treated again with collagenase. The combined filtrates were centrifuged at 580 *g* for 3 min and the resulting cell pellet resuspended in 20 ml of buffer B.

Cell separation

Cell separation was achieved by counterflow centrifugation, with a Beckman elutriator rotor on a Beckman J-6 centrifuge. Cells (up to 10^9) were eluted in buffer B at 880 r.p.m. (234 g_{max}). Two cell populations (F) were obtained at flow rates of 20 ml min^{-1} (F1, 600 ml total volume collected) and 40 ml min^{-1} (F2, 1000 ml), and the remainder of cells was discarded. For this investigation we used F2 cells, consisting of more than 60% mucous cells, about 25% chief cells and less than 2% parietal cells, as determined by cell staining (Heim *et al.*, 1989). Cell viability, as checked by trypan blue exclusion, was >90%.

Total protein and glycoprotein production

Cells (3.6×10^6/1.8 ml) suspended in Dulbecco's modified Eagle's medium (DME), containing HEPES 25 mM instead of $NaHCO_3$, L-leucine 10 μM, bovine serum albumin (1 mg ml^{-1}), penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and gentamicin (50 μg ml^{-1}) were incubated at 37°C in the presence of 0.028 μCi ml^{-1} [^{3}H]-L-leucine, 0.278 μCi ml^{-1} [^{14}C]-GlcNAc and the test substances to be studied (Heim *et al.*, 1989). The cyclo-oxygenase inhibitor indomethacin was present at a concentration of 10 μM in all samples to prevent

[1] Author for correspondence.

indirect effects resulting from interactions with endogenously synthesized prostaglandins.

After 20 h, a time optimal for the simultaneous detection of effects on the incorporation of [^{3}H]-L-leucine and [^{14}C]-GlcNAc (Heim *et al.*, 1990), cellular and released acid precipitable material were separated by centrifugation and precipitated by trichloroacetic acid/phosphotungstic acid (TCA/PTA, final concentration 10%/1%). The samples were washed thoroughly and then solubilized in 1 ml of KOH 0.6 M. Incorporated radioactivity was determined in an LKB 1217 Rackbeta liquid scintillation counter, using quench correction programmes for ^{3}H and ^{14}C double-labelled samples, and served as a measure for total protein and glycoprotein synthesis and release, respectively. Values were expressed as d.p.m./10^6 cells after correcting for unspecific trapping by the incubation medium.

Gel chromatography of released glycoproteins

Cell suspensions were incubated for 20 h as described above in DME containing 1.11 μCi ml^{-1} [^{14}C]-GlcNAc. Cells were separated by centrifugation for 15 s in an Eppendorf centrifuge 3200 and the supernatants subjected to gel chromatography on Sepharose CL-6B in a column of 2.6 cm diameter and 90 cm length. For each column run we applied supernatants of 3×10^7 cells, pooled from three separate cell preparations. The samples (8 ml) were eluted with NaCl 0.2 M, containing 0.02% sodium azide, (Pearson *et al.*, 1980) at a pump controlled flow rate of 33.6 ml h^{-1}.

Fractions of 11.2 ml volume were collected and examined for TCA/PTA precipitable radioactivity. To this end, 1 ml of each fraction was treated with 250 μl TCA/PTA (50%/5%) and processed further as described for the supernatant samples above. Values for each column fraction were expressed as d.p.m./10^6 cells after correcting for unspecific trapping by the incubation medium, which was incubated and subjected to gel chromatography in parallel. The chromatography column was calibrated with dextran blue ($\approx 2 \times 10^6$ daltons, fractions 16–18), ferritin ($\approx 4.4 \times 10^5$ daltons, fractions 28–30), bovine serum albumin ($\approx 6.7 \times 10^4$ daltons, fractions 34–36) and phenol red ($\approx 3.5 \times 10^2$ daltons, fractions 47–49).

Statistics

Incorporation rates were evaluated by analysis of variance (Winer, 1971). If the calculated F-value indicated a probability of $P < 0.05$ then Dunnett's t test (Dunnett, 1955) was performed, using the variance and degree of freedom of experimental error derived from the analysis of variance, to trace out which concentration(s) of a test agent resulted in a significant effect compared to control. Differences with a $P < 0.05$ were considered to be significant. Results were shown as mean ± s.e.mean. The concentration of a test agent at which it was half-maximally effective (EC_{50} value) was estimated graphically.

Substances

The following were used: collagenase type I and V, Dulbecco's modified Eagle's medium deficient in L-leucine, penicillin-streptomycin solution (10,000 units ml^{-1}, 10 mg ml^{-1}), gentamicin solution (50 mg ml^{-1}), histamine dihydrochloride, 3-isobutyl-1-methylxanthine, dibutyryl cyclic AMP sodium salt (Sigma, München, FRG); bovine serum albumin, HEPES (Serva, Heidelberg, FRG); forskolin (Hoechst, Frankfurt, FRG); [4,5-^{3}H]-L-leucine sp. act. 140 Ci mmol^{-1} in 2% ethanol, N-acetyl-[1-^{14}C]-D-glucosamine sp. act. 58.7 mCi mmol^{-1} in 2% ethanol (Amersham, Braunschweig, FRG); indomethacin sodium trihydrate (Merck, Sharp and Dohme, Rahway, U.S.A.), ranitidine hydrochloride (Glaxo, Bad Oldesloe, FRG). All other substances were of analytical grade and obtained from Merck (Darmstadt, FRG).

Results

Total protein and glycoprotein production

Glycoprotein and protein synthesis in and glycoprotein and protein release from the cells were stimulated in a concentration-dependent manner by forskolin (Figure 1) with EC_{50} values of 280 nM, 2 μM, 850 nM, and 2 μM, respectively; by IBMX (Figure 2) with EC_{50} values of 3 μM, 15 μM, 22 μM and 21 μM, respectively, and by db cyclic AMP (Figure 3) with EC_{50} values of 150 μM, 300 μM, 170 μM and 330 μM, respec-

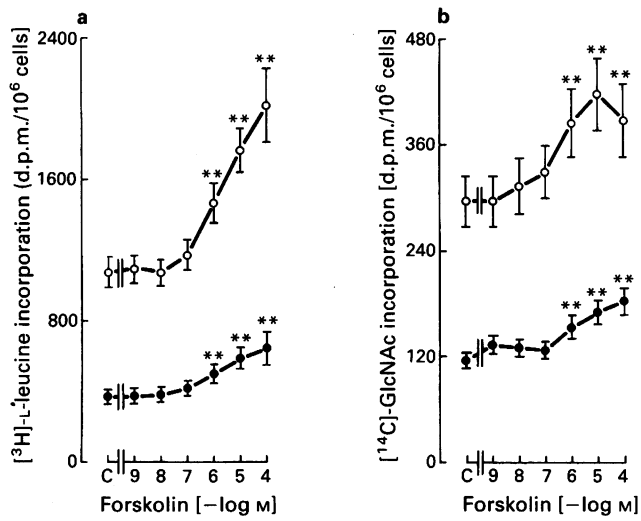

Figure 1 Effects of forskolin on protein (a) and glycoprotein (b) production. Enriched gastric mucous cells were incubated for 20 h with the tracers in the absence (C) or presence of forskolin in the concentrations indicated. Radioactivity incorporated into cellular (○) and released (●) acid precipitable material was determined as described under Methods. Each value represents the mean of 33 separate incubations obtained from 11 different cell preparations and vertical bars represent s.e.mean. ** $P < 0.01$ versus corresponding C.

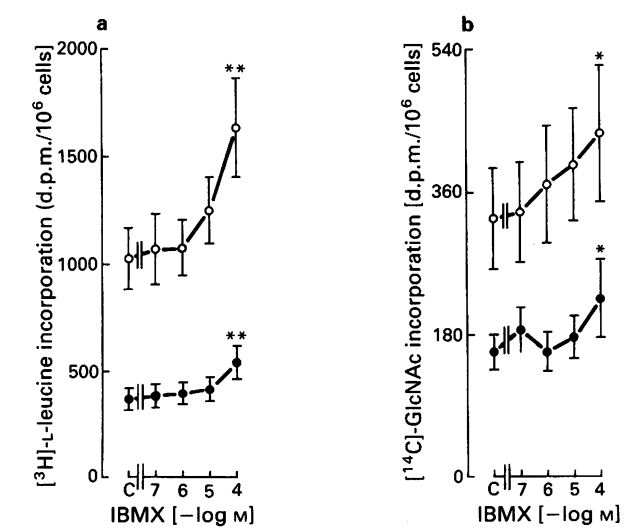

Figure 2 Effects of 3-isobutyl-1-methylxanthine (IBMX) on protein (a) and glycoprotein (b) production. Enriched gastric mucous cells were incubated for 20 h with the tracers in the absence (C) or presence of IBMX in the concentrations indicated. Radioactivity incorporated into cellular (○) and released (●) acid precipitable material was determined as described under Methods. Each value represents the mean of 15 separate incubations obtained from 5 different cell preparations and vertical bars represent s.e.mean. * $P < 0.05$ and ** $P < 0.01$ versus corresponding C, respectively.

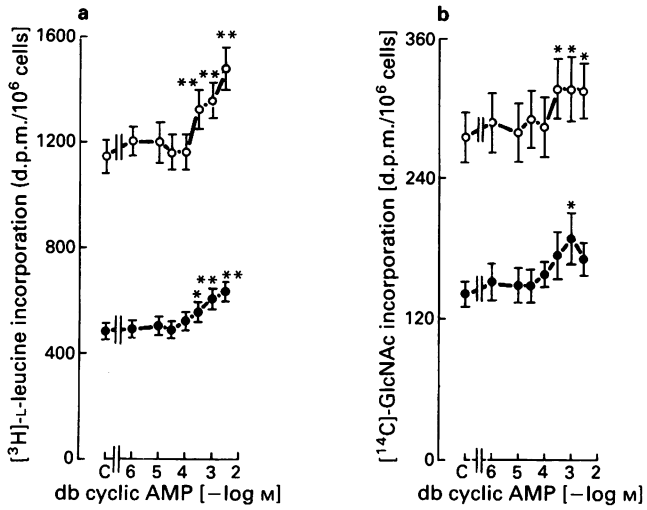

Figure 3 Effects of dibutyryl cyclic AMP (db cyclic AMP) on protein (a) and glycoprotein (b) production. Enriched gastric mucous cells were incubated for 20 h with the tracers in the absence (C) or presence of db cyclic AMP in the concentrations indicated. Radioactivity incorporated into cellular (○) and released (●) acid precipitable material was determined as described under Methods. Each value represents the mean of 24 separate incubations obtained from 8 different cell preparations and vertical cell bars represent s.e.mean. * $P < 0.05$ and ** $P < 0.01$ versus corresponding C, respectively.

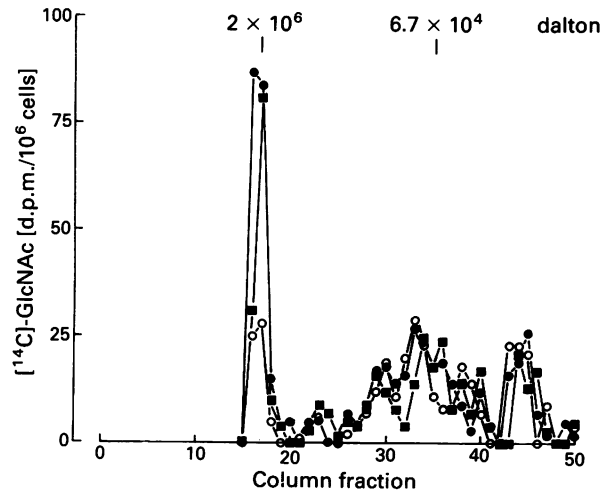

Figure 5 Gel chromatography of supernatants of enriched gastric mucous cells. Cells were incubated for 20 h in the presence of 1.11 μCi ml^{-1} N-acetyl-[^{14}C]-D-glucosamine ([^{14}C]-GlcNAc) without (○) or with forskolin 10 μM (●) or dibutyryl cyclic AMP 1 mM (■). Gel chromatography of cell supernatants and determination of radioactivity incorporated into released acid precipitable material were performed as described under Methods.

tively. Concentrations of these substances higher than those indicated in Figures 1–3 could either not be achieved because of a poor solubility (forskolin) or resulted in a decrease of stimulation (IBMX and db cyclic cAMP, results not shown).

Stimulation was also obtained with histamine as shown in Figure 4. However, effects on overall glycoprotein synthesis and release were small with maximal stimulations of 16% ($P < 0.05$) and 14% (NS) over control, respectively, at 10 μM. In the presence of IBMX at the submaximal concentration of 30 μM (see Figure 2), EC_{50} values for stimulation of protein and glycoprotein synthesis and of protein release by histamine were lowered from 380 nM to <100 nM, from 1 μM to 140 nM, and from 380 nM to 160 nM, respectively, and the EC_{50}-value for stimulation of glycoprotein release was 160 nM.

Released glycoproteins

Forskolin 10 μM, db cyclic AMP 1 mM (Figure 5), IBMX 100 μM (results not shown) and histamine 100 μM (Figure 6) enhanced mainly the release of high molecular weight glycoproteins (column fractions 16–18) with related stimulations of [^{14}C]-GlcNAc incorporation amounting to 221%, 110%, 67% and 108%, respectively, over control. The effect of histamine could be blocked by pretreatment of the cells with the histamine H_2-receptor antagonist, ranitidine, in a concentration of 100 μM (Figure 6).

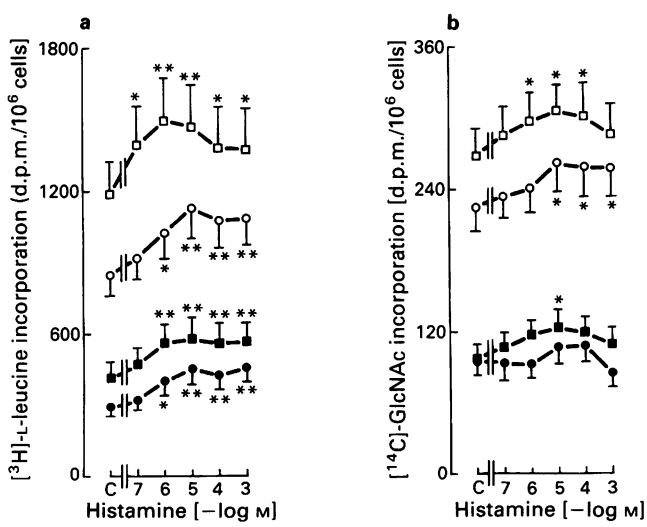

Figure 4 Effects of histamine on protein (a) and glycoprotein (b) production. Enriched gastric mucous cells were pre-incubated for 40 min without (○, ●) or with (□, ■) 3-isobutyl-1-methylxanthine 30 μM. Then another 20 h incubation with the tracers in the absence (C) or presence of histamine in the concentrations indicated followed. Radioactivity incorporated into cellular (○, □) and released (●, ■) acid precipitable material was determined as described under Methods. Each value represents the mean of 21 separate incubations obtained from 7 different cell preparations and vertical bars represent s.e.mean. * $P < 0.05$ and ** $P < 0.01$ versus corresponding C, respectively.

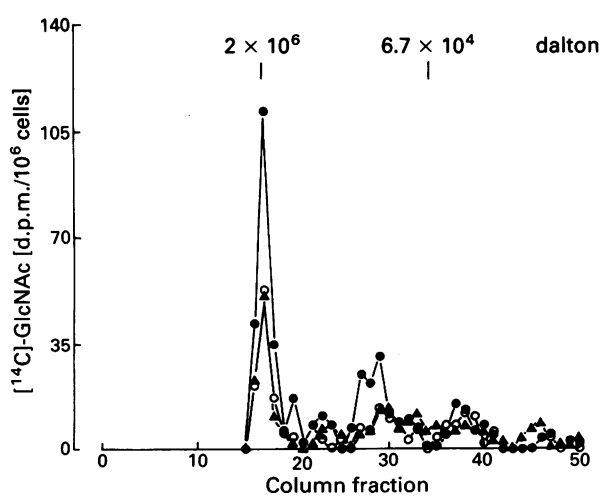

Figure 6 Gel chromatography of supernatants of enriched gastric mucous cells. Cells were pre-incubated for 40 min without (○, ●) or with (▲) ranitidine 100 μM. Then another 20 h incubation in the presence of 1.11 μCi ml^{-1} N-acetyl-[^{14}C]-D-glucosamine ([^{14}C]-GlcNAc) in the absence (○) or presence of histamine 100 μM (●, ▲) followed. Gel chromatography of cell supernatants and determination of radioactivity incorporated into released acid precipitable material were performed as described under Methods.

Discussion

Cyclic AMP is a 'second messenger' for the mediation of hormonal and neuronal effects in various tissues. In the gastric mucosa it has been connected with, among other actions, the stimulation of acid production by parietal cells (Soll & Berglindh, 1987) and the stimulation of pepsinogen synthesis and release (Defize & Hunt, 1988) by chief cells. Our results indicate a role for cyclic AMP in the regulation of mucus production by gastric mucous cells. We could show that agents considered to activate the cyclic AMP 'second messenger' system by acting at the membrane receptor level (histamine), at the adenylate cyclase (forskolin), at cyclic AMP phosphodiesterases (IBMX), or at cyclic AMP-dependent protein kinases (db cyclic AMP), as described in the Introduction, resulted in stimulation of the protein and glycoprotein synthesis in and the release of high molecular weight (mucous) glycoproteins from enriched pig gastric mucous cells.

In the presence of IBMX the potency of histamine for stimulation of these processes was enhanced and the effects of histamine could be blocked by pretreatment of the cells with the histamine H_2-receptor antagonist, ranitidine (Sewing & Hannemann, 1986), providing further evidence that stimulation by histamine was connected with a H_2-receptor-mediated activation of the cyclic AMP system. The test agents stimulated these processes in concentrations known to activate also other putative cyclic AMP-mediated processes in the gastric mucosa. For example, near-maximal stimulation of protein and glycoprotein production and a release of high molecular weight glycoproteins (this paper) and near-maximal stimulation of acid production by parietal cells and pepsinogen secretion from chief cells occurred with forskolin 10 μM (Chew, 1983; Hersey *et al.*, 1983), IBMX 100 μM (Soll & Wollin, 1979; Raufman & Cosowsky, 1988), db cyclic AMP 1 mM (Batzri & Dyer, 1981; Koelz *et al.*, 1982) and histamine 100 μM (Mårdh *et al.*, 1987; Sewing & Beinborn, 1988). In addition, EC_{50} values for stimulation of protein and glycoprotein production by IBMX in our cell system correspond well to IC_{50} values for inhibition of gastric mucosal phosphodiesterases by this compound (Klotz *et al.*, 1977).

Enhancement of overall protein synthesis by activators of the cyclic AMP system is also in accordance with stimulatory effects of forskolin (Murdoch *et al.*, 1982; Barinaga *et al.*, 1985) and db cyclic AMP (Murdoch *et al.*, 1982) on protein synthesis in other tissues and supports the hypothesis of a more general function of the cyclic nucleotide in the regulation of this cell function (Nagamine & Reich, 1985).

Our results indicate that in the pig, stimulation of the cyclic AMP system in different cell types of the gastric mucosa may activate processes both destructive of (acid and pepsinogen secretion) and protective for (mucous secretion) the gastric epithelium. In the pig, histamine may be a physiological activator of parietal, chief and mucous cells, leading via a H_2-receptor stimulated elevation of cellular cyclic AMP levels to a secretion of acid (Mårdh *et al.*, 1987), pepsinogen (Sewing & Beinborn, 1988) and mucous glycoproteins, respectively. Stimulation of mucous secretion by histamine has also been detected in *in vivo* experiments in cats (Vagne & Perret, 1976) and man (Lee *et al.*, 1987). However, the possibility of species differences concerning histamine effects should be kept in mind, since histamine did not stimulate the release of high molecular weight glycoproteins from rabbit gastric mucosal explants (Seidler & Sewing, 1989) and also did not stimulate pepsinogen secretion from rabbit isolated gastric glands (Koelz *et al.*, 1982) or cultured canine chief cells (Defize & Hunt, 1988).

In summary we conclude that in the pig, cyclic AMP-dependent processes are involved in the regulation of protein and glycoprotein synthesis in and the release of high molecular weight (mucous) glycoproteins from gastric non-parietal cells and that histamine may be a physiological activator of this system.

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The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats

J.J. Mosterd & [1]H.H.W. Thijssen

Department of Pharmacology, University of Limburg, Maastricht, The Netherlands

1 The long-term (30 days) effects of a single dose of brodifacoum (0.2 mg kg^{-1}, orally) on blood clotting activity and on liver parameters of the vitamin K cycle were investigated in rats. Maximal effect on blood clotting activity was seen on day one. On day seven blood clotting activity had returned to normal.

2 Liver microsomal vitamin KO reductase activity was maximally suppressed (10% of control activity) on day one, steadily recovered to about 40% on day 15 to remain at that level. The same time course was seen for the number of microsomal warfarin binding sites.

3 The persistent inhibition of the vitamin K cycle was also verified *in vivo*; following vitamin K administration (10 mg kg^{-1}, i.v.) on day 30, the brodifacoum-treated rats accumulated vitamin KO in the liver.

4 Although clotting factor synthesis was normal, brodifacoum-treated rats were highly sensitive to warfarin.

5 Brodifacoum rapidly accumulated in the liver until the saturation of the microsomal binding site. Brodifacoum binding to the target prevented its elimination from the liver; liver content on day 30 was not different from day 7.

6 The results show (1) an over capacity for the hepatocellular vitamin K cycle, (2) a dissociation of the vitamin K epoxidation and the vitamin K-dependent carboxylation, (3) the 'superwarfarin' rodenticides to be extremely persistent due to their binding to the target.

Keywords: Anticoagulation; binding site; brodifacoum; vitamin K cycle; vitamin K epoxide reductase; warfarin

Introduction

In addition to their clinical use as oral anticoagulants, 4-hydroxycoumarins (warfarin and congeners) are applied world-wide as rodenticides. The cumulative ingestion of these agents causes the rodents to bleed to death due to the complete suppression of the hepatic synthesis of vitamin K-dependent clotting factors. However, their use also resulted in the selection of so-called warfarin-resistant species, i.e. kinds with reduced sensitivity to the warfarin action (Drummond, 1966; Greaves & Ayres, 1967).

The resistance is due to molecular or mechanistic alterations of the target enzyme vitamin K epoxide reductase (Zimmerman & Matschiner, 1974; Hildebrandt & Suttie, 1982; Thijssen, 1987). This enzyme regains vitamin K from vitamin K epoxide (vitamin KO) formed out of vitamin K hydroquinone during the carboxylation of glutamic acid residues to gamma-carboxy glutamic acid (Gla) in substrate proteins of clotting factor precursors. Vitamin KO reductase is probably also involved in the reduction of vitamin K to its hydroquinone. The interaction of warfarin and analogues with the sensitive reductase is essentially irreversible; the enzyme inhibitor complex dissociates only during reactivation at high levels of the dithiol reductor (Fasco *et al.*, 1983; Thijssen *et al.*, 1988). The enzymes involved in the recycling of vitamin K reside in the rough endoplasmic reticulum of the hepatocyte. The recycling is known as the vitamin K cycle (for reviews see Olson, 1984; Suttie, 1987).

Superwarfarins have been introduced to cope with the problem of the warfarin resistance. These compounds (e.g. difenacoum, brodifacoum, flocoumafen and others) are characterized by their biological persistence; plasma half lives of brodifacoum of about 500 h (man, Breckenridge *et al.*, 1985), 160 h (rat, Bachmann & Sullivan, 1983), and 60 h (rabbit, Breckenridge *et al.*, 1985) have been reported.

Poisoning, intentional or accidental, with these rodenticides has been reported (Park *et al.*, 1986; Greeff *et al.*, 1987; Weitzel *et al.*, 1990). Vitamin K is the physiological antidote. However, due to their persistence, the administration of vitamin K has to be continued over a prolonged time period. A diagnostic test for the intoxication with warfarin-type of vitamin K antagonists was proposed (Bechtold *et al.*, 1983; Choonara *et al.*, 1985). The test estimates the plasma accumulation of vitamin KO in patients following a pharmacological vitamin K dose. Accumulation is only seen in the case of a challenge with warfarin-type compounds.

In monitoring cases of accidental intoxications with difenacoum and brodifacoum in factory workers, Park *et al.* (1986) observed that the hepatic vitamin K metabolism remained disturbed for more than 18 months. Blood clotting activity, however, was normal from beyond the 5th month after the last 4-hydroxycoumarin exposure. It was suggested that a partial inhibition of vitamin KO reductase may not interfere with the clotting factor synthesis (Park *et al.*, 1986). A threshold for vitamin KO inhibition was also suggested by Choonara *et al.* (1988) to explain a comparable observed dissociation between the inhibition of vitamin KO reductase and clotting factor analysis in healthy volunteers on a low dose (0.2 mg daily) of warfarin.

We studied the prolonged effect of a single dose of brodifacoum in rats on blood coagulation and the hepatic vitamin K cycle. The results show comparable features as found in man after brodifacoum intoxication (see above). The persistent inhibition of the hepatic vitamin KO reductase activity along with a reserve capacity of the vitamin K cycle probably account for the prolonged effect in rats.

Methods

Male Wistar rats (270–300 g) were used. The animals had free access to water and food and were housed in a 12 h on/off, light/dark cycle. The experimental rats were given 1 ml kg^{-1} of 0.2 mg ml^{-1} brodifacoum (Sorex, Widnes, England), dissolved in dimethylsulphoxide (DMSO)/polyethyleneglycol-400/water, 0.02/1/1 (v/v/v), as a single oral dose (control rats

[1] Author for correspondence.

received the vehicle). At various times, groups of 3–4 experimental and 2–3 control rats were killed. Under light ether anaesthesia the abdomen was opened, blood was withdrawn completely from the abdominal aorta and the blood was mixed immediately with 1/9th of its volume of 0.1 M trisodiumcitrate. Plasma was prepared and stored at −70°C. The liver was perfused *in situ* with ice-cold saline via the portal vein and removed. The liver was homogenized in 3 vol of ice-cold buffer (0.02 M Tris-HCl, 0.15 M KCl, 0.25 M sucrose, pH 7.4) by the Potter technique. Microsomes were prepared as described (Thijssen, 1987) and were stored at −70°C. The protein content was measured by the Lowry method, with bovine serum albumin as a reference.

Blood clotting activity was measured by the thrombotest (Nycomed, Oslo) and was expressed as % of control prothrombin complex activity (% PCA).

The vitamin KO reductase activity was measured as described (Thijssen, 1987) with a slight modification: to a reaction mixture of 95 μl 0.02 M Tris, 0.15 M KCl buffer (pH 7.4), containing 0.4 mg of microsomal protein was added 2 μl of vitamin KO, 2 mg ml^{-1} in 1% Triton X-100 in Tris buffer. After a preincubation of 3 min at 30°C the reaction was started by the addition of 5 μl of dithiothreitol solution, 0.1 M in Tris buffer. The reaction was stopped after 10 min incubation by the addition of 1.4 ml of a 0.02 M $AgNO_2$/isopropanol (5/9, v/v) mixture. Extraction and high performance liquid chromatography (h.p.l.c.) assay of vitamin K were as described by Thijssen (1987). The assays were performed in duplicate. Microsomal warfarin binding was assayed as described (Thijssen & Baars, 1989); briefly, the microsomal suspension was diluted 5 fold in 0.02 M Tris-HCl buffer pH 7.4 and 0.5 ml was pipetted in two rows of vessels, one of which contained 20 μg unlabelled racemic warfarin. After 3 min of incubation at 30°C, 5 × 10^4 d.p.m. [^{14}C]-warfarin (151 ng) was added. After 10 min incubation, 2 ml ice-cold Tris-buffer was added and the reaction mixture was filtered immediately over a Millipore HA filter. The filters were rinsed with 2 ml of ice-cold buffer. The filters were counted for ^{14}C by liquid scintillation counting. Specific warfarin binding was obtained by subtracting non-specific binding from total binding. The binding assays were performed in duplicate.

In vivo, the effect on vitamin K metabolism was determined by measuring the liver vitamin KO accumulation, 1 h after the i.v. administration of 10 mg kg^{-1} vitamin K1 (Konakion) via the tail vein to brodifacoum-treated and control rats.

A group of rats (3 control rats and 3–4 brodifacoum-treated rats), which were provided with a permanent catheter in the left femoral artery, received a single dose of 0.1 mg kg^{-1} S-warfarin s.c. At various times, arterial blood samples were taken. Blood coagulation (thrombotest) and S-warfarin contents (h.p.l.c.) were measured.

The brodifacoum content of plasma, liver and liver microsomes was assayed as follows: to 0.3 ml of sample was added 25 μl difenacoum 2 μg ml^{-1} in ethanol as an internal standard. This was mixed with 1 ml ice-cold acetonitrile and centrifuged. The supernatant was used for the brodifacoum assay by h.p.l.c. The following h.p.l.c. system was used: a reversed phase column (Chromosphere C18, Chrompack, 100 × 3 mm); eluent, acetonitrile/0.1% acetic acid 5/3 (v/v); flow 1 ml min^{-1}. Detection was by u.v. monitoring at 313 nm or by fluorometry technique; ext 255 nm, em 375 nm. Both difenacoum and brodifacoum appear as two peaks in the chromatogram, representing the *cis*- and *trans*-isomers. The retention times for difenacoum were 3.5 and 4.3 min and for brodifacoum 6.5 and 7.7 min.

The vitamin KO accumulation in liver was assayed as follows: 1 ml liver homogenate was mixed with 1.4 ml isopropanol. To the mixture was added 0.6 ml hexane containing 2.5 μg of tocopherol acetate for internal standardization. After centrifugation, the hexane phase was evaporated to dryness under a stream of nitrogen at 35°C. The residue of the hexane phase was redissolved in 0.05 ml isopropanol and analysed by h.p.l.c. The following h.p.l.c. system was used: a reversed phase column (Chromosphere C18, Chrompack, 100 × 3 mm); eluent, acetonitrile/isopropanol/water (100/8/1, v/v/v); flow 1.2 ml min^{-1}. Detection was by u.v. monitoring at 250 nm.

Statistics

The following statistical methods were used: (1) Student's *t* test for unpaired observations: this method was used to compare liver vitamin K and vitamin KO concentrations in brodifacoum-treated and control rats, and to compare the effect on the prothrombin times after S-warfarin administration in brodifacoum-treated and control rats. (2) One-way analysis of variance, followed by LSD (least significant difference)-test for brodifacoum concentrations in liver and liver microsomes and for the microsomal vitamin KO reductase activity and warfarin binding capacity during the 30 days of measurement (Sachs, 1984).

Results

The effect of a single oral dose of 0.2 mg kg^{-1} (0.38 μmol kg^{-1}) brodifacoum on blood coagulation and vitamin K metabolism was followed for 30 days.

The effect on the blood coagulation is presented in Figure 1. A maximal effect on PCA was seen on day 1, when the PCA was reduced to about 35%. The prothrombin time returned to normal between day 2 and day 7.

The effect of brodifacoum on the vitamin K metabolism was measured *in vivo* as well as *ex vivo*. The *ex vivo* measured liver microsomal activity of the enzyme vitamin KO reductase in the brodifacoum treated rats as compared to vehicle-treated controls is shown in Figure 2. Vitamin KO reductase activity in control microsomes was 175 ± 34 pmol vitamin K mg^{-1} min^{-1} ($n = 9$). The activity remained inhibited for at least 30 days. On days 1 and 2, about 90% of the enzyme was suppressed and on day 7, when prothrombin times had returned to normal, vitamin KO reductase activity was about 25% of control activity. Between day 7 and day 15 the enzyme activity increased significantly ($P < 0.05$) to a plateau of about 45%. *In vivo*, the effect on vitamin K metabolism was determined by measuring the hepatic vitamin KO accumulation 1 h after the i.v. administration of 10 mg kg^{-1} vitamin K1 on day 8 and on day 30 (Table 1). A significant accumulation of vitamin KO was seen in the brodifacoum rats, indicating that the hepatic vitamin K cycle is still inhibited after 30 days.

Our laboratory showed previously that rat liver microsomal warfarin binding was related to vitamin KO reductase activity. *Ex vivo* the binding sites are dose-dependently reduced following warfarin administration (Thijssen & Baars, 1989). We measured the effect of brodifacoum on microsomal warfarin binding (Figure 3). Microsomal warfarin binding in control microsomes was 43 ± 11 pmol mg^{-1} protein ($n = 9$).

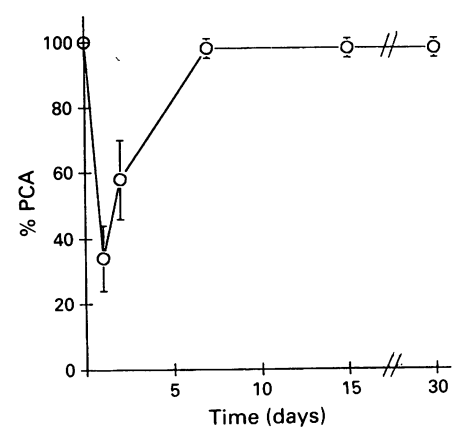

Figure 1 The effect of a single oral dose of brodifacoum (0.2 mg kg^{-1}) on the prothrombin complex activity (% PCA). Mean for groups of 3–4 rats; s.e. shown by vertical bars.

Table 1 Vitamin K and vitamin KO liver tissue levels following vitamin K administration

Day		n	*Vitamin KO* ($\mu g\,g^{-1}$ liver)	*Vitamin K* ($\mu g\,g^{-1}$ liver)	*KO/K ratio*
8	Brodifacoum	4	7.1 ± 0.5**	13.1 ± 0.9**	0.54 ± 0.13*
	Control	3	4.6 ± 1.1	25.6 ± 6.1	0.19 ± 0.06
30	Brodifacoum	4	7.3 ± 1.7*	18.7 ± 4.3*	0.39 ± 0.09**
	Control	3	4.0 ± 0.6	28.6 ± 4.3	0.14 ± 0.03

Liver homogenates were prepared 1 h after vitamin K, 10 mg kg^{-1}, i.v. Data are given as mean ± s.e.
* $P < 0.05$ compared with control group; ** $P < 0.01$ compared with control group.

Within the first 7 days, warfarin binding was strongly suppressed, 5 ± 1, 7 ± 3, 9 ± 2% of control on days 1, 2 and 7. A slight but consistent increase in warfarin binding capacity was seen after day 7; 26 ± 7 and 28 ± 5% on days 15 and 30. The correlation between vitamin KO reductase activity and the residual warfarin binding is given by the equation $y = 1.59x + 3.52$ ($r = 0.978$, $P < 0.001$), where y = % vitamin KO reductase activity and x = % binding capacity.

Brodifacoum contents of plasma, liver and liver microsomes were measured. No brodifacoum was detected in plasma; detection limit, is about 50–100 ng ml^{-1} sample. Liver brodifacoum concentrations initially decreased slightly to reach a steady state after day 7 (Table 2). Microsomal brodifacoum levels appeared to be stable during the 30 days; about 30–40 ng (= 57–76 pmol) mg^{-1} protein. As we recovered 15–20 mg of microsomal protein g^{-1} wet weight of liver, the data show liver brodifacoum to be located mainly in the microsomal fraction. The initial amount (day 1) accumulated in the liver was about 20% of the administered dose. On day 30, about 16% was still present.

The anticoagulant responses of a single S-warfarin dose (0.1 mg kg^{-1}, s.c.) in brodifacoum-treated rats on day 8 and on day 25 were measured. When S-warfarin was administered on day 8, 2 of the 3 brodifacoum rats were completely anticoagulated at 24 h and 32 h (prothrombin times >200 s). Control rats were only slightly anticoagulated. We could not quantify the anticoagulant effect because of this high response. On day 25, the effect also appeared to be more pronounced in the brodifacoum-treated rats than in the control rats (Figure 4). The area under the effect curves (i.e. PT/PT_0 * time) were 40.2 ± 15.3 h (brodifacoum rats, $n = 4$) versus 17.0 ± 10.7 h (control rats, $n = 3$). The difference was not statistically significant ($P = 0.08$, unpaired t test). No difference in S-warfarin kinetics was observed between brodifacoum and control rats.

Discussion

The anticlotting activity of the 4-hydroxycoumarins is considered to be due to the interruption of the hepatic vitamin K cycle resulting in a reduced synthesis of coagulation proteins. This mechanism of action was shown also to operate for the 4-hydroxycoumarin rodenticides like brodifacoum, difenacoum etc. (Leck & Park, 1981; Breckenridge *et al.*, 1985). However, a dissociation between the effect on the vitamin K cycle and on the blood clotting activity was observed in man intoxicated with brodifacoum and difenacoum, suggesting either a reserve capacity for vitamin KO reductase or an alternative mechanism for clotting factor synthesis inhibition (Park *et al.*, 1986). Our study shows the same phenomenon in rats; while the effect on blood clotting activity of brodifacoum (0.2 mg kg^{-1}) was restored after day 7, vitamin KO accumulation after a vitamin K dose was still manifest on day 30. Undoubtedly, the latter is due to the persistent inhibition of the microsomal vitamin KO reductase enzyme. Taking the relation between the vitamin K cycle and the vitamin K-dependent carboxylation for granted, the results show that (1) the vitamin K cycle has a reserve capacity i.e. a partly (25–40%) operating vitamin KO reductase activity suffices to provide the carboxylase with vitamin K cofactor; (2) the epoxidation of a pharmacological dose of vitamin K is not necessarily coupled to the carboxylation reaction. Elimination of the reserve capacity increases the sensitivity for interfering drugs. This is clearly demonstrated by the increased sensitivity of the brodifacoum-treated rats to low doses of warfarin which was still observable at day 25 (Figure 4).

The apparent dissociation between the effect on vitamin KO reductase and blood coagulation activity may partly be caused by the insensitivity of the coagulation tests for small changes in blood clotting factor levels (Choonara *et al.*, 1988). Fully carboxylated clotting factors contain 10–12 Gla residues (Suttie, 1987). The minimal amount of Gla needed for functional activity is not known, not only the number of Gla's but also their position in the molecule is essential (Borowski *et al.*, 1986). For human prothrombin and protein S it was found that the absence of 2 Gla's strongly reduces normal activity (Borowski *et al.*, 1985; Grinnel *et al.*, 1990). In other words, if the full capacity of the vitamin K cycle were needed, a

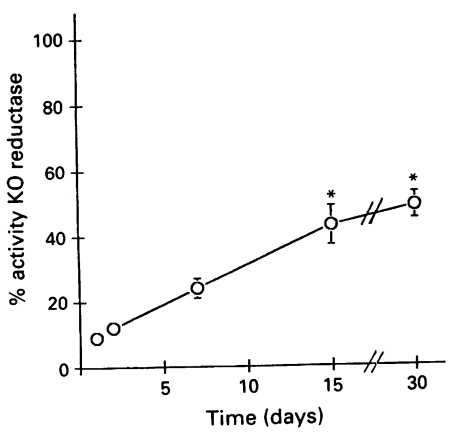

Figure 2 Effect of brodifacoum on liver microsomal vitamin K epoxide reductase activity (% of control rats). Mean for groups of 3–4 rats; s.e. shown by vertical bars. * Significantly different from days 1 and 2; $P < 0.05$.

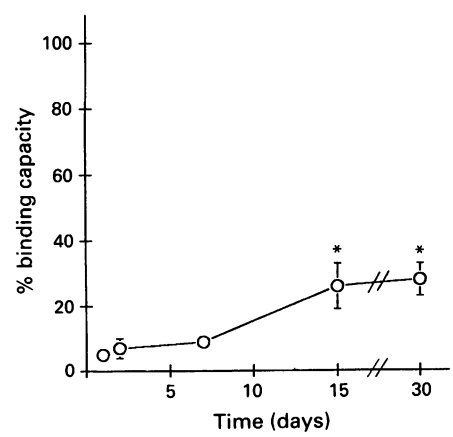

Figure 3 Effect of brodifacoum on the residual warfarin binding capacity (% of control rats). Mean for groups of 3–4 rats; s.e. shown by vertical bars.
* Significantly different from days 1 and 2; $P < 0.05$.

Table 2 Brodifacoum concentrations in liver and liver microsomes

Day	n	Liver ($ng\,g^{-1}$)	Microsomes ($ng\,mg^{-1}$ protein)
1	4	792 ± 28	30 ± 1
2	4	720 ± 40	38 ± 5
7	4	647 ± 36*	37 ± 4
15	3	570 ± 42*	29 ± 8
30	3	623 ± 33*	31 ± 5

Data are mean ± s.e.
*Significantly different from day 1, $P < 0.05$.

20–30% inhibition of vitamin K or vitamin KO reductase would be expected to give a measurable effect in coagulation tests.

Liver microsomes contain a specific saturable binding site for 4-hydroxycoumarins which is related to vitamin KO reductase activity (Thijssen & Baars, 1989). As expected, brodifacoum interferes with the microsomal warfarin binding; it occupies the warfarin binding sites and the persistent reduction in the warfarin binding sites parallels the persistent reduction of reductase activity. However, the reductase activity was throughout higher than expected on the ground of the residual warfarin binding (slope 1.6, see Results). An explanation for this discrepancy is not apparent. It might indicate that additional warfarin binding sites exist, unrelated to the enzyme but occupied by brodifacoum. However, we found in rats, at steady state warfarin dosing, a strict 1 to 1 relation between binding and enzyme activity (Mosterd & Thijssen, unpublished observations).

As the microsomal brodifacoum levels did not decline during the 30 days of observation, the increase in warfarin binding sites, hence in vitamin KO reductase activity, may reflect *de novo* synthesis of enzyme.

Our findings on the kinetics of brodifacoum ($0.2\,mg\,kg^{-1}$) in rat liver are in agreement with those of Bachmann & Sullivan (1983). The compound is extremely slowly eliminated. For the closely related rodenticide, flocoumafen, the elimination half life for rat liver was 220 days (Huckle *et al.*, 1989). Evidently, the main factor determining this persistence is the tight binding to the warfarin binding site. However, warfarin which also binds tightly, is not as persistent; the warfarin elimination half life in rat liver is about 30–40 h (Thijssen & Baars, 1987). The additional factor may be the high lipophilicity of brodifacoum and other similar compounds preventing them from escaping from the binding compartment, i.e. the endoplasmatic reticulum. Furthermore, the systemic clearance may

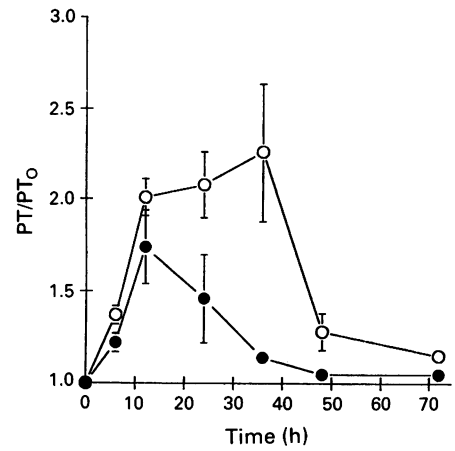

Figure 4 The effect of a single dose of S-warfarin ($0.1\,mg\,kg^{-1}$, s.c.), 25 days after brodifacoum administration, on the prothrombin time: (○) brodifacoum-treated rats ($n = 4$); (●) vehicle-treated rats ($n = 3$); mean with s.e. shown by vertical bars.

be low. However, the brodifacoum response in rats is strongly affected by cyt P450 inducers (attenuation) or inhibitors (potentiation) (Bachmann & Sullivan, 1983).

The data on brodifacoum liver disposition suggest that following the initial liver accumulation, brodifacoum redistributes to reach a steady state of liver distribution between days 2–7. Consistent with the strong binding to the target, no redistribution is seen from the microsomal fraction. Concomitant with the elimination of the 'excess' of brodifacoum the clotting factor synthesis is restored. Apparently brodifacoum is needed in excess of the saturation of the vitamin KO reductase-related binding site, to block completely the supply of vitamin K cofactor.

In summary, the persistence of brodifacoum and similar rodenticides maintain a long-lasting suppression of the hepatic vitamin KO reductase capacity which is reflected by the accumulation of vitamin KO after a pharmacological dose of vitamin K. Vitamin K-dependent clotting factor synthesis is not compromised in parallel due to a 3–4 fold overcapacity of the reductase. A possible consequence of these features is that subjects exposed to the superwarfarins are probably more sensitive to weak inhibitors of the vitamin K cycle; e.g. compounds like salicylates and cephalosporins containing an N-methyl-thiotetrazole side chain (Lipsky, 1988).

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The agonist properties of *m*-chlorophenylbiguanide and 2-methyl-5-hydroxytryptamine on 5-HT_3 receptors in N1E-115 neuroblastoma cells

María-Isabel Sepúlveda, Sarah C.R. Lummis & Ian L. Martin

Molecular Neurobiology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH

1 The effects of 5-HT_3 selective agonists have been studied in whole-cell voltage-clamped N1E-115 neuroblastoma cells.

2 Application of 5-hydroxytryptamine (5-HT) results in the rapid development of a transient inward current at quasiphysiological membrane potentials. This current can be blocked by the 5-HT_3 specific antagonists BRL 43694 and GR67330.

3 Application of 2-methyl-5-HT (2-Me5-HT), a 5-HT_3 selective agonist, produced a qualitatively similar inward current, but with a maximum response only 20% of that produced by 5-HT.

4 In the presence of 100 μM 2-Me5-HT, the upper part of the 5-HT dose-response curve was shifted to the right but reached the same maximum value as in the absence of 2-Me5-HT. 2-Me5-HT appears therefore to be a partial agonist under these conditions.

5 The novel 5-HT_3 agonist, *meta*-chlorophenylbiguanide (mCPBG) is a full agonist, but has a Hill coefficient (1.5) significantly less than that of 5-HT (2.3).

6 Comparison with radioligand binding data show that mCBPG is 100 times less potent than expected; it may therefore exhibit a high affinity for a desensitized state.

Keywords: 5-HT_3 receptor; N1E-115 neuroblastoma cells; partial agonist; agonist; 2-methyl-5-HT; *m*-chlorophenylbiguanide; 5-hydroxytryptamine

Introduction

Receptors for 5-hydroxytryptamine (5-HT) have been classified into three main groups: 5-HT_1, 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986), of which 5-HT_1 and 5-HT_2 receptors mediate their effects via G-proteins. 5-HT_3 receptors directly gate a cation channel (Derkach *et al.*, 1989); activation of 5-HT_3 receptors in vertebrate neurones causes rapid depolarization (Wallis & Nash, 1981; Ireland & Tyers, 1987) and single channel characteristics of these receptors have been described in guinea-pig submucous plexus (Derkach *et al.*, 1989). Interest in 5-HT_3 receptors in the central nervous system has been stimulated by evidence suggesting that drugs acting at this receptor have potential in the treatment of emesis, anxiety and various psychotic illnesses (Costall *et al.*, 1987; Miner *et al.*, 1987; Jones *et al.*, 1988). 5-HT_3 receptors are present at relatively low density in CNS tissue (Kilpatrick *et al.*, 1987), but are present in much greater concentrations in certain neuronal cell lines, including N1E-115 neuroblastoma cells, NG108-15 neuroblastoma-glioma hybrid cells and NCB-20 neuroblastoma hybrid cells (Neijt *et al.*, 1988; Hales *et al.*, 1988; Yakel & Jackson, 1988). In N1E-115 cells, for example, we have shown using ligand binding studies with the specific 5-HT_3 antagonist [^{3}H]-GR65630, that there are approximately 200,000 sites per cell (Lummis *et al.*, 1990). Application of 5-HT or 2-methyl-5-HT (2-Me5-HT), a specific 5-HT_3 agonist, to these cells causes a depolarization which undergoes rapid desensitization; the response can be blocked by 5-HT_3 specific antagonists (Neijt *et al.*, 1988; 1989; Peters & Lambert, 1989). The purpose of this study is to detail the effects of rapid agonist application to N1E-115 cells by use of the whole cell recording mode of the patch-clamp technique.

Methods

Cell culture

N1E-115 mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum and 2 mM glutamine. For electrophysiological experiments, cells were grown in 35 mm dishes for 1–3 days. For binding studies, cells were grown in 10 mm diameter wells in 24 well plates until confluent (2–4 days); the number of cells present was estimated in a sample of wells for each experiment and was routinely 2–4 $\times$ 10^5 cells per well.

Electrophysiological studies

Whole-cell currents were recorded as described by Hamill *et al.* (1981) using a List EPC-7 amplifier (List Medical, West Germany). Patch-pipettes were made from thin-walled borosilicate (hard) glass capillary tubing (Clark Electromedical GC150T-15) with a two-stage vertical pipette-puller (PP-83 Narishige, Japan). The tips of pipettes were fire polished in a microforge before back-filling with intracellular solution. Pipettes used had resistances of 3–5 MΩ. The pipette filling solution, which was filtered through 0.2 μm Millipore filters before use, had the following composition (mM): KCl 140, $MgCl_2$ 2.0, $CaCl_2$ 0.1, EGTA 1.1 (free Ca^{2+} concentration 1.0 $\times$ 10^{-8} M), HEPES 10, pH 7.2.

N1E-115 cells on a 35 mm culture dish were continuously perfused with a solution of the following composition (mM): NaCl 140, KCl 2.8, $MgCl_2$ 2.0, $CaCl_2$ 1.0, glucose 10, HEPES 10, pH 7.2. A flow rate of 3–5 ml min^{-1} was used, allowing the solution in the culture dish to be completely renewed about twice a minute. Cells were viewed on an inverted microscope by phase contrast optics, and those selected for experiments had a rounded, smooth surface and few processes. Only patches with seals equal to or greater than 5 GΩ were used. Cells were routinely clamped at -60 mV and membrane currents in response to agonists measured. Agonists were applied through a microflow system described previously (Suzuki *et al.*, 1990) allowing a complete change of agonist concentration within 100 ms. To calibrate the rate of solution exchange in our experiments we voltage-clamped the cells and monitored current at -60 mV; the current is outward and carried mainly by K^+. The application of a saline solution where Na^+ replaced K^+ led to a decrease in current due to the change in electrochemical gradient across the cell membrane. The time

from the onset of the decrease in current to the establishment of a new stable value was taken as the time for complete solution exchange; this was 75–85 ms. Ionic currents produced by positive charge moving from intracellular to extracellular solutions are shown as positive currents. Experiments were conducted at room temperature (20–22°C).

The signal from the patch-clamp amplifier was viewed on an oscilloscope and simultaneously recorded on videotape for subsequent analysis with an adapted digital audio processor (Sony PCM-701ES). Records were replayed on a chart recorder (Gould 1000S) and the currents elicited by agonists measured from the traces. Alternatively, current traces were digitised by use of Data Translation Laboratory interface on a microcomputer (IBM AT) and analysed using a programme written by J. Dempster (Department of Physiology and Pharmacology, University of Strathclyde, Glasgow).

Binding studies

Radiolabelled ligand binding experiments were performed directly on cells in culture dishes. The culture medium was removed and the dishes were rinsed twice with 0.5 ml of Hanks buffer (composition, mM: NaCl 137, KCl 5.4, $NaHCO_3$ 4.2, $CaCl_2$ 1.25, $MgCl_2$ 0.5, NaH_2PO_4 0.3, $MgSO_4$ 0.2, glucose 23.4, HEPES 5.0, pH 7.4). The cells were then incubated in a total volume of 0.5 ml containing [^{3}H]-GR65630 (0.2 nM unless otherwise stated) and ligands being tested. Non-specific binding was determined with 10 μM mCPBG. Cells were incubated for 30 min at 37°C and the incubation was terminated by rapid removal of incubation medium and the cells washed twice with 0.5 ml buffer. The cells were subsequently removed from the culture plates by incubation with 0.5 ml 1% Triton-X-100, which was then placed in a scintillation vial together with 2 successive rinses of the plate with 0.5 ml distilled water. Radioactivity was determined by scintillation counting. Data were analysed by the computer programmes EBDA and LIGAND (Munson & Rodbard, 1980).

Materials

[^{3}H]-GR65630 (85 Ci mmol^{-1}) was obtained from Amersham, UK. GR67330, *m*-chlorophenylbiguanide (mCPBG) and 2-methyl-5-HT (2-Me5-HT) hydrochloride were synthesized by the Chemistry Research Department, Glaxo Group Research. BRL 43694 was obtained from Glaxo. All other supplies, unless stated, were obtained from commercial sources. GR65630 = 3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone; GR67330 = 1,2,3,9-tetrahydro-3-[(5-methyl-1H-imidazol-4-yl)methyl]-9-methyl-4H-carbazol-4-one; BRL 43694 = endo-4-amino-5-chloro-2-methyl-N-(1-azabicyclo[3,3,1] non-4-yl) benzamide.

Results

5-HT$_3$ receptors in N1E-115 cells

The effect of 5-HT on N1E-115 mouse neuroblastoma cells is shown in Figure 1. 5-HT application resulted in a rapid inward current at a holding potential of −60 mV. The size and direction of the current was dependent upon the holding potential used. As seen in Figure 2, peak responses reversed near 0 mV and showed inward rectification. The response exhibited virtually complete desensitization upon sustained agonist application. The rate of desensitization increased with agonist concentration. The $t_{0.5}$ (time for a response to decay to 50% of its peak value) against concentration are shown in Figure 3. These values are much faster than those previously reported (Neijt *et al.*, 1989); however, these authors used a drug application system where the time needed for exchange of solutions was approximately 2 s. This is at least 20 fold slower than our method. The desensitization which occurs within this 2 s interval is the probable explanation of the discrepancy. Desensitization at 10 μM 5-HT was not voltage-dependent in the range −60 to 40 mV (not shown). The response to 5-HT application was blocked by two selective 5-HT$_3$ receptor antagonists, BRL 43694 and GR67330. Figure 4a illustrates the control response to application of 10 μM 5-HT. After application of 1.0 nM BRL 43694 for 2 min, the response to the same concentration of 5-HT (measured in the continued presence of BRL 43694) was reduced to 13% of the control (Figure 4b). Figure 4c shows that the antagonist effect was reversible, with 87% recovery after 5 min. A similar effect was obtained with GR67330 at a concentration of 0.1 nM

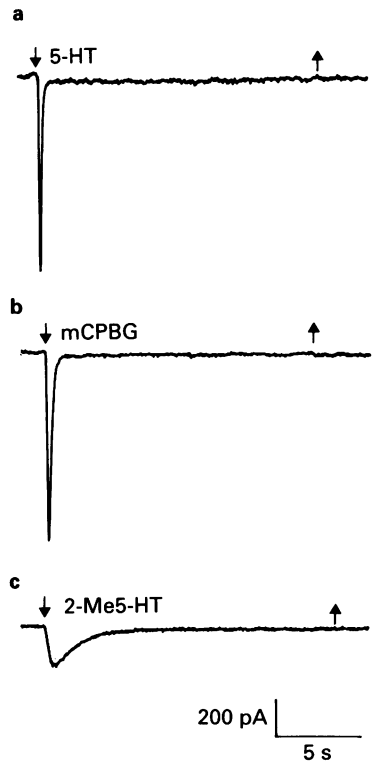

Figure 1 Currents elicited by 10 μM 5-hydroxytryptamine (5-HT, a), 10 μM *m*-chlorophenylbiguanide (mCPBG, b) and 100 μM 2-methyl-5-HT (2-Me5-HT, c) in N1E-115 neuroblastoma cells voltage-clamped at −60 mV. The maximum response to application of saturating concentrations of 5-HT in these three cells was of a similar amplitude. The arrows indicate the time the application was started and finished.

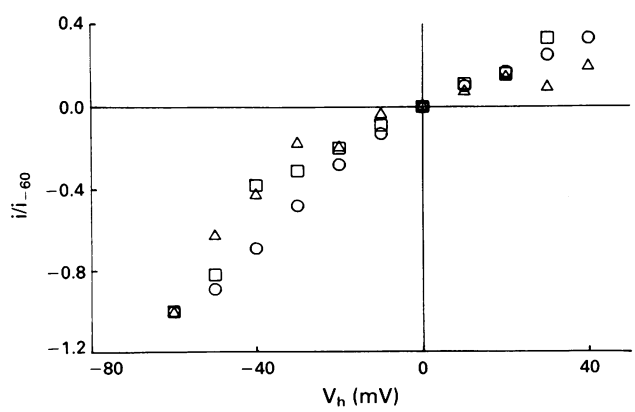

Figure 2 Current-voltage relationship of the ionic currents induced by 10 μM 5-hydroxytryptamine (□), 10 μM *m*-chlorophenylbiguanide (○) and 100 μM 2-methyl-5-HT (△) in three different N1E-115 cells. Amplitudes were normalized for each cell to the amplitude of the inward current elicited by the respective agonist at a holding potential of −60 mV (i/i_{-60}). The responses to the three agonists studied showed inward rectification and reversed at around 0 mV.

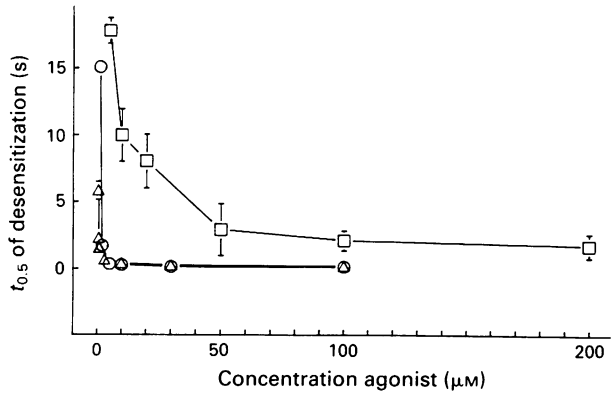

Figure 3 Desensitization of response to 5-hydroxytryptamine (5-HT, ○), *m*-chlorophenylbiguanide (△) and 2-methyl-5-HT (□). The time required for the currents elicited by 5-HT_3 receptor agonists to desensitize to 50% of their peak amplitude ($t_{0.5}$) is plotted against agonist concentration. Shown are mean (n = 3–6) with s.e. indicated by vertical bars.

(results not shown) though complete recovery was not observed.

Selective 5-HT_3 agonists

Two selective 5-HT_3 agonists, 2-Me5-HT (Richardson *et al.*, 1985) and the novel agonist mCPBG (Kilpatrick *et al.*, 1990) were tested on N1E-115 cells. Figure 1 shows that mCPBG and 2-Me5-HT elicit inward currents at −60 mV similar in time course to those observed with 5-HT. Figure 2 shows the dependence on holding potential of the peak currents in response to 2-Me5-HT and mCPBG. Both current-voltage relationships exhibited inward rectification and reversed at around 0 mV. The rate of desensitization for mCPBG (Figure 3) was essentially identical to that of 5-HT, while the rate of desensitization produced by application of 2-Me5-HT was apparently slower. However, the R_{max} (peak current amplitude elicited by agonist at saturating concentration) to 2-Me5-HT was only 20% of that found for 5-HT. At this current the $t_{0.5}$ of the R_{max} to 2-Me5-HT was the same as that of a response to 5-HT of a similar current amplitude. The desensitization was independent of voltage for mCPBG and 5-HT. The data for 2-Me5-HT had a signal to noise ratio which precluded this analysis.

The magnitude of the inward current increased with agonist concentration; the apparent affinity for a particular agonist remained constant between cells but the R_{max} varied significantly (180–950 pA). In order to pool results from individual cells, the responses were normalized to the maximum current elicited by saturating concentrations of agonist.

Figure 5 shows the normalized dose-response curves to 5-HT, mCPBG and 2-Me5-HT. Each point represents the mean responses obtained in 3–8 cells. The dose-response curves shown are theoretical lines generated from parameters obtained from the best non-linear regression fit to the data using the Hill equation (Enzfit, Elsevier computer programme based on equations developed by Marquardt, 1963). The mean EC_{50} (concentration which evokes 50% maximum response) and Hill coefficient (n_H) of the curve fitted to the dose-response data presented in Figure 5 for the three agonists studied were (mean ± s.e.) 4.1 ± 0.6 μM and 2.3 ± 0.2 for 5-HT (n = 6); 1.4 ± 0.2 μM and 1.5 ± 0.2 (n = 3) for mCPBG and 17.0 ± 3.0 μM and 1.2 ± 0.2 (n = 3) for 2-Me5-HT respectively.

Binding studies were performed in intact cells by use of the selective antagonist [^{3}H]-GR65630 as radioligand and 5-HT, 2-Me5-HT and mCPBG as competing ligands. IC_{50} values (the competing ligand concentration which inhibited 50% of the specific radioligand binding) are given in Table 1.

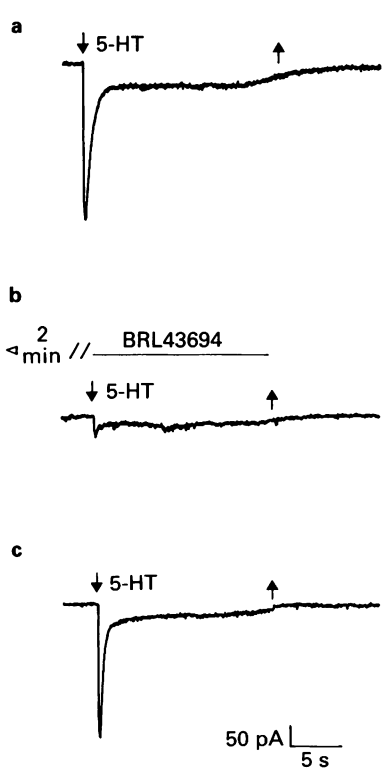

Figure 4 Reversible antagonism of 5-hydroxytryptamine (5-HT, 10 μM)-induced current by BRL 43694. The peak amplitude of the 5-HT-induced inward current (a) was reduced to 13% of its control value by 1.0 nM BRL 43694 applied for 2 min before being co-applied with 10 μM 5-HT (b). Recovery after 5 min of washing with control external solution is depicted in (c).

2-Me5-HT: a possible partial agonist

Responses to saturating concentrations of 5-HT and mCPBG reached similar peak amplitudes when studied in the same cell, whilst the peak responses to 2-Me5-HT were significantly less (Table 1). Figure 6 shows the concentration-dependence of currents elicited by 5-HT and 2-Me5-HT measured in the same cell clamped at −60 mV. The maximum response evoked by 2-Me5-HT was 19.9 ± 2.4% (mean ± s.e., n = 9) of that obtained by saturating concentrations of 5-HT. The responses obtained to increasing concentrations of 5-HT in

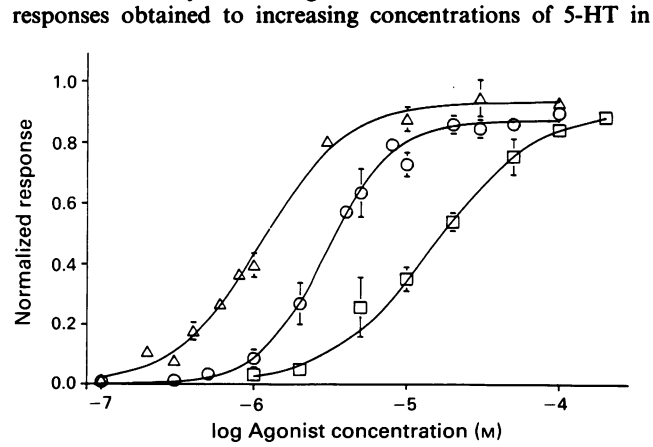

Figure 5 Concentration-response curves of agonist-induced inward currents measured at −60 mV holding potential. Ordinate scale represents the measured peak amplitudes normalized to the maximum response elicited by a saturating concentration of 5-hydroxytryptamine (5-HT, ○), *m*-chlorophenylbiguanide (△) and 2-methyl-5-HT (□). Each point is the mean of measurements at that concentration in 3–6 cells. The line drawn is the best fit to the Hill equation using non-linear computer iterative fit based on the Marquardt (1963) algorithm.

Table 1 Potency and affinity ratios of 5-HT$_3$ agonists

	Electrophysiological data		*Binding data*	
	EC_{50} (μM)	$\frac{R_{max}\ agonist}{R_{max}\ 5\text{-}HT}$	IC_{50} (μM)	$\frac{EC_{50}}{IC_{50}}$
5-HT	4.1 ± 0.6	1	0.6 ± 0.2	7
2-Me5-HT	17.0 ± 0.3	0.2	3.1 ± 0.9	6
mCPBG	1.4 ± 0.2	0.9	0.002 ± 0.0007	700

Data = mean ± s.e. ($n \geq 3$)
2-Me5-HT = 2-methyl-5-hydroxytryptamine; mCPBG = *m*-chlorophenylbiguanide.

the presence of 100 μM 2-Me5-HT are also shown in this figure. In these experiments 5-HT and 2-Me5-HT were co-applied by rapid superfusion followed by washout of both agonists. The apparent EC_{50} for 5-HT increased from 3.1 μM to 18.1 μM in the presence of 100 μM 2-Me5-HT, although the same maximal response was obtained.

Discussion

The 5-HT$_3$ receptor is known to undergo rapid desensitization. In order to ensure that our investigations in N1E-115 cells were not compromised by this fact we have used a technique developed by Suzuki *et al.* (1990) which allows the aqueous environment of the cell to be changed from one defined condition to another within 100 ms. Qualitatively the characteristics we have observed are similar to those reported by others for 5-HT$_3$ receptor responses in N1E-115 (Lambert *et al.*, 1989), NG108-15 (Neijt *et al.*, 1988), NCB-20 (Hales *et al.*, 1988) cells, the rat isolated vagus nerve (Kilpatrick *et al.*, 1990; Ireland & Tyers, 1987) and hippocampal cultured cells (Yakel & Jackson, 1988). The response to 5-HT is mediated by an inward current which reverses at 0 mV under the conditions used and exhibits inward rectification. Specific 5-HT$_3$ receptor antagonists, BRL 43694 and GR67330, inhibit these currents in a reversible manner. Using this rapid application system, we have been able to quantify, in greater detail, the inward currents elicited in these cells in response to the application of 5-HT$_3$ receptor agonists.

There is evidence that the maximal response produced by 2-Me5-HT is significantly less than that obtained with 5-HT in a number of preparations including the rat isolated vagus nerve (Ireland & Tyers, 1987; Kilpatrick *et al.*, 1990), rat cultured hippocampal cells (Yakel & Jackson, 1988) and N1E-115 cells (Neijt *et al.*, 1988). Here we find that the maximal response to 2-Me5-HT is 19.9% of that observed with 5-HT. 2-Me5-HT appears to act as a partial agonist in these cells. The co-application of 2-Me5-HT with 5-HT shifts the upper portion of the dose-response curve for the latter agonist to the right, although 5-HT is still able to produce its own maximum response in the presence of 100 μM 2-Me5-HT. We have also shown that the dose-response curve to 2-Me5HT has a Hill coefficient of 1.2 which is significantly less than that obtained for 5-HT (n_H = 2.3). Desensitization can compromise the determination of Hill coefficients. However, the difference between the Hill coefficients exhibited by these two agonists in this study is unlikely to be due to differences in desensitization produced by these two compounds; we find that the half-time of the desensitization in response to application of 2-Me5-HT is identical to that produced by 5-HT if measured at the appropriate concentrations of the two agonists which produce the same magnitude of inward current.

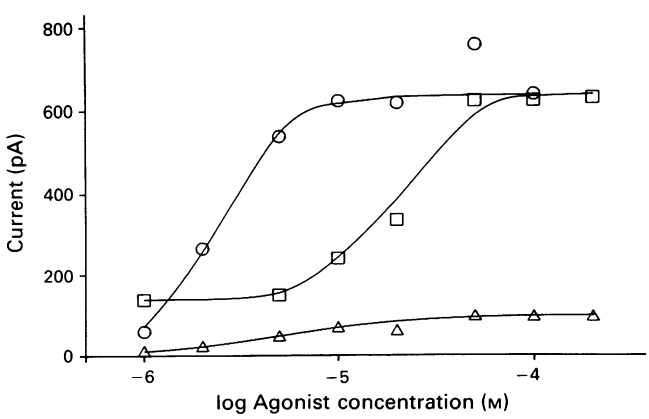

Figure 6 Concentration-response curves for 5-hydroxytryptamine (5-HT, ○), 2-methyl-5-HT (2-Me5-HT, △) and 5-HT in the presence of 100 μM 2-Me5-HT (□) performed in the same cell. The affinity of 5-HT for the receptor in this cell is reduced from 3.1 μM to 18.1 μM in the presence of 2-Me5-HT.

There is a marked disparity between the EC_{50} value obtained for mCPBG in the electrophysiological studies and the IC_{50} value obtained for this compound in the radioligand binding experiments. The ratio EC_{50}/IC_{50} for mCPBG is 700 while for both 5-HT and 2-Me5-HT it is less than ten. This may be explained by the receptor desensitization referred to previously. The 5-HT$_3$ receptor probably belongs to the same structural family as the nicotinic acetylcholine (ACh) receptor, another ligand gated ion channel which conducts cations. Desensitization of the nicotinic ACh receptor induces a conformational change of the receptor protein to a form which has a high affinity for agonists (Changeux *et al.*, 1987) and evidence suggests that the $GABA_A$ receptor may suffer a similar fate (Bristow & Martin, 1989). In the partially desensitized state the receptor may exist with two populations: one with a high affinity for agonists, the desensitized state, and the other with low affinity for agonists, the functionally coupled stated. If similar conformational adjustments occur with the 5-HT$_3$ receptor our data may be rationalized with the assumption that radioligand binding experiments, carried out at 37°C, induce partial receptor desensitization, and that mCPBG exhibits a much higher affinity for the desensitized state than either 5-HT or 2-Me5-HT. Clearly additional experiments will be required to support this argument but it is a tempting speculation in view of the fact that we have observed that the affinity of mCPBG is reduced by about ten fold if the radioligand binding experiments are conducted in N1E-115 cell membranes at 0°C; the affinities for 5-HT and 2-Me5-HT at the lower temperature are not significantly different (unpublished results). Isomerisation of the two receptor conformations would be much slower at lower temperatures.

We have shown that 2-Me5-HT acts as a partial agonist at the 5-HT$_3$ receptor in N1E-115 cells. mCPBG is a full agonist at the same receptor, though of the three agonists investigated, it may exhibit a much higher affinity for the desensitized conformation of the receptor. The desensitization rate observed with this receptor is dependent on the agonist concentration and independent of the membrane potential.

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A comparative study of the prostanoid receptor profile of 9α11β-prostaglandin F_2 and prostaglandin D_2

[1]H. Giles, M.L. Bolofo, [2]S.J. Lydford & G.R. Martin

Analytical Pharmacology Group, Biochemical Sciences, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The aim of this study was to determine the receptor profile of 9α11β-prostaglandin F_2 (PGF_2) and compare it with that of its parent, prostaglandin D_2 (PGD_2). The experiments were designed to overcome the problems associated with the presence of multiple prostanoid receptor sub-types in most tissues; the lack of selective antagonists for each receptor means that conclusions regarding efficacy at FP and EP_2 receptors must remain provisional.

2 At DP receptors in human platelets and rabbit jugular vein, PGD_2 was a full agonist, $p[A_{50}]$ 7.02 ± 0.09 and 6.60 ± 0.12 respectively. 9α11β-PGF_2 was approximately 30–60 fold less potent than PGD_2.

3 9α11β-PGF_2 was a full agonist in the FP receptor containing preparation, cat iris sphincter ($p[A_{50}]$ 7.35 ± 0.09) comparable in potency to PGD_2 ($p[A_{50}]$ 7.15 ± 0.19). Likewise the two prostanoids showed similar potency at the TP receptor in guinea-pig aorta (9α11β-PGF_2 $p[A_{50}]$ 6.00 ± 0.07; PGD_2 6.24 ± 0.08).

4 9α11β-PGF_2 and PGD_2 had efficacy but low potency at EP_1 receptors (guinea-pig oesophageal muscularis mucosa) and demonstrated 2000–3000 fold lower potency than PGE_2 ($p[A_{50}]$ 8.35 ± 0.09). Similarly, in the EP_2 receptor-containing preparation, cat trachea, 9α11β-PGF_2 was 3500 fold less potent and PGD_2 700 fold less potent than PGE_2 ($p[A_{50}]$ 8.06 ± 0.26).

5 9α11β-PGF_2 and PGD_2 (10 μM) were without affinity at the IP receptors on human platelets and had no agonist action in the EP_3 receptor containing preparation, guinea-pig vas deferens.

6 9α11β-PGF_2 is a major metabolite of PGD_2 *in vivo* and this conversion clearly represents an inactivation step since 9α11β-PGF_2 is of considerably lower potency than PGD_2 at DP receptors. However, it is of similar potency to PGD_2 at TP, FP, EP_1 and EP_2 receptors and it may, therefore, contribute to the biological effects which follow PGD_2 administration or endogenous synthesis; its actions at these receptors are likely to be similar to those of PGD_2 itself.

Keywords: 9α11β-prostaglandin F_2; prostaglandin D_2; receptor classification

Introduction

Prostaglandin receptors have been divided into the following sub-types (Kennedy *et al.*, 1982; Coleman *et al.*, 1984) at which one of the natural prostanoids is the most potent: TP (thromboxane) receptors, which mediate platelet aggregation and smooth muscle contraction, FP ($PGF_{2\alpha}$) receptors which also mediate smooth muscle contraction, DP (PGD_2) and IP (prostacyclin) receptors which elicit smooth muscle relaxation and inhibit platelet aggregation, and a heterogeneous group of EP (PGE) receptors: EP_1 and EP_2 which generally mediate smooth muscle contraction and relaxation respectively, and an EP_3 receptor sub-type which can elicit inhibition of autonomic transmitter release and smooth muscle contraction (Coleman *et al.*, 1987a,b).

Originally this classification was based predominantly on agonist potency orders which, although giving qualitative information about possible receptor differences, can be confounded by many factors. A particular problem with this approach in the prostanoid area is that most tissues contain more than one prostaglandin receptor type (for review see Coleman, 1987). Also, many prostaglandins and some synthetic analogues have affinity and efficacy at several of these receptors. This is not surprising considering the very close structural similarity of most of the prostanoids. The use of selective competitive antagonists is therefore particularly important when attempting to quantify the site of agonist action but unfortunately antagonists are not available for all the prostanoid receptors. However, in recent years several competitive antagonists at the TP receptor have been synthesized, including GR 32191 which is highly selective (pA_2 8.79: Lumley *et al.*, 1989), and BM 13.177 (pA_2 6.0: Patscheke *et al.*, 1984; Giles *et al.*, 1989). BW A868C is a potent, competitive and very selective DP receptor antagonist (pK_B 9.26) (Giles *et al.*, 1989) and AH6809 is an antagonist at the EP_1 receptor (pA_2 6.8), although it also has a low affinity for DP and TP receptors (Coleman *et al.*, 1985; Keery & Lumley, 1988). Competitive antagonists at the other receptors have not been described.

9α11β-PGF_2 is a major metabolite of PGD_2 (Liston & Roberts, 1985; Pugliese *et al.*, 1985) capable of causing biological effects in a variety of animal species, including man. Its actions include contraction of bronchial smooth muscle *in vitro* and *in vivo* (Beasley *et al.*, 1987b; Seibert *et al.*, 1987), inhibition of human platelet aggregation (Roberts & Liston, 1985; Pugliese *et al.*, 1985) and increases in urine flow in rats (Stier *et al.*, 1987). It appears to have both vasoconstrictor and dilator properties since it contracts canine and human coronary artery rings (Pugliese *et al.*, 1985; Roberts *et al.*, 1987) and causes rises in systemic blood pressure in rats (Liston & Roberts, 1985), but when injected intradermally into man it causes wheal, flare and increased cutaneous blood flow (Beasley *et al.*, 1987a). These apparently opposite effects may be due to action at more than one prostaglandin receptor, since it is known that PGD_2 itself has efficacy at several subtypes (for review see Giles & Leff, 1988).

This paper attempts to identify the prostaglandin receptors through which 9α11β-PGF_2 exerts its effects and compares its potency at each receptor with that of its parent, PGD_2.

[1] Author for correspondence.

[2] Present address: Dept of Pharmacology, Fisons Research & Development Laboratories, Bakewell Road, Loughborough, Leicestershire LE11 0RH.

Methods

Human platelet assays

The method for preparation of human platelet-rich plasma (PRP) and washed platelets has been described in detail elsewhere (Giles *et al.*, 1989).-Briefly, blood from human volunteers was collected into sodium citrate, PRP was prepared by centrifugation and stored at room temperature for 30 min before use. Washed platelets were prepared from PRP and resuspended in Tyrode buffer containing 1 mM Ca^{2+} and 400 $\mu g\,ml^{-1}$ fibrinogen. Indomethacin 5 $\mu g\,ml^{-1}$ was used in both assays to prevent synthesis of cyclo-oxygenase products which could compromise analysis of exogenous drug effects. Aggregation responses were measured as changes in light transmission in Payton dual channel aggregometers.

Experimental protocol Inhibition of aggregation: Prostaglandin receptor agonists were incubated in the platelet suspension for 6 min before aggregation with adenosine diphosphate (ADP) (20 μM). Antagonists were added 2 min before the agonist. Responses were expressed as percentage inhibition of the control ADP-induced aggregation, which was checked at intervals throughout the experiment. Aggregation responses to U46619 were expressed as a percentage of the maximum control aggregation.

Smooth muscle

Rabbit jugular vein (RbJV) External jugular veins were removed from male New Zealand White rabbits (2.4–3.0 kg) as described previously (Giles *et al.*, 1989). Ring segments approximately 5 mm wide were prepared and subjected to 0.75 g force.

Cat trachea and iris sphincter Cats (Hill Grove Family Farm Ltd.) were killed by an overdose of pentobarbitone sodium 200 $mg\,ml^{-1}$ (Euthesate) (i.v.) and the trachea was removed and stored overnight at 4°C. The iris sphincter muscle was prepared as described in detail by Coleman (1987); two preparations were obtained from each eye. 0.5 g force was applied to iris sphincter, and 1 g to cat trachea.

Guinea-pig aorta Thoracic aortae were removed from male Dunkin Hartley guinea-pigs (275–375 g) killed by a blow to the head. Aortae were cannulated and cleared of adhering connective tissue: 3 mm rings were placed under 1 g force.

Guinea-pig ileum The terminal ileum was removed and the 10 cm section closest to the ileocaecal junction discarded; 2 cm portions of ileum were cleared of adherent tissue and the contents gently flushed out before being suspended longitudinally in organ baths. A force of 1 g was applied.

Guinea-pig oesophageal muscularis mucosa Guinea-pigs (375–500 g) were killed by a blow to the head. The gastric end of the oesophagus was removed. The muscular layers were gently peeled off to leave an inner, white tube of muscularis mucosa which was cut into two longitudinal segments (approx. 1.5 cm) and mounted in under 0.5 g force.

Guinea-pig vas deferens Whole vasa deferentia were removed from guinea-pigs as described previously (Coleman *et al.*, 1987b), and each cut in half and mounted under 0.5 g force. Contractions were elicited with trains of pulses at 5 Hz frequency, 0.5 ms duration, 35 V for 5 s delivered every minute by Harvard Dual Channel stimulators.

Tissues were mounted in 20 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118.41, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10 and $CaCl_2$ 2.50 except for guinea-pig muscularis mucosa where Tyrode solution of the following composition was used (mM): NaCl 136.89, $NaHCO_3$ 11.90, KCl 2.68, NaH_2PO_4 0.42, $MgCl_2$ 1.05, glucose 5.55 and $CaCl_2$ 2.50. Atropine (0.4 μM) was present in guinea-pig muscularis mucosa and ileum assays, and indomethacin (1 $\mu g\,ml^{-1}$) was present in all the bathing media, which were maintained at 37°C (32°C for guinea-pig ileum) and continuously gassed with 95% O_2:5% CO_2. Ring preparations were suspended between two wire hooks (platinum or stainless steel), one attached to a Grass FT03C force displacement transducer and the other to a stationary support in the organ bath. Other preparations were suspended between the transducer and stationary support with cotton thread. Tissue responses were measured as changes in isometric force and recorded on Gould BS 272 pen recorders.

Experimental protocols

Tissues were subjected to an initial force then allowed to stabilize for 60 min, with several exchanges of bathing fluid. During this period muscularis mucosa and ileum preparations were exposed to phenoxybenzamine (0.4 μM) for 30 min (as described by Eglen & Whiting, 1988) which was then removed by several exchanges of bathing fluid. All concentration-effect curves were constructed by cumulative addition of agonists (0.5 $\log_{10}$ increments).

Guinea-pig aorta, ileum, vas deferens, rabbit jugular vein (contraction) and cat iris sphincter Preparations were incubated with antagonist or vehicle for 60 min before construction of a single concentration-effect curve in each preparation. Responses were expressed as increase in g force or, for guinea-pig vas deferens, % inhibition of twitch height.

Guinea-pig muscularis mucosa Tissues were challenged with a maximal concentration of PGE_1 (3 μM) to provide a reference contraction, washed out several times and left for a further 45 min before constructing cumulative concentration-effect curves. Preparations were then washed thoroughly and incubated with antagonist for 45 min and a second curve obtained. In some preparations a third curve was constructed in a similar manner. Responses were calculated as a percentage of the initial PGE_1-induced contraction.

Cat trachea and rabbit jugular vein After establishing a stable contraction with 3 μM bethanechol (cat trachea) or 1 μM histamine (RbJV) relaxation concentration-effect curves were constructed. Preparations were then washed thoroughly and were incubated with antagonists prior to recontraction and construction of a second curve. Responses were expressed as percentage relaxations of the bethanechol/histamine-induced contraction.

Analysis of data

Curve fitting: concentration-effect curves were fitted to a logistic function of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad (1)$$

in which α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively. Location parameters were actually estimated as $-\log_{10}[A_{50}]$ ($p[A_{50}]$) values. Arithmetic means of $p[A_{50}]$ estimates, together with the standard error, are quoted in the text. Differences in α or m were tested for by one-way analysis of variance. Where complete curves could not be obtained the concentration which achieved a response of 50% ($p[A_{50\%}]$) or 30% ($p[A_{30\%}]$) of that for the full agonist were estimated graphically. All logarithmic values quoted in the text are to the base 10.

Analysis of antagonism

Single curve designs A one-way analysis of variance tested for treatment effects on the computed estimates of α and m. If these were unchanged, implying parallel displacement of the

agonist curve, antagonist effects were analysed in accordance with competition theory. When a single concentration of antagonist was used, a concentration-ratio was calculated from the mean p[A_{50}] values, and a pA_2 was estimated from the Schild equation:

$$pA_2 = \log_{10}[B] - \log_{10}\left(\frac{[A_{50}{}^T]}{[A_{50}{}^C]} - 1\right) \quad (2)$$

where [B] is the concentration of anatagonist, pA_2 is the negative logarithm of [B] at which the concentration of A has to be doubled to overcome the antagonist effects of B. [$A_{50}{}^C$] and [$A_{50}{}^T$] are the control and antagonist-treated [A_{50}] values respectively.

For the antagonist study in the platelet assay, computed [A_{50}] values were fitted to a form of the Schild equation which gives equal weight to control and treatment data (Black *et al.*, 1985):

$$\log_{10}[A_{50}] = \log_{10}[A_{50}{}^C] + \log_{10}\left(1 + \frac{[B]^n}{K_B}\right) \quad (3)$$

where K_B is the antagonist equilibrium dissociation constant and n is the parameter equivalent to the slope of a Schild plot. If n was not significantly different from unity, indicating simple competition, it was constrained to this value in order to obtain an estimate of K_B. The dissociation constant was actually estimated as pK_B ($-\log_{10} K_B$). In this experimental design a complete study of antagonism was conducted with each platelet suspension and so equation (3) was used to provide an estimate of pK_B in each case. The arithmetic mean of these values is quoted in the text together with the standard error.

Paired curve design Paired *t* tests were performed to test for differences between computed [A_{50}], α and m estimates obtained in each pair of concentration-effect curves. If antagonists produced a parallel displacement of the curve, a midpoint concentration-ratio was calculated from each curve pair and used to estimate a pA_2 value.

Drugs

The following were used: 9α11β-PGF$_2$ (Ultrafine Chemicals, Manchester, U.K.), PGD$_2$, PGE$_1$, PGE$_2$, PGF$_{2\alpha}$ and U46619 (9,11-dideoxy-9α,11α-methanoepoxyPGF$_{2\alpha}$) (Cayman Chemical, Denver, Colorado, U.S.A.), epoprostenol sodium, Grade 1 (PGI$_2$), BW A868C ((±)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin), BM 13.177 4-[2-(benzenesulphonamido)ethyl]-phenooxyacetic acid (synthesized at Wellcome Research Laboratories, Beckenham, Kent). Fluprostenol sodium was a gift from Coopers Animal Health Limited, Berkhamstead, Herts. AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid) and GR32191 [1α(Z), 2β, 3β, 5α](+)-7-[5-[1,1'-biphenyl]-4-ylmethoxy)-3-hydroxy-2(1-piperidinyl) cyclopentyl]-4-heptonoic acid) were gifts from Glaxo Group Research Limited, Ware, Herts. Indomethacin, adenosine-5-diphosphate, histamine hydrochloride, atropine sulphate and bethanechol chloride (Sigma), phenoxybenzamine hydrochloride (Smith, Kline and French, Welwyn Garden City, Herts), and fibrinogen (Kabi Vitrum, Stockholm, Sweden.)

Prostaglandins, their synthetic analogues, and phenoxybenzamine were dissolved initially in absolute ethanol; indomethacin was dissolved in 10% Na_2CO_3. Other drugs were dissolved in distilled water. Dilutions were made in distilled water and the final concentration of vehicle in the organ bath did not affect tissue responsiveness.

Results

FP receptors

9α11β-PGF$_2$ and PGD$_2$ caused concentration-dependent contractions of cat iris sphincter with similar potency, p[A_{50}] 7.35 ± 0.09 (n = 9), 7.15 ± 0.19 (n = 5) respectively. The maximum responses achieved by PGD$_2$ and 9α11β-PGF$_2$, and by the selective FP receptor agonist, fluprostenol (p[A_{50}] 8.76 ± 0.14, n = 3) were not significantly different (ANOVAR) from that achieved by PGF$_{2\alpha}$ (p[A_{50}] 7.74 ± 0.12, n = 4), suggesting that all four compounds were behaving as full agonists (Figure 1). Consistent with this, 3 μM PGF$_{2\alpha}$ added on completion of each curve elicited little or no further contraction. Responses to 9α11β-PGF$_2$ and to PGD$_2$ were unaffected by the presence of 10 μM BM 13.177 or 5 μM AH6809 (n = 4 for each treatment), indicating that contraction was not mediated via TP or EP$_1$ receptors (data not shown).

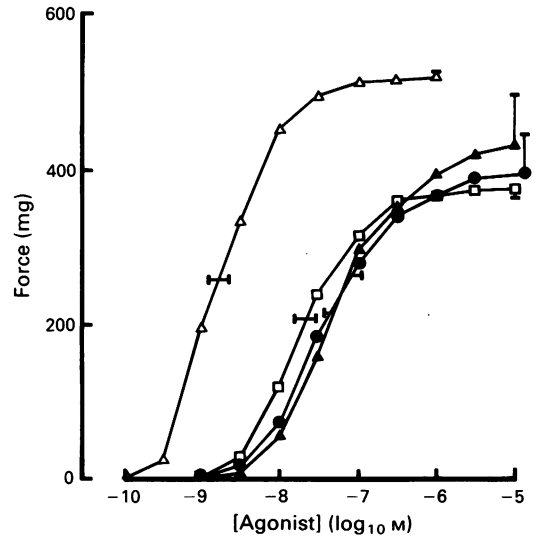

Figure 1 Mean contractile concentration-effect curves for 9α11β-PGF$_2$ (●, n = 9), PGD$_2$ (▲, n = 5), PGF$_{2\alpha}$ (□, n = 4) and fluprostenol (△, n = 3) in the FP receptor containing preparation, cat iris sphincter. Points are means and vertical lines show s.e.mean; horizontal lines show calculated p[A_{50}] ± s.e.mean.

Interestingly, the TP receptor agonist, U46619, also behaved as a full agonist, albeit of low potency, p[A_{50}] 6.15 ± 0.08 (n = 5), and its effects were not antagonized by 10 μM BM 13.177 (n = 4).

TP receptors

9α11β-PGF$_2$ and PGD$_2$ both caused concentration-related contractions of guinea-pig aorta, and were of similar potency, p[A_{50}] 6.00 ± 0.07 (n = 8) and 6.24 ± 0.08 (n = 6) respectively, and while U46619 had similar effects, it was more potent, p[A_{50}] 7.68 ± 0.04 (n = 6) (see Figure 2a). For each agonist, increasing concentrations of BM 13.177 (3–100 μM) caused concentration-dependent rightward shifts of the concentration-effect curves (Figure 2b and c, data for U46619 not shown). Lack of potency meant that the maximum effect to 9α11β-PGF$_2$ and to PGD$_2$ was not reached at 10 μM (highest concentration used), particularly in the presence of the antagonist, therefore Schild analysis was not performed. However, assuming simple competitive antagonism, a pA_2 value of approximately 6.0 was estimated.

In the rabbit jugular vein, 9α11β-PGF$_2$, in the presence of 1 μM BW A868C, also caused concentration-dependent contractions which were antagonized by BM 13.177 (2–100 μM), thus providing further evidence for the efficacy of 9α11β-PGF$_2$ at TP receptors (data not shown).

TP receptors are also present on human platelets. However, 1–10 μM 9α11β-PGF$_2$ (in the presence of 1 μM BW A868C) did not cause aggregation of human washed platelets. On some occasions a shape change reaction was observed which could be blocked by BM 13.177 (100 μM) (Figure 3b). In order to determine whether 9α11β-PGF$_2$ could antagonize TP receptor-mediated responses, the effect of 9α11β-PGF$_2$ on platelet aggregation by U46619 was examined. 9α11β-PGF$_2$

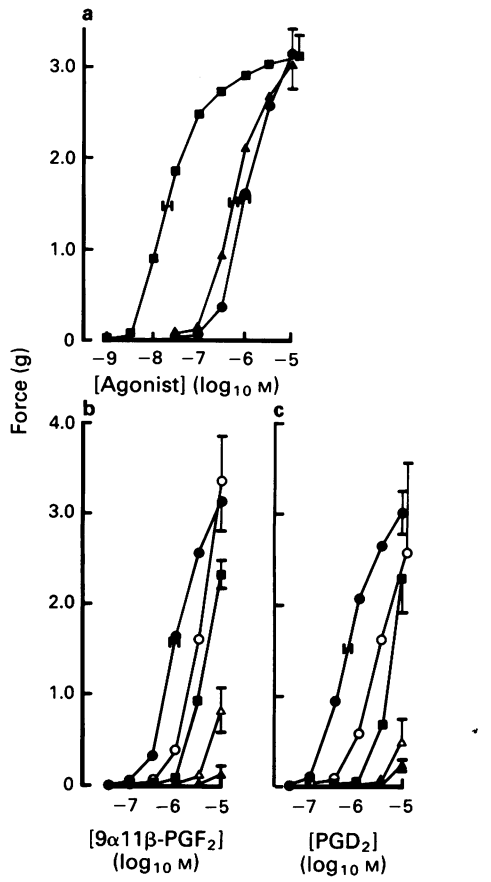

Figure 2 (a) Mean contractile concentration-effect curves for U46619 (■, $n = 6$), 9α11β-PGF_2 (●, $n = 8$) and PGD_2 (▲, $n = 6$) at TP receptors in guinea-pig aorta. (b and c) Antagonist effects of BM 13.177 on 9α11β-PGF_2 (b) and PGD_2 (c) contractile concentration-effect curves in the guinea-pig aorta at the following concentrations of BM 13.177: zero (●), 3 μM (○), 10 μM (■), 30 μM (△), 100 μM (▲). $n = 3$, except for controls where $n = 6$–8.

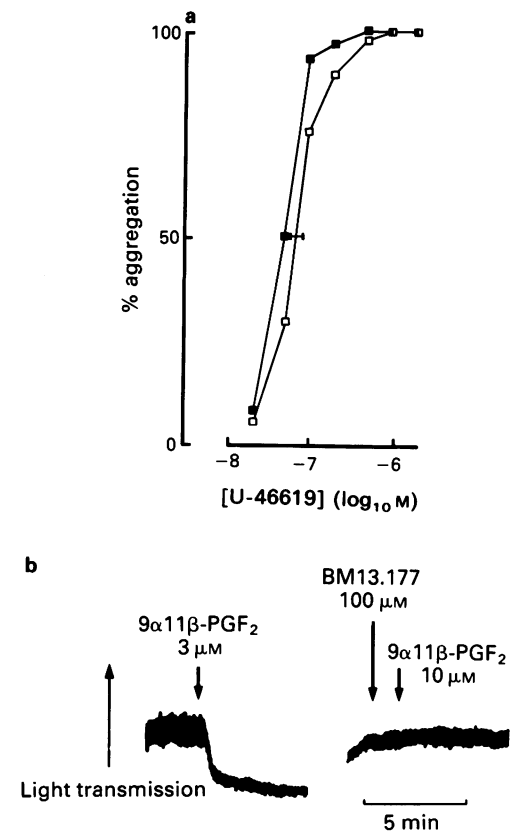

Figure 3 (a) Aggregation to U46619 in human washed platelets in the absence (■) or presence (□) of 10 μM 9α11β-PGF_2 ($n = 6$). Control and treatment curves were generated in platelets from the same donors. (b) Representative recordings showing the decrease in light transmission through a human washed platelet suspension upon addition of 3 μM 9α11β-PGF_2 (left), and the abolition of the response in the presence of 100 μM BM 13.177 (right). All experiments were performed in the presence of 1 μM BW A868C to block DP receptor effects.

10 μM caused a small (0.18 log unit) but consistent rightward displacement of the concentration-effect curves to U46619 (paired t test $P < 0.05$) (Figure 3a).

EP_1 receptors

9α11β-PGF_2 and PGD_2 also caused concentration-related contractions of guinea-pig muscularis mucosa (see Figure 4). Addition of 100 μM BM 13.177 usually caused a small (0.6 log unit) rightward shift of these curves, but in 15–20% of tissues, no agonism by either prostanoid was detected when BM 13.177 was present. When agonism was still evident, the addition of 5 μM AH6809 in addition to 100 μM BM 13.177 resulted in complete inhibition of contractile activity, indicating that the residual agonism, in the presence of the TP receptor antagonist, was due to activation of EP_1 receptors. In all experiments time-matched control curves were superimposable, as were second and third curves in the presence of 100 μM BM 13.177 alone.

The presence of EP_1 receptors in this tissue was confirmed using PGE_1 and PGE_2 which caused concentration-dependent contractions. Incubation with 2 μM AH6809 before the second curve resulted in a parallel rightward shift of 1.12 ± 0.06 log units ($n = 3$) and 1.02 ± 0.04 ($n = 4$) for PGE_1 and PGE_2 curves respectively, providing pA_2 estimates of 6.7–6.8, consistent with activation of EP_1 receptors. First and second control concentration-effect curves were superimposable ($n = 4$) and were unaffected by 10 μM BM 13.177.

Figure 4a shows mean concentration-effect curves at EP_1 receptors for PGE_1, PGE_2 (1st curves), 9α11β-PGF_2 and PGD_2 (2nd curves, in the presence of BM.13177). $p[A_{50}]$s cannot be quoted since 9α11β-PGF_2 and PGD_2 did not achieve maximum effect at 10 μM, the highest concentration used. The average concentration at which 30% of the maximum response to PGE_2 was achieved ($p[A_{30\%}]$) was: PGE_2 8.7; PGE_1 7.7; PGD_2 5.4; 9α11β-PGF_2 5.2.

9α11β-PGF_2 and PGD_2 also caused concentration-dependent contractions of guinea-pig ileum ($p[A_{50}]$ 6.11 ± 0.04, $n = 7$ and 6.03 ± 0.13, $n = 5$ respectively). The maximum response was generally less than 30% of that achieved by PGE_2. However, these effects were not elicited at TP or EP_1 receptors since they were not antagonized by 10 μM BM 13.177 or by 5 μM AH6809. Similar results were obtained with fluprostenol. Responses to PGE_2 were unaffected by 10 μM BM 13.177 but were antagonized by AH6809 (control $p[A_{50}]$ 7.24 ± 0.15, $n = 4$; 2 μM AH6809 6.53 ± 0.10, $n = 3$), and a pA_2 value of 6.4 was estimated consistent with EP_1 receptor activation.

EP_2 receptors

Although 0.3–10 μM 9α11β-PGF_2 or PGD_2 caused concentration-dependent relaxations of bethanechol-contracted cat trachea, a maximum response was not achieved even at 10 μM. Mean concentration-effect curves for both agonists, and for PGE_2, are shown in Figure 5. Average $p[A_{30\%}]$s were: 9α11β-PGF_2 5.15, PGD_2 5.85, PGE_2 8.7. Second concentration-effect curves, constructed in the presence of vehicle or 1 μM BW A868C, were superimposable ($n = 3$, data not shown).

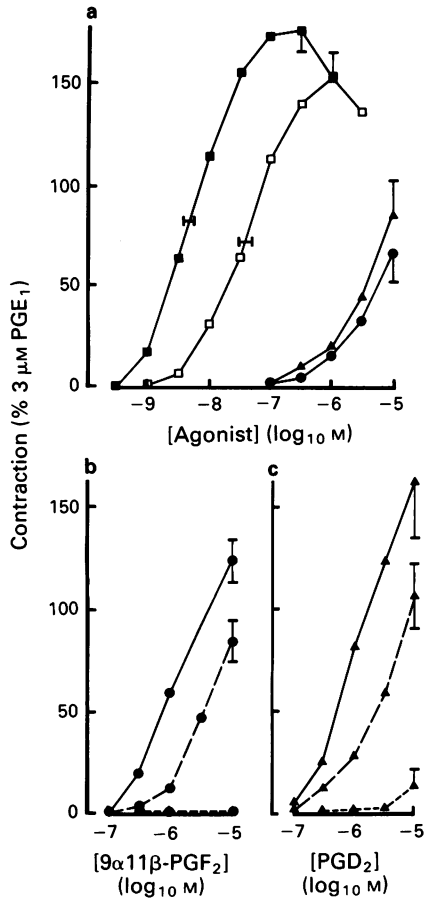

Figure 4 (a) Mean contractile concentration-effect curves for PGE_2 (■, $n = 11$), PGE_1 (□, $n = 6$), PGD_2 (▲, $n = 6$) and 9α11β-PGF_2 (●, $n = 6$) at EP_1 receptors in guinea-pig oesophageal muscularis mucosa. Curves to PGD_2 and 9α11β-PGF_2 were obtained in the presence of 100 μM BM 13.177 to block TP receptor mediated effects. (b and c) Mean contractile concentration-effect curves to 9α11β-PGF_2 (b) ($n = 4$) and PGD_2 (c) ($n = 5$) in guinea-pig oesophageal muscularis mucosa. A triple curve design was followed. First curves (———) were in the absence of antagonists, second curves (– – – –) were in the presence of 100 μM BM 13.177 and third curves (--------) in the presence of 100 μM BM 13.177 and 5 μM AH6809.

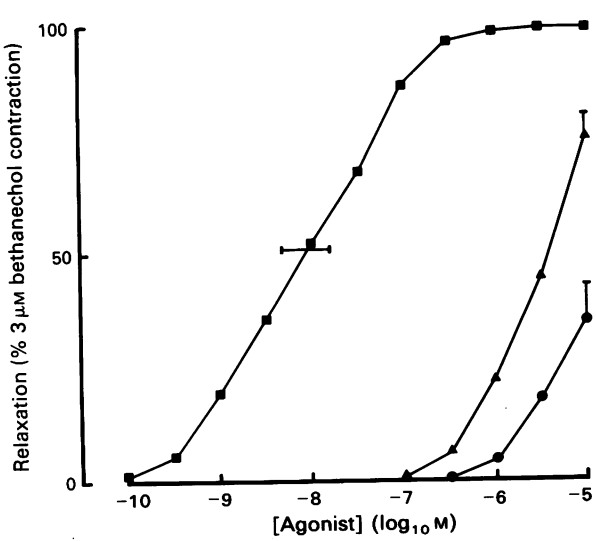

Figure 5 Mean relaxant concentration-effect curves for PGE_2 (■, $n = 5$), PGD_2 (▲, $n = 7$) and 9α11β-PGF_2 (●, $n = 8$) at EP_2 receptors in cat trachea. Tissues were pre-contracted with 3 μM bethanechol and responses are expressed as % relaxation of the bethanechol-induced contraction.

EP_3 receptors

Both 9α11β-PGF_2 and PGD_2 (1–10 μM) were without effect on field-stimulated guinea-pig vas-deferens. In contrast, PGE_2 (1–100 nM) elicited concentration-dependent inhibitions of twitch which were unaffected by the presence of 2 μM AH6809.

DP receptors

Effects at DP receptors were examined in a human washed platelet suspension, in the presence of the selective TP receptor antagonist, GR32191. This antagonist has no effect on PGD_2-mediated inhibition of platelet aggregation (Lumley *et al.*, 1989) or on inhibition of ADP-induced platelet aggregation by the DP receptor agonist, BW 245C ($n = 3$, data not shown), indicating that GR32191 has little or no affinity for DP receptors. BM 13.177 was not used in this assay since 100 μM 13.177 produced a small (0.1–0.3 log unit), but reproducible, non-specific rightward shift of concentration-effect curves to both IP-(prostacyclin) and DP-(BW245C, PGD_2, 9α11β-PGF_2) receptor agonists (data not shown).

Both PGD_2 and 9α11β-PGF_2 caused inhibition of ADP-induced human platelet aggregation (Figure 6a) but the

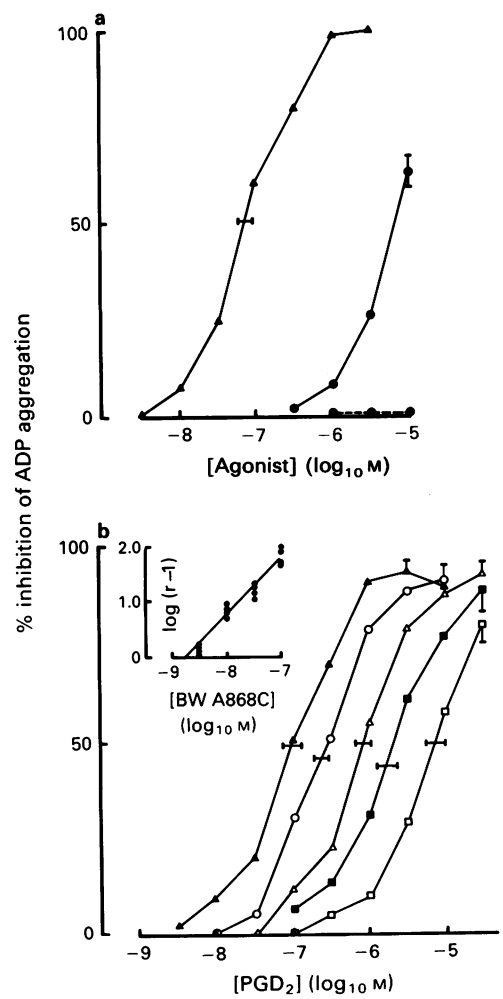

Figure 6 (a) Inhibition of ADP-induced platelet aggregation ($n = 3$) at DP receptors in human washed platelets by PGD_2 (▲), and 9α11β-PGF_2 in the absence (●) or presence (● – – – ●) of 0.1 μM BW A868C. Curves to both agonists were generated in platelets from the same donors. (b) Antagonism by BW A868C of PGD_2-induced inhibition of platelet aggregation in human washed platelets at the following concentrations of BW A868C: zero (▲), 3 nM (○), 10 nM (△), 30 nM (■), 100 nM (□), ($n = 3$). Inset: Schild plot for antagonism of PGD_2 by BW A868C. The line through the data has a slope of unity and was calculated as described in the methods section. All experiments were performed in the presence of 1 μM GR32191 to block TP receptor effects.

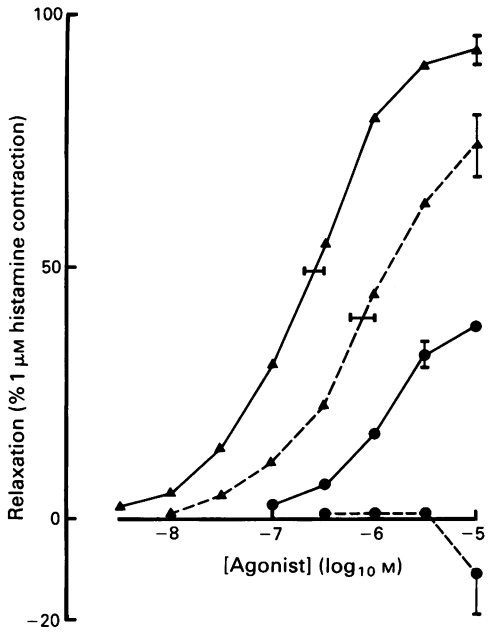

Figure 7 Antagonism by BW A868C of relaxations to PGD_2 (▲, $n = 4$) and 9α11β-PGF_2 (●, $n = 6$) in rabbit jugular vein rings pre-contracted with 1 μM histamine. Concentration-effect curves were obtained in a paired design: 1st curves (———) were controls, and second curves (– – – –) were in the presence of 1 μM BW A868C. 1st and 2nd control curves were superimposable (data not shown). All curves were generated in the presence of 100 μM BM 13.177 to block TP receptor effects.

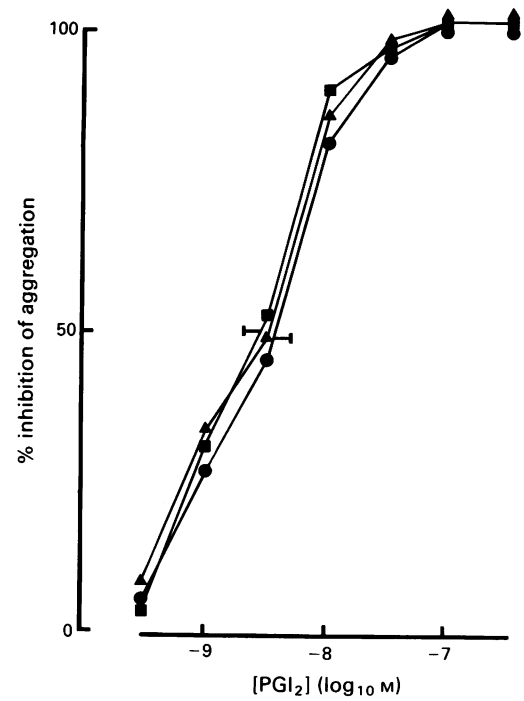

Figure 8 Inhibition of ADP-induced platelet aggregation in human washed platelets by PGI_2 in the absence (■) or presence of 10 μM PGD_2 (▲) or 10 μM 9α11β-PGF_2 (●) $n = 3$. All curves were obtained in the presence of 1 μM BW A868C and 1 μM GR 32191 to block DP and TP effects respectively. All curves were generated in platelets from the same donors.

maximum response to 9α11β-PGF_2 could not be achieved at 10 μM. PGD_2, p[A_{50}] 7.02 ± 0.09, $n = 4$, was approximately 1.8 log units more potent, and elicited 90–100% inhibition of aggregation. Similar results were obtained in PRP. BW A868C 0.1 μM abolished responses to 9α11β-PGF_2 and resulted in a 1.83 log unit right-shift of the PGD_2 curve. A subsequent study demonstrated that BW A868C (3–100 nM) caused a progressive rightward displacement of PGD_2 concentration-effect curves (Figure 6b), with no significant change in slope or asymptote. Analysis of computed [A_{50}] values using Equation 3 showed that the Schild plot slope parameter (n) was not significantly different from unity (1.08 ± 0.06), and provided a pK_B estimate of 8.79 ± 0.01 (Figure 6b).

PGD_2 and 9α11β-PGF_2 also caused concentration-dependent relaxation of histamine-contracted rabbit jugular vein (Figure 7). PGD_2 was approximately 1.5 log units more potent (p[A_{50}] 6.60 ± 0.12, $n = 6$) than 9α11β-PGF_2 (responses evident at 0.3–10 μM). 9α11β-PGF_2 curves were sometimes bell-shaped, with contractile effects at 10 μM. Figure 7 shows that these contractions were more evident in the presence of 1 μM BW A868C, which abolished the relaxations. BW A868C, 1 μM, also antagonized responses to PGD_2. However, the degree of rightward shift was limited to 0.57 ± 0.09 log units ($n = 4$), indicating the presence of a second receptor type mediating relaxation.

IP receptors

The actions of 9α11β-PGF_2 and PGD_2 at the IP receptors in human washed platelets were examined in the presence of 1 μM BW A868C and 1 μM GR32191 to block DP- and TP-receptor effects respectively. Both these antagonists were without effect on PGI_2 concentration-effect curves ($n = 3$). PGD_2 or 9α11β-PGF_2 alone (10 μM in each case) caused no pro- or anti-aggregatory activity, nor did they alter inhibition of aggregation by PGI_2 (Figure 8).

Discussion

The purpose of these experiments was to determine the prostanoid receptor actions of PGD_2 and its primary metabolite 9α11β-PGF_2. Quantification of agonist action ideally requires estimation of affinity and efficacy at each receptor type, since these measurements dissect out the information contained in the potency (p[A_{50}]) values. The most reliable method for estimating these parameters involves irreversible receptor blockade (Furchgott, 1966; Furchgott & Bursztyn, 1967) or alternatively, for partial agonists, comparison or interaction with a full agonist (Barlow *et al.*, 1967; Jenkinson, 1979). Unfortunately no irreversible antagonists at prostanoid receptors are available and there was no clear evidence that PGD_2 or 9α11β-PGF_2 behaved as a partial agonist in any of the assays described in this paper. Therefore in this study, agonist relative potencies and, where available, selective receptor antagonists were used in an attempt to characterize the receptor types involved. Particular care was taken to avoid confounding factors such as agonist uptake processes (Kenakin, 1980), agonist loss due to binding to another site or agonist action through more than one receptor (see Kenakin, 1984). For example, studies on platelets were carried out in plasma-free medium to eliminate agonist plasma binding; concentration-effect curve shapes were examined carefully for evidence of biphasic or bell-shaped curves, or for shallow slopes, all of which may be indicative of activation of more than one receptor.

Because most of the tissues studied did contain heterogeneous populations of prostaglandin receptors, selective competitive antagonists were used both to block unwanted effects and to identify sites of agonist action. The validity of the conclusions drawn in this study are dependent upon the selectivity of these antagonists. In this context BW A868C, BM 13.177, GR 32191 and AH 6809 were used to demonstrate that 9α11β-PGF_2 had efficacy at DP, TP and EP_1 receptors and to reveal effects of 9α11β-PGF_2 and PGD_2 at other prostanoid receptors. Formal Schild analysis was not always pos-

sible because concentration-effect curves to 9α11β-PGF$_2$ could not be fully defined, due to lack of potency. Nevertheless, the selectivity of BW A868C and GR 32191 has been rigorously demonstrated (Giles *et al.*, 1989; Lumley *et al.*, 1989). BM13.177 is also reported to be without actions at other receptors and results from this study demonstrated that it does not have significant affinity at either FP or EP$_1$ receptors, since agonist concentration-effect curves in cat iris sphincter (FP) and in guinea-pig ileum (EP$_1$) were unaffected by 100 μM BM 13.177. The small effect in human platelets reported in this study was presumably non-specific and not prostanoid receptor-mediated since 100 μM antagonized to the same extent both IP and DP agonism. This non-specific effect was not seen in the smooth muscle assays and its underlying mechanism(s) remains unknown. AH 6809 is less selective but the concentrations (2–5 μM) used in these studies are claimed to be selective for EP$_1$ receptors (Coleman *et al.*, 1985).

Inhibition of platelet aggregation by PGD$_2$ and 9α11β-PGF$_2$ was blocked by BW A868C and the potency of PGD$_2$ in the platelet assay allowed a pK$_B$ estimate of 8.79 to be made for BW A868C. This value is similar to, although slightly lower than that of 9.26 previously obtained at DP receptors when BW 245C was used as the agonist (Giles *et al.*, 1988). Previously it has been demonstrated in human platelets that responses to PGD$_2$ and 9α11β-PGF$_2$ are antagonized by AH6809 at concentrations compatible with its affinity for DP receptors (Keery & Lumley, 1988), indicating that responses to these prostanoids are DP receptor-mediated in this assay. The present study, using the more selective DP receptor antagonist, BW A868C, confirms these findings. 9α11β-PGF$_2$ was considerably less potent than PGD$_2$ at DP receptors, 1.8 log units on the platelets, similar to that previously reported by Pugliese *et al.* (1985) and approximately 1.5 log units less potent in RbJV.

At TP receptors in guinea-pig aorta, 9α11β-PGF$_2$ was of similar or slightly lower potency than PGD$_2$ and the pA$_2$ estimates of 6.0 for BM 13.177 obtained from experiments using both agonists were consistent with those obtained previously, when U46619 was used as the agonist (Patscheke *et al.*, 1984; Giles *et al.*, 1989). 9α11β-PGF$_2$ also contracted RbJV via TP receptors and it therefore seems possible that the vascular smooth muscle contractions previously described in coronary arteries (Pugliese *et al.*, 1985; Roberts *et al.*, 1987), as well as the increases in blood pressure observed following intravenous administration of 9α11β-PGF$_2$ to rats (Liston & Roberts, 1985) could be due to TP receptor activation. 9α11β-PGF$_2$ has also been found to activate TP receptors in guinea-pig and human isolated bronchial smooth muscle (Featherstone *et al.*, 1988; Coleman & Sheldrick, 1989). Agonist effects of 9α11β-PGF$_2$ at platelet TP receptors were minimal or absent, presumably because the receptor density, or the efficiency of transduction, was lower in the platelet than the vasculature. Nevertheless, 9α11β-PGF$_2$ blocked U46619-induced aggregation consistent with an affinity of approximately 10 μM at this receptor.

EP$_1$ receptor activity was measured in guinea-pig muscularis mucosa. In the presence of 100 μM BM 13.177 to block TP receptor effects, contractions to 9α11β-PGF$_2$ and PGD$_2$ were abolished by 5 μM AH6809. Because these EP$_1$ receptor-mediated responses were only evident at 1–10 μM, it was not possible to estimate a pA$_2$ for AH6809; however, this concentration of antagonist should be selective for EP$_1$ receptors. Interestingly, EP$_1$ receptor-mediated responses to 9α11β-PGF$_2$ and PGD$_2$ were not always present and there were variations of nearly tenfold in the p[A$_{50}$] for both PGE$_1$ and PGE$_2$, possibly due to between-tissue variation in EP$_1$ receptor density. This would be compatible with the observations of Eglen & Whiting (1988) who identified both EP$_1$ and TP receptors in this tissue but found that the TP receptor antagonist SQ29,548 behaved as a simple competitive antagonist of PGD$_2$ with a Schild plot slope of 0.98. However, PGD$_2$ contractions resistant to TP receptor antagonism have been observed in guinea-pig trachea (Jones *et al.*, 1982) and the authors suggested that these were mediated via an EP receptor. Likewise, in an earlier paper, contractions of guinea-pig colonic circular muscle to PGD$_2$ were blocked by the EP$_1$ receptor antagonist, SC-19220 (Bennett & Sanger, 1978).

Conclusions regarding agonist activity at FP and EP$_2$ receptors must remain more tentative. Cat iris sphincter muscle is frequently used to assay for activity at FP receptors (Coleman, 1987), and the effect of the putative selective FP receptor agonist, fluprostenol (Dukes *et al.*, 1974; Coleman *et al.*, 1984) as well as the natural prostanoids tested were clearly not mediated through EP$_1$ or TP receptors because agonism was resistant to blockade by BM 13.177 or AH6809. Assuming that all the agonists were acting via the same FP receptors, then 9α11β-PGF$_2$ and PGD$_2$ were only slightly less potent than the endogenous agonist, PGF$_{2\alpha}$, and both were full agonists. However, in the absence of a competitive antagonist or of binding data these conclusions must remain provisional.

Similar caveats apply to the data generated in cat trachea which is reported to contain a homogeneous population of EP$_2$ receptors (Coleman, 1987). Both 9α11β-PGF$_2$ and PGD$_2$ were agonists in this tissue, although of much lower potency than PGE$_2$. Relaxations were not via DP receptors since they were unaffected by BW A868C, or by IP receptors since at 10 μM both PGD$_2$ and 9α11β-PGF$_2$ lacked efficacy and affinity at IP receptors (Figure 8). Further evidence for the activation of EP receptors mediating smooth muscle relaxation was provided by the BW A868C-resistant relaxations elicited by PGD$_2$ in the rabbit jugular vein, a tissue previously reported to contain EP receptors mediating relaxation (Giles *et al.*, 1989; Lawrence *et al.*, 1989). However, if these receptors are the same as those in cat trachea then 9α11β-PGF$_2$ should also have displayed BW A868C-resistant relaxations in RbJV, since it relaxed cat trachea with only slightly less potency than PGD$_2$. This was not observed, but it is possible that relaxations were masked by contractile effects resulting from high concentrations of 9α11β-PGF$_2$ overcoming the TP receptor blockade (Figure 7). Further studies, in the presence of a TP receptor antagonist of higher affinity are necessary to validate this hypothesis.

IP and EP$_3$ receptors were the only sites at which 9α11β-PGF$_2$ and PGD$_2$ did not display efficacy and, at least for the IP receptor, neither compound had significant affinity. In the case of PGD$_2$ this confirms binding studies which have demonstrated that it has no affinity for the prostacyclin binding site on human platelets (Siegl *et al.*, 1979; Schafer *et al.*, 1979).

Some of the pitfalls of attempting to quantify the receptor profile of a ligand in tissues containing several receptor types have been evident in this study. For example, longitudinal guinea-pig ileum is the preparation more commonly used to assess EP$_1$ receptor activity (Coleman, 1987) and the results of the present study were in agreement with previous findings (Coleman *et al.*, 1985; Eglen & Whiting, 1988) that the PGE$_2$ concentration-effect curves in guinea-pig ileum are antagonized by AH6809 at concentrations consistent with the most potent effects of PGE$_2$ being due to EP$_1$ receptor activation, thus establishing the presence of EP$_1$ receptors in this tissue. However, the most potent effects of 9α11β-PGF$_2$ and PGD$_2$ were clearly not mediated via EP$_1$ receptors because they were unaffected by AH6809. Therefore, their affinity and efficacy at EP$_1$ receptors could not be assessed in this tissue. Their actions were not due to TP receptor activation, since they were unaffected by BM 13.177 but may have been via FP receptors because fluprostenol also caused contractions. Since other studies (Lawrence & Jones, 1989; Eglen & Whiting, 1989) have suggested that EP$_3$ receptors may also elicit contractions in this tissue, it is clear that guinea-pig ileum does not contain a homogeneous EP$_1$-receptor population mediating direct contractile effects and therefore is not an ideal assay for assessing agonist action at the EP$_1$ receptor. In the present study, agonist effects of 9α11β-PGF$_2$ and PGD$_2$ at EP$_1$ receptors were examined in guinea-pig muscularis

mucosa but these were revealed only when more potent TP-receptor effects were blocked by BM 13.177. Similarly, multiple receptor populations in RbJV meant that the efficacy of PGD_2 at EP receptors was not revealed until both a DP and a TP receptor antagonist were present. Even in the presence of TP receptor blockade, interpretation of relaxant effects by 9α11β-PGF_2 was confounded by contractile effects at high concentrations. Thus, in Figure 7 9α11β-PGF_2 appears to behave as a partial agonist at DP receptors whereas this depression of the curve asymptote is more likely to be due to an opposing contractile effect. The importance of using competitive antagonists to confirm the site of agonist action was highlighted by the study with U46619 in cat iris sphincter. U46619 is a potent TP receptor agonist (Coleman *et al.*, 1980) commonly used to identify the presence of TP receptors, but in the cat iris sphincter it had considerable efficacy at contractile receptors which were resistant to TP receptor blockade (in agreement with previous work, Kennedy *et al.*, 1983), presumably FP receptors.

Results from this study demonstrate that 9α11β-PGF_2 generated from the metabolism of PGD_2 is unlikely to elicit receptor-mediated effects distinct from those of its parent compound, since it does not have a significantly greater potency than PGD_2 at any prostaglandin receptor. Indeed, at DP-receptors, at which PGD_2 is the most potent prostaglandin and at which it is likely to exert its major effect, 9α11β-PGF_2 is considerably less active and the conversion clearly represents an inactivation step. Since 9α11β-PGF_2 is of similar potency to PGD_2 at other prostanoid receptors it does have the potential to contribute to the *in vivo* biological responses seen following administration of PGD_2, particularly if a significant proportion of PGD_2 is converted to 9α11β-PGF_2. Theoretically, 9α11β-PGF_2 could activate any of the receptors at which it has efficacy, assuming a high enough receptor concentration is present. Realistically, it is likely to activate those at which it has a similar potency to that of the endogenous ligand i.e. FP and possibly TP receptors. However, it clearly can activate relaxant vascular receptors and the final effect in any vessel or vascular bed will be dependent upon the density of relaxant DP (and EP_2) receptors relative to the density of contractile TP receptors; in human skin it seems that the relaxant receptors must predominate since intravenous 9α11β-PGF_2 causes increased blood flow, accompanied by wheal and flare (Beasley *et al.*, 1987a). 9α11β-PGF_2 may, therefore, contribute to the vasodepression elicited by PGD_2 *in vivo* via either DP or EP_2 receptors, since the most potent vasodilator effects of intravenous PGD_2 administered to rats and guinea-pigs are via DP receptors, but BW A868C-resistant vasodepression can also be demonstrated (Cambridge *et al.*, 1988).

Although 9α11β-PGF_2 and PGD_2 in common with most other prostaglandins have efficacy at several prostanoid receptors it is interesting to consider whether prostaglandins really are non-selective *in vivo* under physiological conditions. Each prostaglandin tends to be most potent at one receptor type and it may be that, under normal conditions, the concentrations achieved are not high enough to activate the other receptor types, particularly since many of these compounds have such a short biological half-life. It is also possible that the potentially deleterious effects of (pathologically) high concentrations of prostaglandins (e.g. PGD_2 release during mast cell degranulation) could be offset by the activation of receptors which mediate an opposing effect.

In conclusion, these results demonstrate that 9α11β-PGF_2 is capable of agonist action at several prostanoid receptors although, in the absence of competitive antagonists, conclusions regarding efficacy at EP_2 and FP receptors must remain tentative. Both 9α11β-PGF_2 and PGD_2 behaved as full agonists at FP receptors in cat iris sphincter and at TP receptors in RbJV and guinea-pig aorta. They also had efficacy, but low affinity at EP_1 and EP_2 receptors, but lacked significant efficacy at EP_3 receptors and IP receptors. Both agonists were of similar potency at FP, TP, EP_1 and EP_2 receptors. However, at DP receptors PGD_2 was approximately 80 times more potent.

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Transepithelial water movement in response to carbamazepine, chlorpropamide and demeclocycline in toad urinary bladder

M.R. Hirji & [1]J.C. Mucklow

Department of Medicine, School of Postgraduate Medicine and Biological Sciences, University of Keele, Thornburrow Drive, Hartshill, Stoke on Trent

1 Osmotic water movement across toad isolated hemibladders was measured by a gravimetric method.

2 The influence of carbamazepine, chlorpropamide and demeclocycline on the antidiuretic hormone (ADH)-induced water flow rate was examined.

3 No antidiuretic activity due to carbamazepine alone was observed but a slight inhibition due to ADH-induced water flow was observed in the presence of carbamazepine over a selected dose-range. This was unexpected and is inconsistent with data from *in vivo* studies in man.

4 Chlorpropamide potentiated ADH-induced water flow, in keeping with the hypothesis that chlorpropamide sensitizes the renal tubules to ADH-induced water flow.

5 Demeclocycline inhibited ADH-induced water flow. The mechanism of action remains unclear.

Keywords: Toad bladder; hydrosmotic water flow; carbamazepine; chlorpropamide; demeclocycline; antidiuretic hormone

Introduction

Several pharmacological agents are known to influence water handling by increasing or decreasing the effect of antidiuretic hormone (ADH). Their precise site and mechanism of action is fairly certain in some instances but speculative in others. Chlorpropamide (CPM) for example, appears to sensitize the renal tubule to the effect of endogenous ADH (Gilman *et al.*, 1985) and demeclocycline (DMC) blocks reversibly the increase in tubular permeability induced by both ADH and adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Singer & Rotenberg, 1973).

The antidiuretic effect of carbamazepine (CBZ) is well documented (Stephens *et al.*, 1977), but the mode of action remains unclear. Fraham *et al.* (1969) showed an increase in plasma antidiuretic activity after CBZ therapy but Meinders *et al.* (1974) could find no increase in plasma arginine-vasopressin (AVP) concentration in patients receiving CBZ and Hanefield *et al.* (1970) found no evidence of increased AVP production by the hypothalamus in response to CBZ. It has been suggested that carbamazepine increases renal responsiveness to ADH but may also act upon circulatory osmoreceptors (Stephens *et al.*, 1978).

Our understanding of the cellular mechanism of action of neurohypophyseal hormone has been derived extensively from studies on the toad urinary bladder, which serves as an excellent model epithelium for investigation of the role of the mammalian renal nephron in salt and water homeostasis (Leaf *et al.*, 1958) but has not been used widely to investigate pharmacological effects.

The urinary bladder of *Bufo marinus* is a bilobed structure and may contain as much as 100–150 ml of fluid when fully distended. The size of the organ makes it possible to obtain large sheets of tissue for gravimetric determination of water movement where one cannulated lobe serves as an experimental sac with the other as a control (Bentley, 1958). The simpler histological structure compared with mammalian renal tubules or amphibian skin assists interpretation of the results. We have used this preparation to investigate the effects of certain drugs upon water transport across the epithelial membrane.

[1] Author for correspondence at present address: Dept. of Clinical Pharmacology, City General Hospital, Newcastle Rd, Stoke on Trent ST4 6QG.

Methods

All studies were carried out on the isolated urinary bladder of the toad, *Bufo marinus* (Mexican), obtained from Xenopus Ltd, Surrey. The toads were fed once a week on house crickets and allowed continuous free access to tap water. They were killed by double pithing and their bilobed urinary bladders were resected.

Measurement of osmotic water movement

Osmotic water movement across isolated hemibladders was measured gravimetrically in paired experiments as described by Bentley (1958). Each hemibladder was fixed with surgical thread to the end of a short length (4 cm) of plastic tubing (0.5 cm o.d.) forming a bag, and rinsed inside and out with incubation medium (composition, mM: NaCl 90, KCl 3.5, $NaHCO_3$ 25, Mg_2SO_4 0.5 KH_2PO_4 0.5, $CaCl_2$ 1, glucose 6, gentamicin 5 mg l^{-1}, 1 N-2-hydroxyethylpiperazine-N-2 ethanesulphonic acid (HEPES) 10 mM, 22 mosmol) pH 7.4–7.5 under air at 22–25°C. Each hemibladder was filled with 5 ml quarter strength incubation medium and suspended in a bath containing 50 ml of aerated full strength incubation medium.

Experimental procedure

After an initial 1 h period of equilibration, the hemibladders were emptied, rinsed, refilled with quarter strength incubation medium and resuspended in freshly aerated full strength incubation medium so that their tops were just covered. Periodic weighting was carried out on an analytical balance at 20 min intervals during a 1 h experimental period. The hemibladder was out of the bath for no more than 15 s during each weighing. Any weight lost was taken to indicate the amount of fluid lost from the lumen by transfer across the bladder membrane.

Arginine vasopressin

Once the hemibladder sacs were equilibrated and steady 20 min interval water flow rates obtained, one of the paired bladder sacs was exposed to a fixed dose of AVP for 20 min. The second bladder sac from the same toad served as a control. The rate of transepithelial water movement was estimated in mg min^{-1} and expressed as $\mu l\ min^{-1}$ water flow rate for each of the 6 doses of AVP tested.

Pharmacological agents

After obtaining a steady reading, for each of the two bladder sacs, one bladder sac was exposed to the test agent such as CBZ, CPM or DMC (applied to the serosal side) while the second bladder served as a control. After 20 min of exposure of one bladder sac to the test agent, both the bladder sacs were exposed to 50 nM AVP (applied to the serosal surface). When changing bathing solutions or washing out the test agents, the two hemibladders were treated identically. The weight change of each hemibladder was measured at 20 min intervals after exposure to AVP and the 40 min values used. The rate of transepithelial water movement was thus estimated in mg min^{-1}, and the rates of mean weight change in the paired hemibladders were compared and expressed as $\mu l\ min^{-1}$ water flow rate.

Although the hydration status of the toads may influence the responsiveness of the hemibladder to AVP, the error introduced was minimized by using toads of the same batch within each experiment. Also each toad provided its own internal control. With the test agents, the experimental protocol was designed to examine the difference between the control and the experimental preparation and not the absolute water flow rate values due to AVP.

Results are expressed throughout as mean $\pm$ s.d. ($\pm$s.e.mean on the graphs). Statistical significance was evaluated by Student's *t* test.

Reagents

Fresh solutions of each test compound were made up daily. As CBZ is practically insoluble in incubation medium, the compound was first dissolved in 5% ethanol (Analar) to give a 1 mM stock solution from which serial dilutions were made with incubation medium. Chlorpropamide is insoluble both in water and ethanol and the aqueous compound was prepared in McIlvaine's standard buffer pH 7.4 (McIlvaine, 1921). Demeclocycline and vasopressin are both water-soluble and millimolar concentrated stock solutions of each were prepared in de-ionised water and diluted with normal strength incubation medium; all solutions were kept refrigerated until required.

Results

Effect of arginine vasopressin alone

The mean rate of osmotic water movement across the isolated control hemibladder was $1.44 \pm 0.19\ \mu l\ min^{-1}$. The presence of AVP significantly raised the basal rate. The lowest dose of AVP applied (0.5 nM) produced an almost twenty fold increase in hydrosmotic response (Figure 1). A peak water flow rate of $53.4 \pm 13.63\ \mu l\ min^{-1}$ ($n = 8$; $P < 0.001$) was observed in the presence of 10 nM AVP. Further increase in AVP dose from 10 to 100 nM resulted in a gradual reduction of water movement to $27.01 \pm 17.45\ \mu l\ min^{-1}$ ($n = 8$; $P < 0.001$), identical to the hydrosmotic response observed at 0.5 nM.

Effect of chlorpropamide

CPM alone showed no antidiuretic activity. The test hemibladders that were incubated for 20 min with CPM, before addition of AVP (50 nM), showed a significant potentiation of the response due to AVP (Figure 2) for CPM doses above 50 μM ($n = 6$; $P < 0.001$). The increased ADH response in the presence of CPM was dose-dependent and exceeded the control value by $59 \pm 9.3\%$ ($n = 6$; $P < 0.001$) at a CPM concentration of 500 μM.

Effect of demeclocycline

DMC alone showed no change in the basal water flow rate across the hemibladders. There was a significant inhibition in

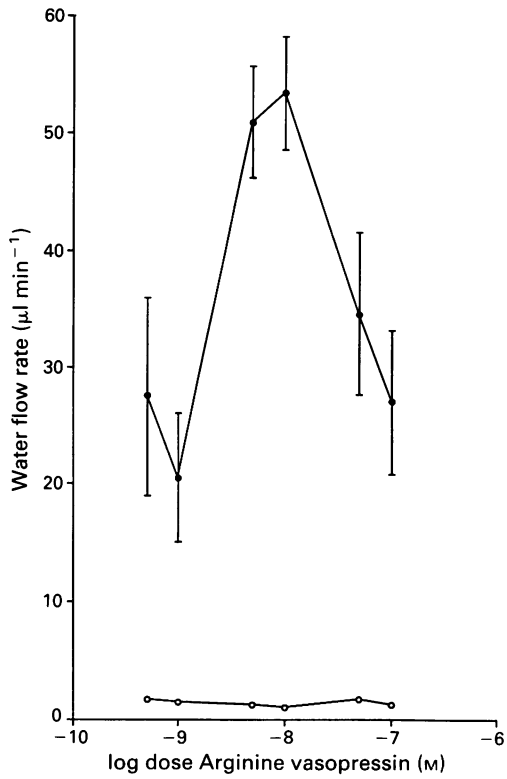

Figure 1 Effect of increasing doses of arginine vasopressin on the hydrosmotic response of the toad bladder. Experimental (●), control (○); mean of eight paired experiments with s.e.mean shown by vertical bars.

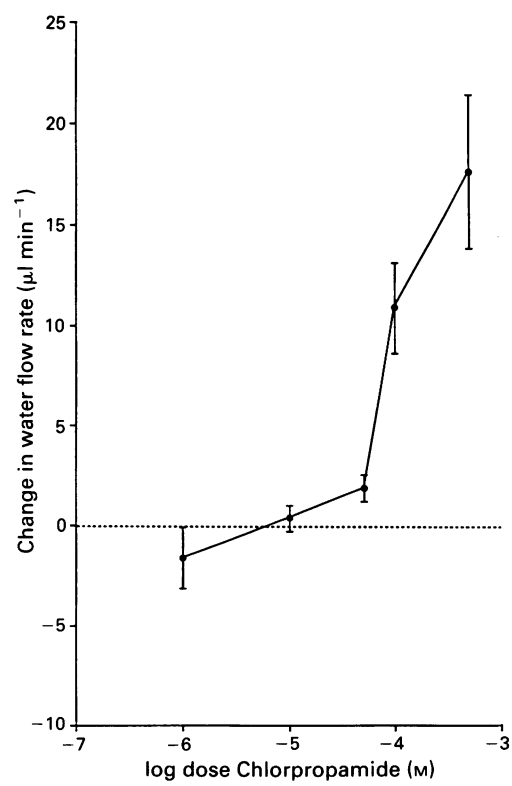

Figure 2 Effect of increasing doses of chlorpropamide on the hydrosmotic response to a single 50 nM dose of arginine vasopressin. The difference between the control and the experimental preparations at each dose is shown as the mean of six paired experiments; s.e.mean shown by vertical bars.

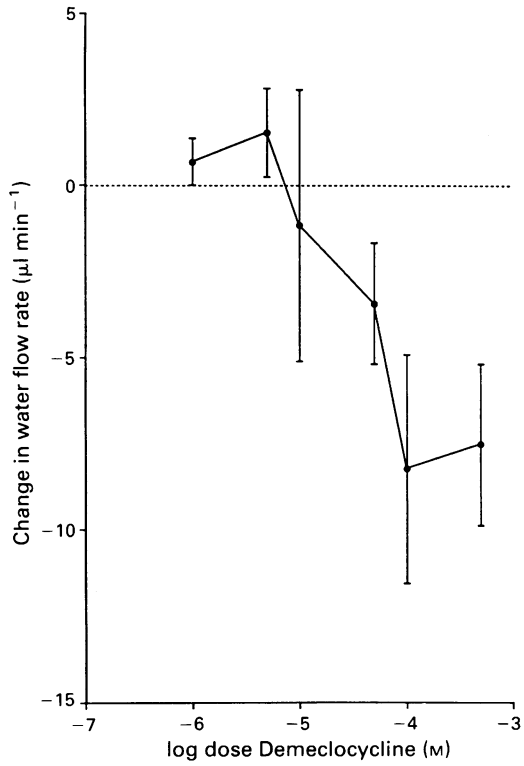

Figure 3 Effect of increasing doses of demeclocyline on the hydrosmotic response to a single 50 nM dose of arginine vasopressin. The difference between the control and the experimental preparations at each dose is shown as the mean of six paired experiments; s.e.mean shown by vertical bars.

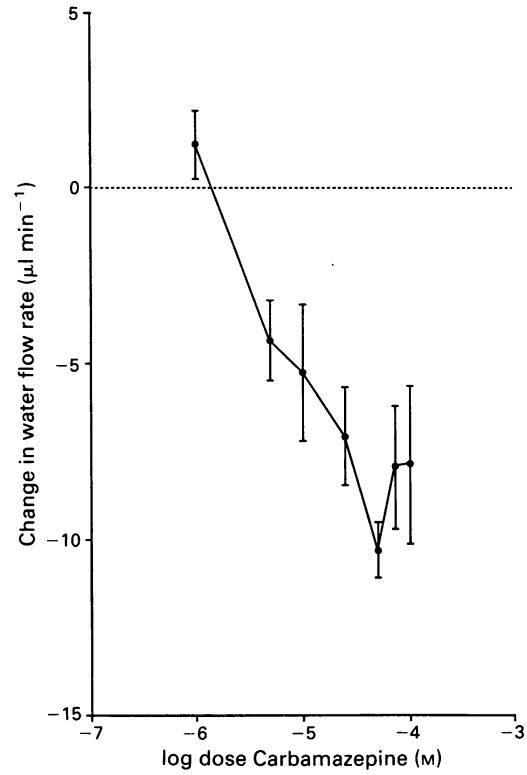

Figure 4 Effect of increasing doses of carbamazepine on the hydrosmotic response to a single 50 nM dose of arginine vasopressin. The difference between the control and the experimental preparations at each dose is shown as the mean of six paired experiments; s.e.mean shown by vertical bars.

the response to AVP (50 nM) when the test hemibladders were incubated for 20 min with DMC. The inhibitory influence of DMC on hydrosmotic responses in the paired hemibladder is shown in Figure 3. The differences between treated and untreated bladders become apparent at doses of 50 μM to 500 μM ($n = 6$; $P < 0.001$). The inhibition was dose-dependent, test and the control values differing by $37 \pm 5.7\%$ ($n = 6$; $P < 0.001$) at a DMC concentration of 500 μM.

Effect of carbamazepine

The basal rate of osmotic water movement across isolated hemibladders in the absence of AVP was not influenced by exposure of tissue to CBZ, 1 to 100 μM. The mean basal weight loss was 0.71 ± 4.78 l min^{-1} in the control bladder and 1.08 ± 3.19 μl min^{-1} in the test bladder ($n = 6$) with no significant differences between test and control. The increased rate of water movement elicited by AVP 50 nM was consistently reduced in hemibladders previously exposed for 20 min on their serosal surface to CBZ 5 μM to 500 μM (Figure 4). The inhibition was dose-dependent and reached $42 \pm 3.4\%$ ($n = 6$, $P < 0.001$) after 20 min before exposure to CBZ, 25 μM. Further increase in CBZ doses resulted in lesser rather than greater inhibition. Three preparations failed to respond to CBZ treatment over the entire dose-range.

Discussion

Effect of arginine vasopressin

Mammalian antidiuretic hormone is acknowledged to be the primary hormonal regulator of water excretion and stimulator of active sodium transport. In amphibian urinary bladder, its mechanism of action is thought to be similar to its action on the mammalian renal collecting ducts (Eggena & Ma, 1986). Vasopressin binds initially to V_2 receptors located in the basolateral cell membrane and activates adenylate cyclase, resulting in the generation of cyclic AMP which serves as an intracellular messenger for both the hydrosmotic and natriferic action of the hormone. The mechanism of the events that follow cyclic AMP generation is not clear but cytoskeletal organelles, such as microtubules and actin-containing microfilaments, are thought to be involved in producing specific changes in the surface architecture (Taylor *et al.*, 1987).

We have confirmed that toad hemibladder tissue is highly sensitive to the hydrosmotic effect of AVP and that the preparation can be used to relate the antidiuretic activity of other pharmacological agents to AVP.

Effect of chlorpropamide

Inappropriate secretion of antidiuretic hormone and hyponatraemia due to CPM has been reported (Moses *et al.*, 1973) but others have suggested that there is no direct action on the pituitary or hypothalamus resulting in release of ADH (Meinders *et al.*, 1975). The most favoured explanation for the effect of CPM is that the drug increases the sensitivity of target epithelium to ADH (Beck *et al.*, 1974).

We have shown that CPM enhances the effect of AVP on hydrosmotic water flow across the hemibladder and the data support the hypothesis that CPM increases renal responsiveness to ADH. The mechanism of action at cellular level is unclear and reports are contradictory. It has been suggested that CPM decreases the inhibitory effect of prostaglandins on ADH-dependent generation of cyclic AMP (Ozer & Sharp, 1973) but Omachi *et al.* (1974) have shown that CPM lowers cyclic AMP levels in toad urinary bladder in response to ADH. Ozer & Sharp, (1973) have interpreted the potentiating effect of CPM upon ADH in toad urinary bladder as the consequence of raising levels of intracellular cyclic AMP; while Kusano *et al.* (1983) proposed that CPM enhances the effect of ADH by a mechanism that increases the osmotic driving force for water reabsorption in collecting tubules.

Effect of demeclocycline

DMC has been shown to inhibit the hydrosmotic effect of ADH in man (Singer & Rotenberg, 1973; Feldman & Singer, 1974). Our findings indicate that the presence of DMC inhibits the increase in hydrosmotic water flow due to ADH and confirms the work of Feldman & Singer, (1974), who used Dominican Republic toad bladder incubated with DMC in Ringer solution. Although the mechanism is not clear, DMC probably impairs ADH-induced cyclic AMP generation and also impairs cyclic AMP action (Singer & Rotenberg, 1973). However, other possibilities, such as the physicochemical properties of DMC and its ability to bind to cell membrane proteins involved in water transport, need to be explored.

Effect of carbamazepine

The antidiuretic effect of CBZ was first described in 1966 (Braunhofer & Eroffnet, 1966) and further reports of hyponatraemia and water intoxication have since appeared (Lahr, 1985). Suggested mechanisms have included enhancement of renal sensitivity to AVP (Smith *et al.*, 1977; Stephens *et al.*, 1978), direct stimulation of AVP release (Kimura *et al.*, 1974; Smith *et al.*, 1977) and impaired AVP degradation (Smith *et al.*, 1977). The reports on the mechanism of action of CBZ are contradictory and unclear (Meinders *et al.*, 1974; Wales, 1975).

Our results have failed to show any enhancing effect, direct or AVP-mediated, upon hydrosmotic movement of water across toad isolated hemibladder. It is not clear from our investigations why CBZ should inhibit the effect of ADH as observed between the CBZ doses 10 μM to 75 μM and why 3 toads failed to respond to CBZ.

CBZ might be exerting its influence indirectly on renal tubular performance in man by acting at sites other than AVP receptors, such as on the adrenal gland to alter circulating aldosterone levels or the release of adrenocorticotrophic hormone (ACTH). There is a possibility that a metabolite of CBZ, such as 10, 11 epoxy-carbamazepine (CBZ-E) or 10, 11 dihydroxy-carbamazepine could possess antidiuretic activity or sensitize renal tubules to AVP.

The water flow rates observed in Figure 1 in response to 50 nM AVP were considerably higher than those observed in later experiments involving the three pharmacological agents. The explanation for this is likely to be due in part to the fact that different batches of toads were used during the course of the overall study; moreover, the dose-response curve for AVP was obtained at a different time of the year (May-June) from the testing of the three pharmacological agents (August-December). Bentley (1966) also reported variations in the toad bladder membrane performance at different times of the year. Nevertheless, the use of matched hemibladders permitted controlled observations at every dose.

The experiments described in this paper demonstrate that toad bladder is a suitable biological preparation for the study of antidiuretic hormone and other pharmacological agents that affect water transport *in vitro*. The technical simplicity, high sensitivity to antidiuretic hormone and the reproducibility of results enhance the value of the hemibladder preparation in studies of agents that are thought to influence water transport.

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Alkaline phosphatase prevents platelet stimulation by thromboxane-mimetics

[1]*Mohamed Hatmi, †Bernard Haye, **J. Michel Gavaret, *B. Boris Vargaftig & **Claude Jacquemin

*Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur/INSERM No. 285; 25 rue du Dr. Roux 75015 Paris; †Laboratoire de Biochimie, Université de Reims, 51062 Reims Cedex and **Unité INSERM No. 96; Hôpital Kremlin Bicêtre, 78 rue du G. Leclerc, 94275 Le Kremlin Bicêtre, France

1 The effects of alkaline phosphatase on platelet aggregation, secretion and thromboxane B_2 (TxB_2) generation induced by the full dose-range of common platelet agonists were studied in human platelet-rich plasma and washed platelets.

2 Platelet aggregation and adenosine 5'-triphosphate (ATP) secretion induced by threshold and supramaximal concentrations of arachidonate and stable TxA_2 and prostaglandin endoperoxide-mimetics (compounds U46619 and EP171) were abolished in the presence of alkaline phosphatase (0.5–1 u ml^{-1}), even though the synthesis of TxB_2 persisted. In contrast, platelet aggregation by PAF-acether and by supramaximal concentrations of thrombin as well as the primary wave of aggregation by adenosine diphosphate (ADP) and adrenaline were unaffected by alkaline phosphatase under conditions where the secondary wave of aggregation by ADP was blocked.

3 Alkaline phosphatase, unlike prostacyclin, failed to raise the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of the platelets. Also, the pretreatment of platelets by inorganic phosphate or by ATP plus creatine phosphate/creatine phosphokinase reversed the inhibitory effect of alkaline phosphatase.

4 Experiments performed in the guinea-pig *in vivo* showed that alkaline phosphatase was effective on thrombocytopenia induced by arachidonate.

5 Our results provide the first direct evidence for a specific inhibitory effect of alkaline phosphatase at a site sensitive to TxA_2 and prostaglandin endoperoxides and suggest that its phosphorylation/dephosphorylation state may play an important role in modulating platelet activation. These results also suggest the presence of ecto-protein kinases on membrane platelets.

Keywords: Alkaline phosphatase; platelet activation; arachidonate; thromboxane A_2; prostaglandin endoperoxide-mimetics; ecto-protein kinases

Introduction

Platelets change shape, aggregate and release their granule contents in response to a wide variety of activating agents (Holmsen, 1965; Caen & Michel, 1972; Kinlough-Rathbone *et al.*, 1977), but the mechanisms involved in these reactions are not completely understood. Platelet activation, by many external stimuli, is associated with phosphorylation and dephosphorylation reactions (Lyons *et al.*, 1975; Haslam & Lynham, 1977; Haslam *et al.*, 1979; Daniel *et al.*, 1981; Imaoka *et al.*, 1983; Schafer *et al.*, 1986; Touqui *et al.*, 1986). This has led us to study the possibility that the dephosphorylating enzyme alkaline phosphatase, by removing phosphate groups from the external platelet membrane, might modulate the platelet function.

In this paper, alkaline phosphatase was used to induce the dephosphorylation of the external surface of the platelets which may be related to its activation. Our data show that alkaline phosphatase prevents platelet aggregation and secretion by thromboxane-mimetics and suggest the dephosphorylation of ecto-proteins.

Methods

Preparation of human platelets

Blood obtained from healthy human volunteers, who had not received any medication for at least 10 days, was mixed with citrate (0.38%, final concentration) to prevent coagulation. Platelet-rich plasma (PRP) was obtained by standard differential centrifugation (200 *g*, 20 min). In order to wash platelets, blood was anticoagulated with 1/6th of its final volume of citric acid-citrate-dextrose (7, 93 and 139 mM, respectively, pH 6.4) containing heparin (20 u ml^{-1}). PRP prepared as above was centrifuged (1700 *g*, 15 min, 37°C) and the platelet pellet was resuspended and washed twice with a modified Tyrode buffer containing bovine serum albumin (0.35%), according to Mustard *et al.* (1972), in which apyrase was omitted and prostacyclin (PGI_2, 1 nM) was added for the first two steps of washing. The platelets were resuspended in the same buffer, their number was determined with a Coulter Counter ZBI and adjusted to a final concentration of 5×10^8 cells ml^{-1}.

Platelet stimulation

Platelet aggregation was measured turbidimetrically (Born, 1962) at 37°C in an Icare aggregometer (Marseille, France) on 0.4 ml samples of PRP or washed platelets, stirred at 1100 r.p.m. Platelets were challenged with different agonists alone or in the presence of alkaline phosphatase. When washed platelets were used (except for thrombin), fibrinogen was added at a final concentration of 0.28 mg ml^{-1}. ATP secretion was determined at 3 min, after adding the stimulating agent, by the bioluminescence technique (luciferine-luciferase) (Holmsen *et al.*, 1972) using a Pico-ATP device from Jobin Yvon (Paris, France). At 5 min, when arachidonate was used, 100 μl samples were collected for the further radioimmunoassay of thromboxane B_2 (TxB_2) bioformation.

Radioimmunoassay of thromboxane B_2

TxB_2 was determined according to Sors *et al.* (1978). Briefly, platelet samples containing TxB_2 were diluted in radioimmunoassay phosphate-gelatin buffer, together with [^{125}I]-TxB_2

[1] Author for correspondence.

and TxB_2-antiserum. After overnight incubation at 4°C, bound fractions were separated by precipitation with polyethylene glycol 6000 and after centrifugation the pellet radioactivity was counted.

Measurement of adenosine 3':5'-cyclic monophosphate (cyclic AMP)

The samples were treated with perchloric acid 1 N and, following appropriate dilution in 0.1 M sodium citrate buffer at pH 6.2, acetylated and assayed for cyclic AMP by radioimmunoassay as described by Cailla *et al.* (1973). The total amount of cyclic AMP was expressed in pmol mg^{-1} of protein measured by method of Lowry *et al.* (1951).

Evaluation of thrombocytopenia and bronchoconstriction in guinea-pigs in vivo

Thrombocytopenia and bronchoconstriction in anaesthetized guinea-pigs were studied as described by Lefort & Vargaftig (1978). 5-Hydroxytryptamine (5-HT, 3 μg/animal) was used as a standard bronchoconstrictor agent. Arachidonate (750 $\mu g\,kg^{-1}$) or PAF (33 $ng\,kg^{-1}$) was given i.v. 60 min after the injection of alkaline phosphatase (AP, 7 $u\,ml^{-1}$) or its solvent (Tris). When arachidonate was studied, aspirin (acetylsalicylic acid, 20 $mg\,kg^{-1}$) was used instead of alkaline phosphatase. The bronchopulmonary resistance to inflation (bronchoconstriction) following the administration of the agonists was expressed in cmH_2O, as its peak value.

Statistical analysis

Statistical significance was analysed by Student's t test for unpaired data. P values of less than 0.05 were considered to be significant. Results are expressed as means ± s.e.mean.

Materials

The stable endoperoxide/thromboxane A_2 (TxA_2) analogues U46619 and EP171 were a gift from Upjohn Co (Kalamazoo, U.S.A.) and from Prof. R.L. Jones, Department of Pharmacology (University of Edinburgh Medical School, Great Britain), respectively. Arachidonic acid (AA), adenosine diphosphate (ADP), adrenaline, prostacyclin (PGI_2), cyclic AMP, kidney bovine alakaline phosphatase, bovine serum albumin (Fraction V), bovine thrombin, creatine phosphate (CP) and creatine phosphokinase (CPK) were purchased from Sigma. Heparin was from Laboratories Choay (Paris, France). PAF (1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was from Bachem (Bubendorf, Switzerland) and collagen from Hormon Chemie (Münich, Federal Republic of Germany), Fibrinogen (Grade L) from Kabi (Stockholm, Sweden) was treated with diisopropyl fluorophosphate (DFP) by standard procedures to inactivate coagulant contaminants. Stock solutions of arachidonate, U46619 and EP171 in ethanol were stored at −20°C, dilutions being freshly prepared before each experiment. PGI_2 was dissolved in 0.05 M Tris buffer, pH 8.5. Thrombin was solubilized in Michaelis Owren-Koller buffer (30 mM sodium veronal, 44 mM sodium acetate, 108 mM NaCl; pH was adjusted to 7.3 with HCl 0.1 N).

Results

When plasma-free washed human platelets were incubated with alkaline phosphatase (0.1–1 $u\,ml^{-1}$) as indicated in the legend of Figure 1a and b, both ATP secretion and aggregation triggered by the natural (arachidonate-derived) and by the synthetic prostaglandin endoperoxide and thromboxane analogues U46619 (1 μM) and EP171 (0.01 μM) were blocked (Figure 2). As shown in Figure 1a and b, inhibition of aggregation induced by arachidonate and by compound EP171 was concentration-dependent; these figures also show that denatured (heated) alkaline phosphatase loses its ability to

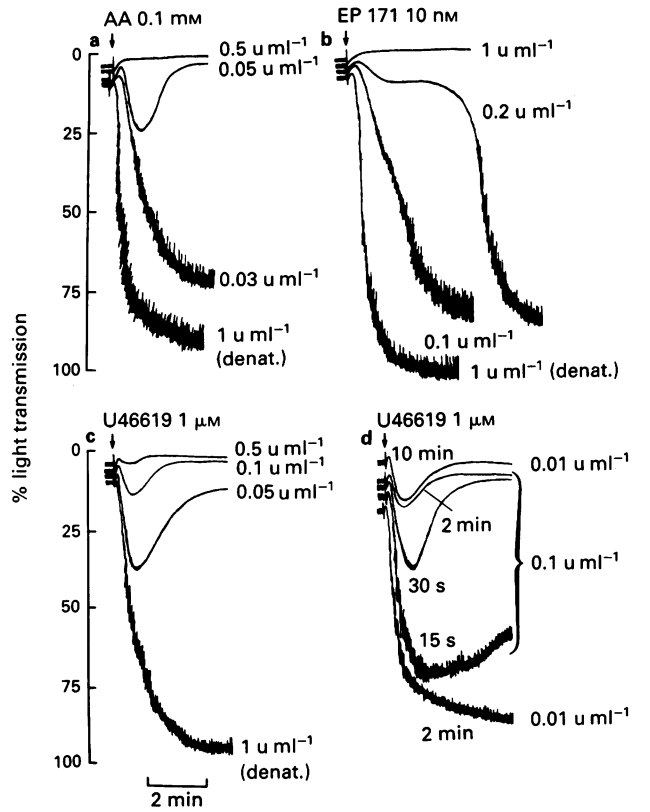

Figure 1 Representative tracings of platelet aggregation by arachidonate (AA) and by the thromboxane and endoperoxide analogues (U46619 and EP171). Inhibition by alkaline phosphatase (AP) of AA (a), EP171 (b) and U46619 (c and d) -induced platelet aggregation. Heating of AP at 100°C for 2 min removed antagonistic activity (denat.), the curves were not different from control. A time course experiment is shown in (d). At a concentration of 0.01 $u\,ml^{-1}$, AP failed to block aggregation by U46619 when incubated with the platelets for 2 min, but became effective when incubation was prolonged to 10 min. The concentration of 0.1 $u\,ml^{-1}$ of AP was already effective when incubated with the platelets for 2 min, and showed a reduced activity when the duration of incubation was shorter (30 or 15 s). Generation of thromboxane B_2 (TxB_2), the stable degradation product of TxA_2, was assayed as described in Methods. Platelets pre-exposed to 0.5 $u\,ml^{-1}$ AP and then stimulated with 0.1 mM AA generated amounts of TxB_2 (439.7 ± 88.2 $ng\,ml^{-1}$) not significantly different from control platelets (495.4 ± 95.2), (means ± s.e.mean, $n = 4$).

interfere with platelet function. Inhibition of aggregation by compound U46619 was concentration- and time-dependent when alkaline phosphatase was used at the sub-maximal concentration of 0.1 $u\,ml^{-1}$ for 15 s–2 min (Figure 1c and d). Alkaline phosphatase also blocked aggregation by arachidonate, U46619 and EP171 when tested on platelet-rich plasma (PRP; data not shown).

Aggregation of human platelets by ADP and adrenaline occurs in the form of a double wave. The secondary wave of aggregation and the accompanying release reaction are inhibited by aspirin-like drugs (O'Brien, 1968; Mills *et al.*, 1974) and accordingly, they are attributed to the formation of pro-aggregatory metabolites of arachidonate, TxA_2 and prostaglandin endoperoxides (Holmsen, 1977; Lages & Weiss, 1980; and Meyers *et al.*, 1979). The primary wave of aggregation by ADP persisted in the presence of alkaline phosphatase, but the secondary wave and the accompanying ATP secretion were abolished. Thus, in three experiments, maximal aggregation by 10 μM ADP was 78% of maximal in the absence, and only 30% of maximal in the presence of alkaline phosphatase. In contrast, aggregation triggered by adrenaline (0.1 μM) and by the phospholipid PAF (1 μM) was not modified by alkaline phosphatase (Figure 3). Effectiveness of alkaline phosphatase against arachidonate as well as its stable mimetics U46619 and EP171 suggested that alkaline phosphatase acts

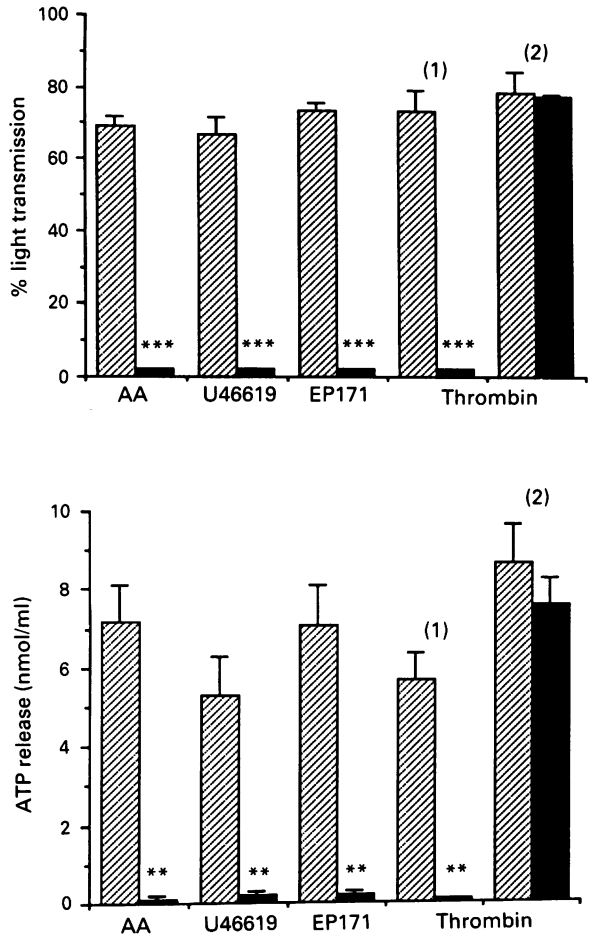

Figure 2 Aggregation and release of ATP after platelet stimulation by arachidonate (AA, 100 μM), U46619 (1 μM), EP171 (0.01 μM) or thrombin ((1) 0.1 u ml^{-1}, (2) 1 u ml^{-1}) in absence (hatched columns) or presence (stippled columns) of alkaline phosphatase (AP, 0.5–1 u ml^{-1}) for 2 min. Platelet aggregation was determined as in % light transmission across the platelet suspension described in Methods. Release of ATP was monitored with the luciferin-luciferase technique and expressed in nmol ml^{-1}. Results are the mean (s.e.mean shown by vertical bars) of three separate experiments. Significantly different (**$P < 0.01$; ***$P < 0.001$) when compared to control platelets.

as an antagonist rather than as a cyclo-oxygenase inhibitor and indeed, alkaline phosphatase failed to interfere with the formation of TxA_2 from arachidonate (see legend of Figure 1).

Platelets exposed to alkaline phosphatase and then washed and resuspended behaved as controls, demonstrating that its effects are reversible. Platelet aggregation and ATP secretion induced by low concentrations of thrombin (0.1 u ml^{-1}) were inhibited by their preexposure to alkaline phosphatase, whereas responses to high concentrations of thrombin (1 u ml^{-1}) were unaffected (Figure 2). Similar results were obtained with collagen (data not shown). Finally, as shown in

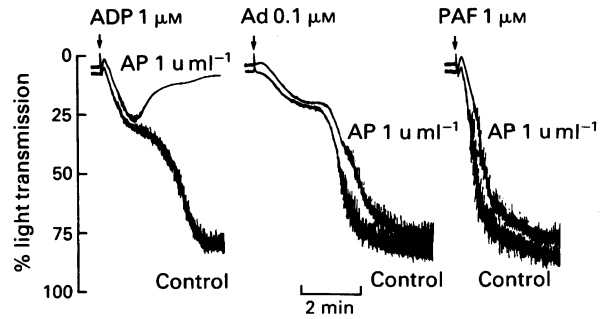

Figure 3 Representative tracings of platelet aggregation in platelet-rich plasma induced by ADP (1 μM), adrenaline (Ad; 0.1 μM) or PAF (1 μM) and inhibition by alkaline phosphatase (AP; 1 u ml^{-1}) added 2 min before the addition of each agonist. In control platelets, aggregation was performed in the presence of alkaline phosphatase solvent (Tris).

Table 1, alkaline phosphatase, unlike prostacyclin, did not raise basal cyclic AMP levels. Exposure of platelets to ATP (a substrate for phosphorylation reaction) and to the enzyme system creatine phosphate (CP)/creatine phosphokinase (CPK) that is used to convert released ADP to ATP (Kinlough-Rathbone *et al.*, 1977) abolished the alkaline phosphatase inhibiting activity on U46619-induced platelet aggregation. Comparable results were obtained when inorganic phosphate, an inhibitor of alkaline phosphatase activity, was used (Figure 4).

In the *in vivo* experiments, thrombocytopenia induced by the administration of arachidonate to the guinea-pigs was suppressed for more than 2 h after the i.v. administration of alkaline phosphatase (7 u ml^{-1} of blood; the volume of blood was estimated to 1/10th of body weight). In contrast, this enzyme did not interfere with bronchoconstriction (22 $\pm$ 3 cmH_2O for alkaline phosphatase-pretreated animals versus 23 $\pm$ 3 cmH_2O for control animals; $n = 3$ for each group) and thrombocytopenia (8 $\pm$ 4% reduction of counts for pretreated animals versus 48 $\pm$ 4 for control animals; $n = 3$ for each group) due to the i.v. administration of arachidonate. As expected, aspirin at 20 mg kg^{-1}, i.v. abolished both bronchoconstriction and thrombocytopenia induced by arachidonate. Alkaline phosphatase also failed to interfere with bronchoconstriction (25 $\pm$ 2 cmH_2O for pretreated animals versus 24 $\pm$ 4 cmH_2O for control animals; $n = 3$ for each group) and thrombocytopenia (74 $\pm$ 6% reduction of counts for pretreated animals versus 81 $\pm$ 5 for control animals; $n = 3$ for each group) due to PAF (Figure 5).

Discussion

Alkaline phosphatase behaves as a selective antagonist of platelet activation by the pro-aggregatory metabolites of arachidonate, by its mimetics and releasing agents (low thrombin and collagen). The enzymatic activity of alkaline phosphatase was required for its effectiveness, since denatured enzyme (which loses the ability to dephosphorylate *p*-nitro-

Table 1 Effects of alkaline phosphatase (AP) and prostacyclin (PGI_2) on the formation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) by platelets

	Cyclic AMP (pmol mg^{-1} protein)		
	Solvent	*AP*	*PGI_2*
Saline	21.4 $\pm$ 4.7	27.5 $\pm$ 6.7	388.2 $\pm$ 39.3***
U46619	23.4 $\pm$ 5.8	26.8 $\pm$ 7.7	348.8 $\pm$ 40.6***

Aliquots of suspended platelets were stirred at 37°C and incubated with PGI_2 (1 μM), AP (1 u ml^{-1}) or the solvent (Tris) alone or in the presence of U46619 (1 μM). The reaction was stopped after 2 min by perchloric acid 1 N and the cyclic AMP content was measured as previously described.

The results are the mean $\pm$ s.e.mean of four separate experiments. Significantly different (***$P < 0.001$) when compared to solvent-treated platelets (control).

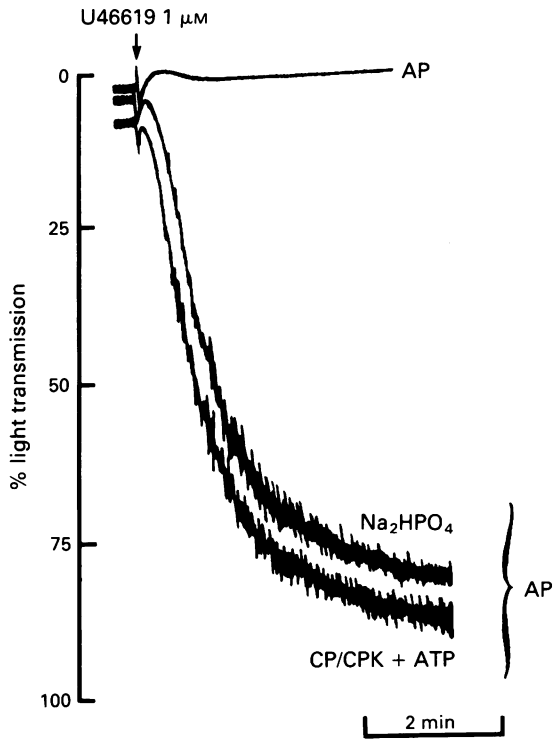

Figure 4 Representative tracings of platelet aggregation by the endoperoxide analogue U46619. Reversal by inorganic phosphate (Na_2HPO_4, 1 mM) and by ATP (0.01 mM) plus creatine phosphate (CP)/creatine phosphokinase (CPK) (0.7 mM and 14 u ml^{-1}, respectively) of the inhibitory effect of alkaline phosphatase. Washed platelets were incubated with alkaline phosphatase alone and in the presence of inorganic phosphate or ATP plus CP/CPK for 2 min before the addition of U46619 (1 μM).

phenylphosphate) failed to modify platelet responsiveness. In agreement with the ability of alkaline phosphatase to suppress the effects of arachidonate, activation of plasma-free platelets by low concentrations of thrombin (0.1 u ml^{-1}) which is accounted for by cyclo-oxygenase metabolites (Packham *et al.*, 1973; Vargaftig, 1977; Lapetina *et al.*, 1978), was blocked by alkaline phosphatase, whereas activation by higher concentrations, known to be cyclo-oxygenase-independent (Packham

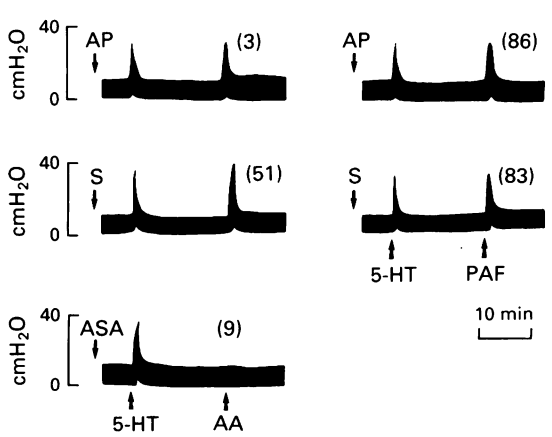

Figure 5 Inhibition by alkaline phosphatase (AP) of thrombocytopenia, but not of bronchoconstriction, due to the administration of arachidonate (AA) to the guinea-pig. Typical tracings of bronchoconstriction (upwards pen deflection, in cmH_2O) and thrombocytopenia (in parentheses, % reduction of number of free platelet counts in blood, 1 min after AA or PAF administration) representative of three separate experiments each. For these tracings, five pentobarbitone-anaesthetized guinea-pigs were challenged with 5-hydroxytryptamine (5-HT; i.v. at 3 μg/animal) as a standard bronchoconstrictor agent. AA (750 μg kg^{-1}, left panel) or PAF (33 ng kg^{-1}, right panel) were given i.v. 60 min after the injection of AP (7 u ml^{-1}), its solvent (Tris) or aspirin (ASA, 20 mg kg^{-1}, only when AA was used).

et al., 1973; Vargaftig, 1977; Lapetina *et al.*, 1978), persisted (Figure 3). Comparable results were obtained with collagen, since reduced concentrations (0.5–2 μg ml^{-1}), but not higher concentration (5 μg ml^{-1}), were also inhibited by alkaline phosphatase (data not shown).

Overall, the antagonism by alkaline phosphatase of platelet activation by some, but not all, stimulating agents and the ineffectiveness of the heated enzyme rules out a non-specific mode of action for alkaline phosphatase, such as a detergent effect. In agreement, alkaline phosphatase also failed to deaggregate platelets, when added 2 min or later after the stimulating agents, under conditions where prostacyclin and bivalent ion chelation with EDTA are effective. Finally, unlike prostacyclin, alkaline phosphatase did not raise basal platelet cyclic AMP levels (Table 1), ruling out the possibility that inhibition involves the second messenger system.

As noticed above, platelets exposed to alkaline phosphatase and then washed and resuspended behaved as controls, demonstrating that its effects are reversible. This finding also argues against the possibility that alkaline phosphatase was contaminated with proteases which could cleave irreversibly either the TxA_2 receptor or other membrane proteins to inhibit platelet aggregation. Reversibility also supports the concept that the sites of interaction of the platelet with the arachidonate metabolites and its mimetics involve the external surface of the cell. This antagonism to the effects of TxA_2 should explain the interference of alkaline phosphatase with the second wave of ADP-induced aggregation, which is suppressed by thromboxane agonists and cyclo-oxygenase inhibitors (O'Brien, 1968; Mills *et al.*, 1974; Armstrong *et al.*, 1985). Those inhibitors are also effective against the second wave of adrenaline-induced aggregation (O'Brien, 1968; Armstrong *et al.*, 1985), which was surprisingly not modified by alkaline phosphatase. This unexpected dissociation between ADP and adrenaline is probably accounted for by different locations of their receptor site(s) within the membrane and/or by divergent transduction mechanisms underlying platelet activation even though in both cases cyclo-oxygenase-dependent metabolites of arachidonate are involved. It is likely that these sites participate with the second wave of aggregation triggered by ADP, since they are alkaline phosphatase-sensitive, whereas equivalent (also cyclo-oxygenase-dependent) sites for adrenaline, were less accessible and are thus probably located more internally within the cell.

The reversibility of the inhibitory effect of alkaline phosphatase as well as its reversion by ATP + CP/CPK or by Na_2HPO_4, support the hypothesis that strategic phosphate groups were accessible from the outside to protein-kinase and phosphatase activities, respectively. Besides, in the *in vivo* experiments in guinea-pigs, bronchoconstriction following the injection of arachidonate, unlike thrombocytopenia, was not suppressed by alkaline phosphatase. The protective effect of alkaline phosphatase seems specific since bronchoconstriction and thrombocytopenia following the i.v. injection of PAF were not modified by this enzyme. As expected, aspirin which suppresses the formation of bioactive metabolites of arachidonate via cyclo-oxygenase, inhibited altogether bronchoconstriction and thrombocytopenia induced by arachidonate.

Our results indicate that alkaline phosphatase interferes selectively *in vitro* and *in vivo* with platelet activation, probably by modifying the phosphorylation/dephosphorylation state of the recognition site for the prostaglandin endoperoxides/TxA_2, but we cannot rule out a direct interaction with this site, by a competitive mechanism. Both possibilities are in fact not in contradiction, since the enzymatic activity requires prior enzyme fixation to the site of action. In addition, our results suggest that ecto-protein kinases exist on platelets.

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Dissociation between the effects of zymosan on the systemic and pulmonary vessels of the rat

[1]Jacques Damas & Denise Lagneaux

Physiologie humaine, Université de Liège, 17 Place Delcour, B-4020 Liège, Belgium

1 Zymosan, an activator of the alternative complement pathway, (2 to 16 mg kg^{-1}) injected intravenously via the tail vein of anaesthetized rats, dose-dependently increased the vascular permeability of lung parenchyma, as measured by the accumulation of ^{125}I-labelled albumin in lungs.

2 Pretreatment of the animals with cyclo-oxygenase inhibitors, indomethacin or ketoprofen (3 mg kg^{-1}) or with the lipoxygenase and cyclo-oxygenase inhibitor, BW755C (40 mg kg^{-1}) abolished the vascular permeability changes induced by zymosan (16 mg kg^{-1}). Neither, the PAF antagonist, WEB 2086 (10 mg kg^{-1}) nor the antagonists of mast cell amines, mepyramine and methysergide (3 mg kg^{-1}) affected the plasma exudation in lungs. Zymosan did not induce any accumulation of labelled albumin in lungs of rats made leucopenic by rabbit anti-neutrophil serum.

3 Zymosan (16 mg kg^{-1}) increased the haematocrit. This increase was not modified by indomethacin but reduced by WEB 2086.

3 Intravenous injection of zymosan (3 and 8 mg kg^{-1}) in anaesthetized rats transiently increased right ventricular blood pressure and pulmonary arterial pressure, accelerated respiratory rate and decreased systemic blood pressure.

5 WEB 2086 largely reduced the systemic hypotension but did not affect the increase of pulmonary vascular resistance. Indomethacin inhibited the increase of blood pressure in the right ventricle and the modification of the respiratory rate. This drug did not inhibit but increased the systemic hypotension induced by zymosan.

6 Zymosan (16 mg kg^{-1}) reduced serum complement haemolytic activity by 46%.

7 These data suggest that the pulmonary vascular changes induced by intravascular complement activation with zymosan in rats are mediated by neutrophils and prostanoids while the systemic vascular effects depend mainly on PAF.

Keywords: Zymosan; leukocytes; PAF; prostanoids; rat systemic and pulmonary vessels

Introduction

Intravascular activation of complement induces thrombocytopenia, intrapulmonary sequestration of neutrophils, lung injury and systemic hypotension in several animal species (Hammerschmidt, 1983; Hugli *et al.*, 1987). In rats, intravenous injection of zymosan, an activator of the alternative complement pathway (Gewurz & Lint, 1977), induces neutropenia, thrombocytopenia, decrease of the mean systemic blood pressure, peripheral plasma extravasation with paw oedema (Damas, 1991). These vascular effects of zymosan in rats are largely reduced by a platelet activating factor (PAF) antagonist and abolished by pretreatment of the animals with a combination of drugs consisting of antagonists for PAF, mass cell amines and a lipoxygenase inhibitor (Damas, 1991). These results suggest that PAF is the main factor responsible for the systemic effects of zymosan but in addition, that mast cell amines and lipoxygenase products are also involved in the rat.

On the other hand, lung injury is observed after activation of complement with cobra venom factor in rats (Till *et al.*, 1982; Ward *et al.*, 1985). Therefore, we examined the pulmonary vascular effects of zymosan in rats. These effects were estimated by measuring the accumulation of ^{125}I-labelled albumin in pulmonary parenchyma and by recording haemodynamic changes.

Methods

Experiments were performed on Wistar rats of either sex. They were divided in two groups. The animals of the first group (body weight 230 g) were used for studies of plasma exudation. Some of these animals were depleted of circulating blood leukocytes by the intraperitoneal injection of 1 ml serum obtained from rabbits immunized with rat neutrophils according to Till *et al.* (1982). These depleted rats were used for the experiments 12 h later. At this time, the blood neutrophil content was <500 neutrophils ml^{-1} blood as determined by total and differential blood cell counts. Simultaneously, total cell count was reduced by $32 \pm 3.2\%$ ($n = 6$). Platelet count was not modified. The animals were treated intraperitoneally with saline or with a test drug. Thirty to sixty min later, anaesthesia was induced by intraperitoneal sodium pentobarbitone (35 mg kg^{-1}). ^{125}I-labelled bovine serum albumin (New England Nuclear) mixed with saline was injected intravenously into the tail vein either simultaneously or at different intervals after zymosan. Fifteen min later, a blood sample was withdrawn by cardiac puncture. The animals were exsanguinated with aortic trans-section and lungs perfused through the right ventricle with 10 ml of saline to remove blood remaining in the pulmonary vascular system. Then, the lungs were withdrawn and separated from the trachea, thymus and mediastinal tissues. The lungs were weighed. Blood samples were centrifuged at 1500 *g* for 10 min and plasma was separated. The radioactivity of plasma samples or lungs was counted with a gamma spectrometer (Packard Instruments Co.) and the results were calculated as μl of extravasated plasma into the lungs during the 15 min delay. The haematocrit of the blood samples was measured with capillary tubes centrifuged in a Haemofuge (Heraeus).

A second group of rats (body weight 340 g) was used to record cardiovascular and respiratory changes induced by zymosan. Some animals received no pretreatment (saline-treated rats), others were pretreated with WEB 2086 or indomethacin. They were anaesthetized with pentobarbitone (40 mg kg^{-1}, i.p.), placed in a supine position and tracheotomized. A catheter was inserted through a femoral vein into

[1] Author for correspondence.

the inferior vena cava for drug administration. After heparinization (10 mg kg^{-1}), a second catheter was introduced into a femoral artery and connected to an electromanometric transducer (Elema) to record blood pressure. Similarly the pulmonary arterial pressure (PAP) or the right ventricular pressure (RVP) was obtained by the floating catheter technique. A Silastic tube (o.d. 0.9, i.d. 0.5 mm) with a lateral opening was inserted in the right jugular vein and pushed under manometric control into the pulmonary artery or remained in the right ventricle when the catheterization provoked important disturbances in heart rate. The position of the catheter was checked at the end of the experiments. A home-made Fleisch pneumotachograph (PTG) was inserted at the end of the tracheal cannula and connected to a differential pressure transducer (Validyne DP 45) with a CD 101 demodulator and an analog integrator (Pneumometer Elema) in order to obtain respiratory flow rate and tidal volume (Vt). The outputs of the manometers were fed into an ink-jet multichannel recorder (Mingograph EEG) for continuous monitoring (5 mm s^{-1}) or calculations (50 mm s^{-1}). All data were means from 10 breaths, blood pressures were measured at the end-expiratory point, heart rate was the mean value of 20 beats. After a stabilization period of about 10 min, control values were recorded before and after (1, 3, 5, 10, 15 and 30 min) three successive injections of zymosan (3, 3 and 8 mg kg^{-1}) performed at 30 min interval.

Finally, serum complement activity was determined in some animals by a haemolytic assay. Washed sheep erythrocytes (Biomérieux, Belgium) were diluted to 20% in complement fixation buffer (Pasteur, Belgium) and sensitized by incubation with 1/100 sheep haemolysin (Biomérieux) for 10 min at 37°C. The sensitized erythrocytes were further diluted to 4% and incubated with an equal volume of diluted rat serum for 30 min at 37°C. The reaction was stopped by centrifugation and the degree of haemolysis was measured spectrophotometrically (541 nm).

The pharmacological agents used were as follows: mepyramine maleate and ketoprofen from Specia (Brussels, Belgium), BW755C (3-amino-1-(*m*-(trifluoromethyl)-phenyl)-2-pyrazoline) from Wellcome (Beckenham, England), methysergide bimaleate from Sandoz (Brussels, Belgium) and indomethacin from Merck Sharp and Dohme (Brussels, Belgium). Zymosan was purchased from Sigma (St Louis, MI, U.S.A.), heparin from Leo (Therabel, Belgium). WEB 2086 (3-(4-2-chlorophenyl) - 9 - methyl - 6H - thieno(3,2 - f)(1,2,4)triazolo - (4,3 - a) - diazepin-2-yl)-1-(4-morpholinyl)-1-propanon) was a kind gift of Boehringer Ingelheim KG (Germany). Indomethacin was dissolved in Tris HCl (0.15 M, pH 7.4). All the other drugs were dissolved in saline, with the aid of HCl 0.1 N for WEB 2086. Zymosan was boiled, washed and suspended in saline before use. Indomethacin, ketoprofen and BW755C were given intraperitonealy 30 to 60 min before zymosan.

Results are expressed as the mean ± s.e. mean. Student's paired *t* test was used to determine statistically significant differences in the same group. Comparison between groups was made by Student's *t* test for unpaired values and confirmed by analysis of variance. A *P* value less than 0.05 was regarded as significant.

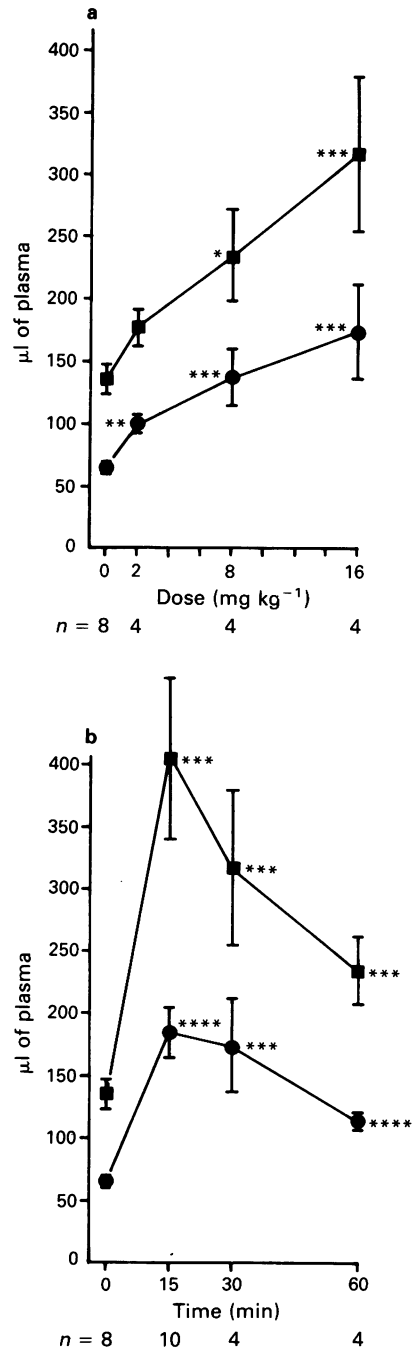

Figure 1 Exudation of ^{125}I-labelled albumin in lungs after intravenous injection of zymosan, as a function of dose (a) and as a function of time after 16 mg kg^{-1} (b). Exudation was expressed as µl of plasma per g of tissue (●) or as µl of plasma per lungs of one animal (■). Values measured in saline-treated rats are control values: 0 mg kg^{-1} and time 0. *n* = number of animals. Vertical bars indicate s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

Results

Vascular permeability

The effects of zymosan on the vascular permeability in lungs were examined by measuring the accumulation of ^{125}I-labelled albumin in the pulmonary parenchyma. Labelled albumin was injected 15 min after the intravenous administration of increasing doses (2 to 16 mg kg^{-1}) of zymosan and the animals were killed 15 min later. A group of rats received saline instead of zymosan and were used as controls. As shown in Figure 1, zymosan dose-dependently increased the extravasation of albumin. In other animals, labelled albumin was injected at various times after zymosan (16 mg kg^{-1}) and the rats were killed 15 min later. The increase of vascular permeability induced by zymosan was observed over the 1 h time period after its administration. Maximal effects were obtained during the first 15 min: at this time, the extravasation of albumin was increased by 301% (Figure 1).

The accumulation of ^{125}I-labelled albumin in lung parenchyma during the first 15 min after zymosan injection (16 mg kg^{-1}) was abolished by indomethacin (3 mg kg^{-1}) or ketoprofen (3 mg kg^{-1}), two cyclo-oxygenase inhibitors (Shen, 1979) and by BW755C (40 mg kg^{-1}), the dual inhibitor of cyclo-oxygenases and lipoxygenases (Higgs *et al.*, 1979).

However, WEB 2086 (10 mg kg^{-1}), a PAF antagonist (Cazals-Stenzels *et al.*, 1987), and the antagonists of mast cell amines, methysergide and mepyramine (3 mg kg^{-1}), did not modify the effects of zymosan in the lungs. The increase of permeability induced by zymosan was not observed in rats made leucopenic with a rabbit anti-neutrophil serum (Figure 2).

We estimated the effects of zymosan on the permeability of the systemic vessels by measurement of changes in the haematocrit. The haematocrit of blood samples withdrawn 15 min after saline injection was: 35.8 ± 2.4% ($n = 6$). This value was increased by zymosan (16 mg kg^{-1}): 54.8 ± 0.8% ($n = 6$, $P < 0.001$), indicating a large plasma extravasation. This increase was inhibited by WEB 2086 (10 mg kg^{-1}): 43.3 ± 2.2% ($n = 6$, $P < 0.001$) but was not modified by indomethacin (3 mg kg^{-1}): 57.0 ± 1.5% ($n = 6$).

Haemodynamic effects of zymosan

In the second series of experiments, cardiovascular and respiratory changes induced by zymosan were recorded. One min after the intravenous administration of zymosan (3 mg kg^{-1}), a rise of right ventricular systolic or of arterial pulmonary blood pressures was noted. Simultaneously, breathing rate was accelerated but with a reduced tidal volume so that no change of total ventilation was found. All these parameters returned to control levels before 3 min while systemic arterial blood pressure decreased to a minimum value (51% of control level) 3 to 5 min after the injection.

More pronounced changes of respiratory rate and of pulmonary arterial pressure were observed one min after 8 mg kg^{-1} of zymosan (Figure 3). Then, systemic blood pressure decreased to 33 and 37% of systolic and diastolic control levels respectively. Simultaneously, the right ventricular systolic pressure was transiently decreased to 60% of the control value. Right ventricular diastolic pressure did not change significantly after each dose of zymosan.

Figures 4 and 5 summarize the evolution of these responses after 3 and 8 mg kg^{-1} of zymosan and compare these effects with those obtained in rats pretreated with WEB 2086 or with indomethacin. WEB 2086 did not inhibit the increase in the right ventricular pressure nor the modifications of the breathing pattern induced by zymosan. But, WEB 2086 largely reduced the drop of the systemic blood pressure and suppressed the simultaneous decrease of the right ventricular systolic pressure. The rise of right ventricular systolic pressure and tachypnoea were abolished by indomethacin but this inhibitor increased the systemic hypotension and the fall of right ventricular pressure observed after the larger dose of zymosan. These later changes were accompanied by a significant relative bradycardia in indomethacin-treated rats.

Complement depletion

Previously, we observed that 4 and 10 mg kg^{-1} of zymosan induced a partial depletion of serum complement haemolytic activity (Damas, 1991). We measured this activity in some animals treated with 16 mg kg^{-1} in order to estimate the importance of the activation of the complement system. In these animals, complement haemolytic activity was reduced to 10.2 ± 0.6 u CH_{50} ml^{-1} ($n = 8$; $P < 0.001$) or 54% of control values: 19.0 ± 0.9 u CH_{50} ml^{-1} ($n = 6$).

Discussion

After intravenous injection of zymosan in rats, the first modification was a transient increase in pulmonary vascular resistances as measured by pulmonary arterial pressures and systolic right ventricular pressure. This was followed by a fall in systemic blood pressure and an increase in the permeability of pulmonary and systemic vessels. Simultaneously, a neutropenia could be measured (Damas, 1991). All these effects are the result of the activation of the complement alternative

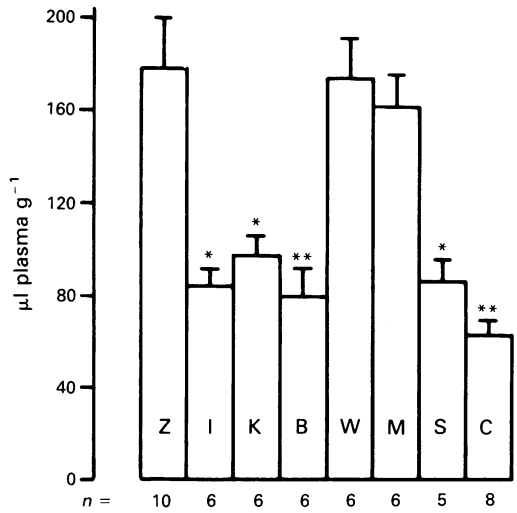

Figure 2 Modification by different drugs of the exudation of ^{125}I-labelled albumin in lungs induced by zymosan (16 mg kg^{-1}). Exudation of albumin was expressed as µl of plasma per g of tissue in rats pretreated with saline (Z), indomethacin (I, 3 mg kg^{-1}), ketoprofen (K, 3 mg kg^{-1}), BW755C (B, 40 mg kg^{-1}), WEB 2086 (W, 10 mg kg^{-1}), methysergide and mepyramine (M, 3 mg kg^{-1}) or anti-neutrophil serum (S). Control rats (C) were not treated with zymosan but with saline. Vertical bars indicate s.e.mean n = number of animals. * $P < 0.02$; ** $P < 0.005$; *** $P < 0.001$.

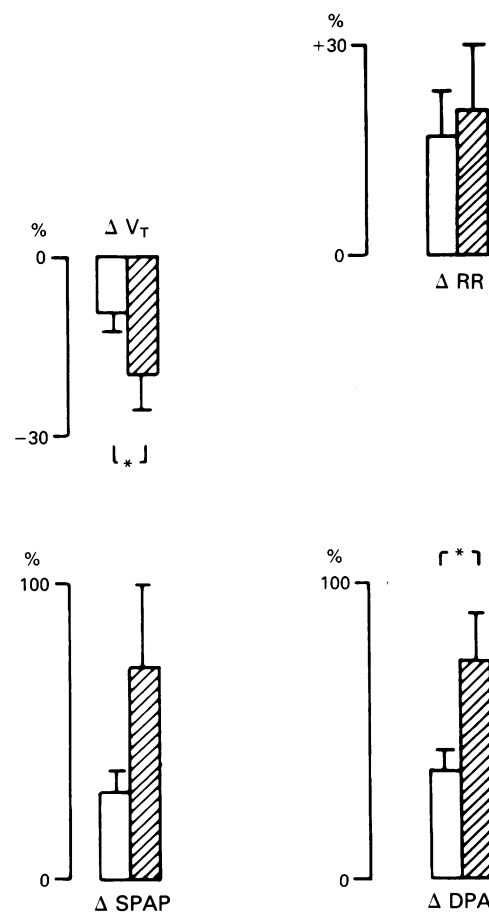

Figure 3 Variations expressed as percentage of control levels of tidal volume (Vt), respiratory rate (RR) and systolic (SPAP) and diastolic pulmonary arterial pressures (DPAP) recorded at the first min after intravenous injection of zymosan 3 mg kg^{-1} (open columns, $n = 4$) or 8 mg kg^{-1} (hatched columns, $n = 4$). Vertical bars indicate s.e.mean. * $P < 0.05$.

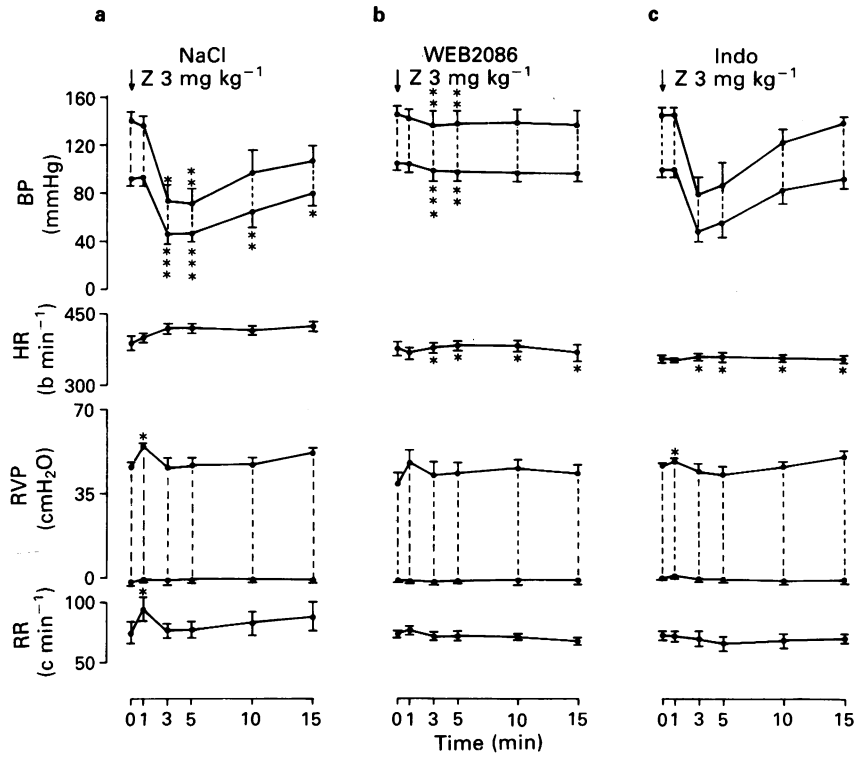

Figure 4 Changes in systemic blood pressure (BP), heart rate (HR), right ventricular pressure (RVP) and respiratory rate (RR) following i.v. injection of zymosan (Z, 3 mg kg^{-1}) in saline-treated rats (a, $n = 8$) or in rats pretreated with WEB 2086 (10 mg kg^{-1}, $n = 6$, b) or with indomethacin (3 mg kg^{-1}, $n = 5$, c). Values are mean with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, in comparison either with the initial values in saline-treated rats (a) or with the corresponding values in saline-treated rats (b and c).

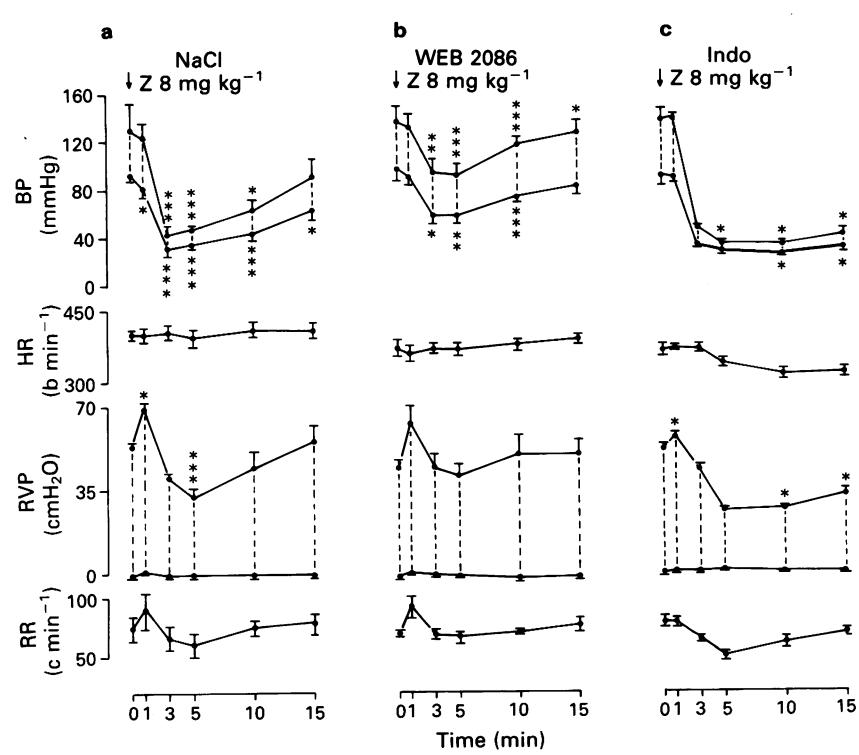

Figure 5 Changes in systemic blood pressure (BP), heart rate (HR), right ventricular pressure (RVP) and respiratory rate (RR) following i.v. injection of zymosan (Z, 8 mg kg^{-1}) in saline-treated rats ($n = 8$, a) or in rats pretreated with WEB 2086 (10 mg kg^{-1}, $n = 6$, b) or with indomethacin (3 mg kg^{-1}, $n = 5$, c). Values are mean with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, in comparison either with the initial values in saline-treated rats (a) or with the corresponding values in saline-treated rats (b and c).

pathway as indicated by the reduction of serum complement haemolytic activity. Moreover, opsonization of zymosan in the vascular tree could be another stimulus for neutrophils which accumulated in the lungs (Till *et al.*, 1982; Kaslovsky *et al.*, 1990).

The vascular changes in lungs, increase in resistances and permeability, were inhibited by the cyclo-oxygenase inhibitors, indomethacin and ketoprofen. Similar results were obtained in sheep (Cooper *et al.*, 1980; McDonald *et al.*, 1983) and formation of prostanoids during intravascular activation of the complement system has been reported in sheep and rabbits (Cooper *et al.*, 1980; Perkowski *et al.*, 1983; Bult *et al.*, 1985). The prostanoids responsible for these changes in rat probably originate from leukocytes as leucopenia suppressed the accumulation of labelled albumin in lungs. Moreover, arachidonic acid is mainly converted to prostacyclin (PGI_2) by cyclo-oxygenase of rat pulmonary parenchyma (Pace-Asciak & Rangaraj, 1977). PGI_2 does not induce any increase in vascular resistance of rat pulmonary circulation (Damas & Troquet, 1978). However, the thromboxane mimic, U-44069 has vasopressor effects in rat pulmonary circulation and, at high doses, induces pulmonary oedema (Damas *et al.*, 1979). Indeed, neutrophils stimulated by opsonized zymosan release thromboxane A_2 and other prostanoids *in vitro* (Weissmann *et al.*, 1979). All arachidonic acid metabolites which are generated by the cyclo-oxygenase pathway increase pulmonary vascular resistance, apart from PGI_2 (Gryglewski, 1987).

In a different model of lung injury, the accumulation of albumin in rat lung parenchyma induced by cobra venom factor, another complement activator, was inhibited by neutrophil depletion, catalase and hydroxyl radical scavengers (Till *et al.*, 1982; Ward *et al.*, 1985; Morganroth *et al.*, 1986). However, this enzyme and these drugs had no effect on the increase in pulmonary vascular resistance (Morganroth *et al.*, 1986) which, in our study, was suppressed by cyclo-oxygenase inhibitors. These results taken together suggest that complement activation stimulates neutrophils which release prostanoids and free radicals. The former agents would produce pulmonary hypertension while the latter would modify vascular permeability, but, acting together, both would trigger plasma exudation in lung parenchyma. Similar conclusions were drawn from study of the mediators responsible for lung injury induced by zymosan-activated plasma in sheep (Perkowski *et al.*, 1983).

On the other hand, systemic hypotension and plasma extravasation were not inhibited by indomethacin. These systemic effects were largely reduced by a PAF antagonist which had no influence on the pulmonary changes induced by zymosan. Thus, in rats, the vascular reactivity of the pulmonary vessels to complement activation contrasted with the sensitivity of the systemic vessels. Recently, Sirois *et al.* (1990) reported that PAF increased vascular permeability in rat trachea and bronchi, as in other systemic vessels, but not in rat lungs. Our results agree with this observation.

Haemodynamic changes induced by zymosan in rats resulted from the release of several mediators, anaphylatoxins, PAF, prostanoids (this work), lipoxygenase products and mast cell amines (Damas, 1991). The systemic hypotension depends mainly on the release of PAF which induces systemic vasodilatation in rats (Feuerstein, 1989). However, after the high dose of zymosan, a simultaneous decrease of right ventricular systolic pressure is observed. This reduction would result either from a direct effect of PAF on cardiac muscles, as already described in some circumstances *in vitro* (Feuerstein, 1989), or from a decrease of cardiac filling due to haemoconcentration and transient accumulation of blood in vasodilated systemic vessels stimulated by PAF. Indeed, the reduction of right ventricular systolic pressure was inhibited by the PAF antagonist.

Indomethacin suppressed the initial rise of right ventricular systolic pressure but increased the fall in systemic blood pressure. Thus the increase in pulmonary vascular resistance, which was terminated before the beginning of systemic hypotension in untreated rats, was not important enough to provoke a reduction in cardiac output. The aggravation of systemic hypotension decreased coronary perfusion and could reinforce cardiac impairment inducing the relative bradycardia observed in indomethacin-treated rats. The influence of indomethacin on the systemic vessels would depend on the suppression of the inhibitory influence of prostaglandins on the release of mediators from mast cells (Raud, 1989) and leukocytes (Weissmann *et al.*, 1979).

In several mammalian species, intravascular activation of the complement system induces systemic hypotension which is inhibited by cyclo-oxygenase inhibitors (Hugli *et al.*, 1987). However, in rats, this hypotension is not reduced by these drugs but depends mainly on the release of PAF, although prostanoids trigger vascular changes in the lungs.

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Endogenous nitric oxide modulates adrenergic neural vasoconstriction in guinea-pig pulmonary artery

S.F. Liu, D.E. Crawley, T.W. Evans & [1]P.J. Barnes

Department of Thoracic Medicine, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY

1 Electrical field stimulation (EFS) of guinea-pig isolated pulmonary artery induced a frequency-dependent contraction. This was abolished by tetrodotoxin (1 μM) and prevented by phentolamine and prazosin (both 1 μM), indicating a role for α_1-adrenoceptors activated by noradrenaline (NA) released from perivascular adrenergic nerves.

2 L-N^G-monomethyl arginine (L-NMMA, 0.3–100 μM) caused a concentration-dependent enhancement of the EFS-induced contraction with a 3.4 ± 0.5 fold increase at 100 μM ($n = 6$). The augmenting effect of 30 μM L-NMMA on the contraction to EFS was completely reversed by 100–300 μM L-arginine, but not by an identical concentration of D-arginine.

3 The contractile response to exogenous NA was similarly enhanced by 30 μM L-NMMA (2.9 ± 0.6 fold increase, $n = 5$).

4 The contractile responses to exogenous phenylephrine and prostaglandin $F_{2\alpha}$ which matched the contraction to EFS (4 Hz) were equally augmented by 30 μM L-NMMA.

5 In vessel rings submaximally contracted with the thromboxane analogue U44069 (2 μM), the selective α_2-adrenoceptor agonist UK14304 induced concentration-dependent relaxation, which was abolished by removal of endothelium. NA had little relaxant effect on these precontracted vessel rings unless in the presence of prazosin (1 μM).

6 Indomethacin had no significant effect on the contractile response to EFS or NA, indicating that vasodilator cyclo-oxygenase products such as prostacyclin are not involved in modulating these responses.

7 Our results suggest that endogenous nitric oxide inhibits the contractile response to adrenergic nerve stimulation in the guinea-pig pulmonary artery by a postjunctional mechanism, but release of prostacyclin does not modulate these responses. Basal release of nitric oxide from endothelial cells may account for this inhibition.

Keywords: Pulmonary artery; innervation; adrenoceptor; nitric oxide; prostacyclin; endothelium; EDRF

Introduction

Endothelium plays an important modulatory role in the response of vascular smooth muscle to a variety of stimuli (De Mey *et al.*, 1982; Furchgott, 1984; Bullock *et al.*, 1986). Endothelium has an inhibitory effect on the contractile responses to α-adrenoceptor stimulation either by exogenous administration of noradrenaline (Cocks & Angus, 1983) and other α-adrenoceptor agonists (Egleme *et al.*, 1984; Lues & Schumann, 1984; Martin *et al.*, 1986) or by adrenergic nerve stimulation in rabbit carotid artery (Tesfamariam *et al.*, 1987; Cohen & Weisbrod, 1988) and rat caudal artery (Hynes *et al.*, 1988). This inhibitory effect of endothelium on the contractile response to adrenergic nerve stimulation is believed to be due to the release of relaxing factor(s) from endothelial cells (Tesfamariam *et al.*, 1987; Tesfamariam & Halpern, 1987; Cohen & Weisbrod, 1988). The factor(s) responsible for the inhibition of neurogenic adrenergic vasoconstriction has not yet been identified.

The pulmonary circulation differs markedly from the systemic circulation in several aspects; the different response to acute hypoxia, which causes contraction in pulmonary, but relaxation in systemic vessels (Staub, 1985) is well known. There are also differences in other control mechanisms (Fishman, 1990), the distribution (Hyman *et al.*, 1989) and affinities (Shaul *et al.*, 1990) of adrenoceptors, and in the response to some peptides, such as vasoactive intestinal polypeptide (Sata *et al.*, 1986). Although the presence of endothelial α_2-adrenoceptors has been demonstrated in the systemic vessels of several regions (Angus *et al.*, 1986; Bullock *et al.*, 1986; Vanhoutte & Miller, 1989), the presence of these receptors on pulmonary artery is still uncertain (Vanhoutte & Miller, 1989).

The two major relaxing factors released from vascular endothelium are prostacyclin (PGI_2) and endothelium-derived relaxing factor (EDRF). EDRF has been characterized as nitric oxide (NO) or a related compound (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Moncada *et al.*, 1988; 1989), which is synthesized from L-arginine (Palmer *et al.*, 1988a,b; Schmidt *et al.*, 1988; Moncada *et al.*, 1989) in vascular endothelial cells. The L-arginine analogue L-N^G-monomethyl arginine (L-NMMA) is a specific inhibitor of NO production (Palmer *et al.*, 1988b; Moncada *et al.*, 1989; Johns *et al.*, 1990). Using the inhibitor and precursor of NO formation and the PGI_2 synthesis inhibitor indomethacin, we have explored the modulatory role of endogenous NO and PGI_2 on the contractile response to adrenergic nerve stimulation in guinea-pig pulmonary artery. We also investigated the possible presence of endothelial α_2-adrenoceptors.

Methods

Tissue preparation

Male Dunkin-Hartley guinea-pigs (300–350 g) were killed by cervical dislocation and pulmonary arteries were rapidly removed. The two branches of the vessels were carefully dissected, cut into rings 2 mm in length, mounted over a pair of rigid wires and suspended in an organ bath containing 2 ml Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, glucose 5.6, $NaHCO_3$ 25.5, EDTA 0.027 and ascorbic acid 0.03. One wire was fixed and the other attached to a force transducer (FT.03 Grass Instruments, Quincy, U.S.A.).

[1] Author for correspondence.

Changes in isometric force were recorded on a polygraph (Grass Model 7). The KH solution was maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. Initially, the vessel rings were repeatedly stimulated by EFS (50 V, 0.2 ms duration, 16 Hz, 15 s) starting from zero resting tension, until the maximum response to EFS was achieved at an increment of 100 mg, and the optimal resting tension determined. Rings were then allowed to equilibrate at this tension (700 mg) in the bath for at least 60 min and washed with fresh KH solution every 20 min during the equilibration period.

Nerve stimulation

Electrical field stimulation (EFS) was applied by two platinum wire electrodes positioned at each end of the vessel ring and connected to a Grass S88 stimulator (Grass Instruments, Quincy, U.S.A.). To activate the intramural nerves without inducing a myogenic response, voltage-duration curves were performed in the presence and absence of 0.3 μM tetrodotoxin by recording the smallest detectable response (<2% of maximal response) and optimal parameters (50 V, 0.2 ms duration) determined in a preliminary study. Frequency-response relationships were constructed in a frequency range of 1–16 Hz, each stimulation being applied for 15 s every 4 min. To assess the nature of the contractile response to EFS, the vessel rings were incubated with tetrodotoxin (1 μM), phentolamine (1 μM) or prazosin (1 μM) for 15 min and stimulated at 1–16 Hz or 8–24 Hz in separate experiments. To assess the effects of endogenous NO and PGI_2 on the adrenergic contraction, vessel rings were stimulated with fixed electrical stimuli (50 V, 0.2 ms, 4 Hz) at 4 min intervals. When responses were constant, the vessel rings were incubated with inhibitory agents for 10 min and a further 3–4 stimulations performed. In the reversibility study, L- or D-arginine were added to the organ bath after the maximal effect of a dose of L-NMMA was achieved. To clarify the relative pre- or postjunctional action of endogenous NO and PGI_2, the effect of 30 μM L-NMMA and 1 μM indomethacin on the matched contractions induced by EFS (4 Hz) and exogenous noradrenaline (NA) were compared in paired rings from the same vessel. Comparisons were also made among the matched contractions induced by EFS, NA, phenylephrine (PE) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in paired vessel rings. To match the EFS-induced contraction, variable concentrations of NA (0.1–0.3 μM), PE (0.08–0.1 μM) and $PGF_{2\alpha}$ (0.3–0.6 μM) were used.

Concentration-response curves

To determine the effect of L-NMMA on contraction to exogenous NA, concentration-response curves to NA were constructed in the presence and absence of 30 μM L-NMMA. In the relaxation study, paired vessel rings were precontracted with 2 μM U44069 and concentration-relaxation curves to NA in the absence and presence of prazosin (1 μM), prazosin plus propranolol (both 1 μM) and prazosin plus yohimbine (both 1 μM), and to UK14304 in the presence and absence of an intact endothelium or 30 μM L-NMMA were obtained.

Drugs

The following drugs were used: noradrenaline hydrochloride, prazosin hydrochloride, yohimbine hydrochloride, indomethacin, U44069 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$), tetrodotoxin, phentolamine hydrochloride, propranolol hydrochloride, L-arginine hydrochloride, D-arginine hydrochloride, sodium nitroprusside (Sigma, Poole, Dorset), prostaglandin $F_{2\alpha}$ solution (Upjohn, Crawley, Sussex), UK14304 (Pfizer, Sandwich, Kent) and L-N^G-monomethyl arginine (a generous gift from Dr S. Moncada, Wellcome Research Laboratory, Beckenham, Kent).

Analysis of results

Contraction is presented in absolute tension or expressed as a percentage of its maximum. An individual concentration-response curve to NA was fitted and the EC_{50} value estimated by use of a computer programme (Graph PAD InPlot, Graph PAD Software, San Diego, CA, U.S.A.). Relaxation was expressed as a percentage of the U44069-induced contraction or the maximum relaxation to nitroprusside (in the endothelial denudation study). The contractile responses to EFS and other vasoconstrictors in the presence of the inhibitors or their vehicles were compared with the responses before adding these inhibitors or vehicles, and expressed as percentage augmentation. Values were presented as mean ± s.e.mean and n indicates the number of animals in each group. Statistical analysis of results was performed by use of Student's t test or one way analysis of variance following by t test with Bonferroni correction, when multiple comparisons were made. For data which were abnormally distributed or with unequal variance, a Mann-Whitney U test was used. A P value < 0.05 was considered to be significant.

Results

Adrenergic response to electrical field stimulation

EFS (50 V, 0.2 ms duration, 1–16 Hz, 15 s) induced a frequency-dependent contraction of the guinea-pig pulmonary artery rings at resting tension. This contraction was abolished by 1 μM tetrodotoxin and antagonized by phentolamine and prazosin (both 1 μM), indicating that it was due to the activation of α_1-adrenoceptors by neurally released NA from perivascular sympathetic nerves. The EFS-elicited contraction was 25.0 ± 5.3, 44.2 ± 6.9, 78.6 ± 8.4, 112.1 ± 9.2 and 161.2 ± 13.2 mg at 1, 2, 4, 8 and 16 Hz respectively (n = 6).

Effects of L-N^G-monomethyl arginine and L-arginine on electrical field stimulation-induced contraction

L-NMMA (0.3–100 μM) enhanced the EFS-induced contractile responses in a concentration-dependent manner (Figure 1) and increased basal tone, especially at high concentrations. The increase in basal tone caused by L-NMMA was 59.4 ± 3.0, 98.3 ± 15.0 and 126.3 ± 28.9 mg at concentrations of 10, 30 and 100 μM respectively ($P < 0.02$, compared with control, n = 6). The augmentation of EFS-induced contraction

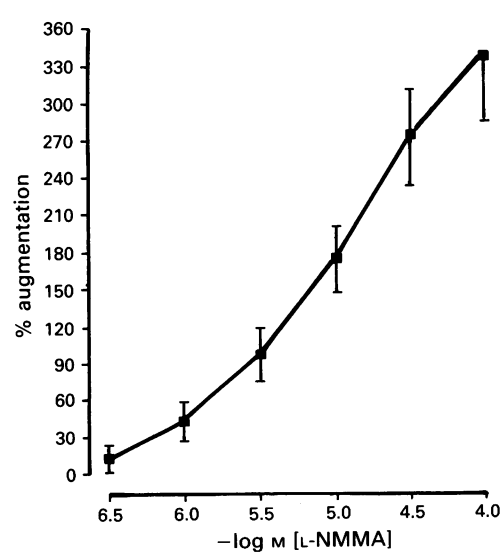

Figure 1 Effect of L-N^G-monomethyl arginine (L-NMMA) on the contractile response to adrenergic nerve stimulation (50 V, 0.2 ms duration, 4 Hz for 15 s) in guinea-pig pulmonary artery rings, showing concentration-dependent augmentation of the adrenergic neurogenic vasoconstriction. Mean of 6 animals with s.e.mean shown by vertical bars.

by 30 μM L-NMMA was completely reversed by 100–300 μM L-arginine, although an identical concentration of D-arginine was ineffective (Figure 2).

Effects of L-N^G-monomethyl arginine on noradrenaline-induced contraction

NA caused a concentration-dependent contraction of pulmonary vessel rings at resting tension. Rings pretreated with 30 μM L-NMMA had a lower threshold concentration of NA than the control rings (15 ± 6 vs 162 ± 59 nM, $P < 0.05$, $n = 5$). The pD_2 values for control and L-NMMA-treated rings was 6.39 ± 0.18 and 7.08 ± 0.05, respectively ($P < 0.03$, $n = 5$), representing a 4.9 fold increase in sensitivity to NA in L-NMMA pretreated rings. The maximal tension generation was 717 ± 123 and 860 ± 53 mg for control and L-NMMA group, which was not significantly different ($P > 0.05$, $n = 5$).

Comparison of the effects of L-N^G-monomethyl arginine on electrical field stimulation- and noradrenaline-induced contractions

To investigate the relative pre- and postjunctional effect of L-NMMA, we compared the augmenting effects of 30 μM L-NMMA on the contractile response to EFS (4 Hz) and a matched contractile response to NA (0.1–0.3 μM), which was 61.2 ± 8.9 and 61.2 ± 10.3 mg respectively ($P > 0.05$, $n = 6$) before L-NMMA treatment. L-NMMA augmented EFS- and NA-induced contraction equally (Figure 3), suggesting that L-NMMA enhanced adrenergic responses via a postjunctional mechanism.

Vasorelaxant response to UK14304 and noradrenaline

To determine the presence and the possible role of α_2-adrenoceptors in this vessel, vasorelaxant responses of U44069-precontracted vessel rings with and without endothelium to the selective α_2-adrenoceptor agonist UK14304 and endothelium intact rings to NA in the absence and presence of various blockers, were determined. U44069 raised the vascular tone to 405 ± 46, 378 ± 53 and 548 ± 52 mg in the rings with and without endothelium, and in the presence of 30 μM L-NMMA respectively ($n = 5$). UK14304 induced a concentration-dependent relaxation of the U44069-precontracted rings (Figure 4). This relaxation was abolished by endothelial denudation. Pretreatment of the vessel rings with 30 μM L-NMMA mimicked the effect of endothelium denudation (Figure 4), indicating that α_2-adrenoceptors located on endothelial cells mediate vasorelaxation by stimulation of NO release. The U44069-produced contraction was 715 ± 69, 727 ± 29, 623 ± 69 and 718 ± 118 mg in control, prazosin, prazosin plus propranolol and prazosin plus yohimbine groups, respectively ($P > 0.05$, $n = 5$). NA had little relaxant effect even on the precontracted rings. The maximal relaxation achieved at the concentration of 3 μM was a 4.0 ± 3.1% reduction of the U44069-elicited tension. In the presence of prazosin (1 μM), NA induced a concentration-

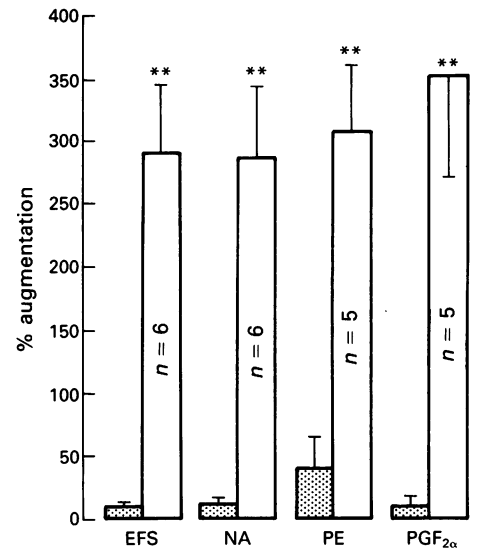

Figure 3 Comparison of the effect of L-N^G-monomethyl arginine (L-NMMA) on matched contractile responses to adrenergic nerve stimulation (EFS, 50 V, 0.2 ms, 4 Hz for 15 s), exogenous noradrenaline (NA), phenylephrine (PE) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Controls are shown in stippled columns and L-NMMA (30 μM) in open columns. ** $P < 0.01$, compared with control.

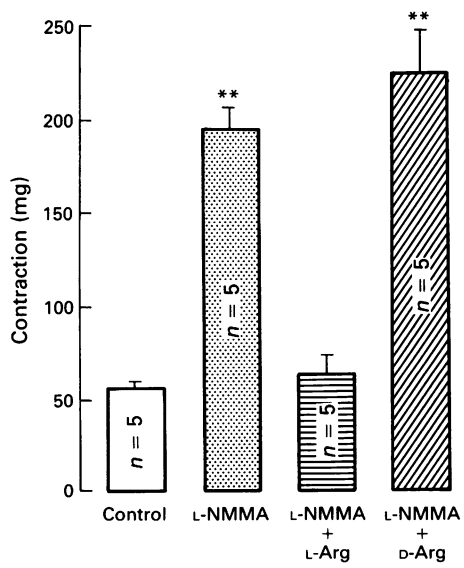

Figure 2 Reversibility of 30 μM L-N^G-monomethyl arginine (L-NMMA)-induced augmentations of adrenergic contraction to electrical field stimulation (EFS, 50 V, 0.2 ms, 4 Hz for 15 s) by L- and D-arginine. L-arginine (L-Arg) 100–300 μM, but not identical concentrations of D-arginine (D-Arg) completely reversed the L-NMMA effect. **: $P < 0.01$, compared with control.

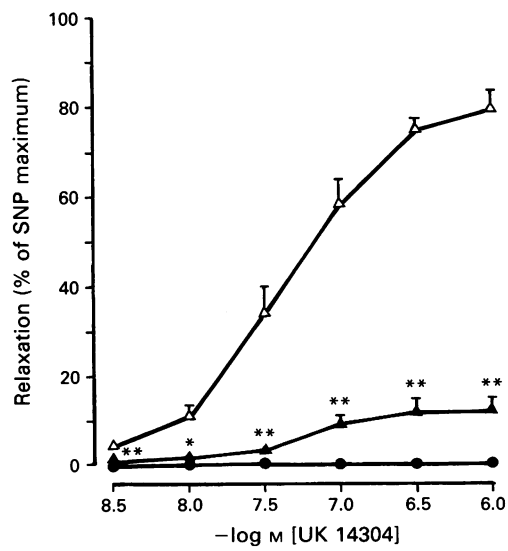

Figure 4 Effects of endothelium removal and L-N^G-monomethyl arginine (L-NMMA) on the relaxant response of the U44069-precontracted vessel rings to UK14304. Relaxation was expressed as percentage of nitroprusside (SNP) maximum relaxation: (△) with (control), (●) without intact endothelium and (▲) in the presence of 30 μM L-NMMA. * $P < 0.05$ and ** $P < 0.01$, compared with control rings. Mean ± s.e.mean of 5 animals with s.e.mean shown by vertical bars.

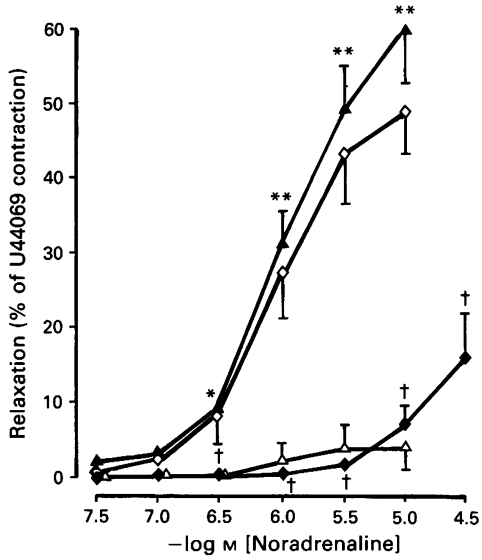

Figure 5 Relaxation of U44069 precontracted vessel rings to noradrenaline (NA) in the absence (△, control) and presence of prazosin (▲, 1 μM), prazosin plus propranolol (◇, both 1 μM) and prazosin plus yohimbine (◆, both 1 μM). Relaxation was expressed as percentage of U44069 (2 μM)-induced contraction. NA caused little relaxant effect (△) except in the presence of prazosin (▲). * $P < 0.05$ and ** $P < 0.01$, compared with control. † $P < 0.01$, compared with prazosin pretreated rings, $n = 5$ in all groups.

dependent reduction of the U44069-induced vascular tone. This relaxation was significantly antagonized by yohimbine (1 μM), but unaffected by propranolol (1 μM, Figure 5), suggesting that although α_2-adrenoceptors are present on these vessels, they play little role in mediating the response to NA.

Effects of L-N^G-monomethyl arginine on the contractions to other vasoconstrictors

The contraction induced by a single concentration of PE and $PGF_{2\alpha}$ was respectively 62.5 ± 12.0 and 68.0 ± 16.3 mg before treatment with L-NMMA ($P > 0.05$, $n = 5$). These contractions, which were similar to the contractile response to EFS, were enhanced to the same extent by 30 μM L-NMMA (Figure 3).

Effects of indomethacin

Indomethacin slightly, but not significantly enhanced the contractile responses to EFS at concentrations of 1 and 3 μM but had no effect at 10 μM. At a concentration of 30 μM, indomethacin slightly enhanced EFS-induced contraction in 4 of the 6 tested vessel rings from 6 guinea-pigs, and slightly inhibited the EFS-induced contraction in 2 rings. Indomethacin (1 μM) slightly enhanced the contraction to NA. These results suggest that endogenous cyclo-oxygenase products are not important in modulating response to adrenergic nerve stimulation.

Discussion

Removal of endothelium increases vasoconstrictor responses to α-adrenoceptor agonists (Cocks & Angus, 1983; Egleme *et al.*, 1984; Lues & Schumann, 1984; Carrier & White, 1985; Martin *et al.*, 1986) and to adrenergic nerve stimulation (Tesfamariam *et al.*, 1987; Cohen & Weisbrod, 1988; Hynes *et al.*, 1988). However, it has not yet been established which factor is responsible for this inhibitory action of endothelium. We now report that the NO synthase inhibitor L-NMMA enhances the contractile response to adrenergic nerve stimulation concentration-dependently in guinea-pig pulmonary artery. This augmentation is completely reversed by L-arginine but not D-arginine. By contrast, this neurogenic contraction is unaffected by indomethacin. These results indicate that endogenous NO but not PGI_2 modulates the adrenergic neurogenic vasoconstriction.

It has been reported that NO can be released from inhibitory non-adrenergic, non-cholinergic nerve (i-NANC) endings of several tissues (Gillespie *et al.*, 1989; Tucker *et al.*, 1990; Li & Rand, 1991), including cerebral arteries (Toda & Okamura, 1990). It is possible that stimulation by EFS of i-NANC nerves, which have been demonstrated in this vessel (Liu *et al.*, 1991b), induces NO release, or alternatively, stimulation of i-NANC nerves releases mediator(s), which act on endothelial cells to stimulate NO release (Liu *et al.*, 1991b). However, release of NO from i-NANC is unlikely. Firstly, we found, in our previous study, that NO mediating the i-NANC relaxation is released from endothelium (Liu *et al.*, 1991b). Secondly, we demonstrated in the present study that EFS- and exogenous NA-induced contractions were augmented by L-NMMA to an equal extent. It is possible that different mechanisms in uptake and access to receptors of endogenous and exogenous NA and the presence of a negative feedback mechanism in endogenous NA release in response to EFS may make a strict comparison of EFS- and exogenous NA-induced contraction difficult. However, the equal augmentation of EFS- and NA-induced contractions by L-NMMA at least indicates that prejunctional action of NO is not a major mechanism. Taken together with the equal augmentation of matched contractions to PE and $PGF_{2\alpha}$, our results suggest L-NMMA augments adrenergic responses via a common mechanism at a postjunctional level. The possibility that a mediator from i-NANC nerves stimulates NO release cannot be totally excluded in the present study, but it would exert a postjunctional effect. Thus, our results suggest that L-NMMA augments the contractile response to adrenergic nerve stimulation by a postjunctional mechanism.

The presence of endothelial α_2-adrenoceptors in guinea-pig pulmonary artery, as in systemic vessels (Vanhoutte & Miller, 1989), is indicated by the demonstration that the selective α_2-adrenoceptor agonist UK14304 and nonselective agonist NA, in the presence of an α_1-adrenoceptor antagonist, induced a concentration-dependent relaxation of precontracted vessel rings. This relaxation was unaffected by the β-adrenoceptor antagonist, propranolol, but was significantly antagonized by the α_2-adrenoceptor antagonist yohimbine. We also provide evidence to indicate that activation of these receptors induce vasorelaxation by stimulating endothelium-derived NO release. Release of EDRF can offset NA-induced contraction (Cocks & Angus, 1983; Angus *et al.*, 1986; Vanhoutte & Miller, 1989). It is possible that NA from adrenergic nerves stimulates α_2-adrenoceptors to induce NO release, which then inhibits the contractile response to EFS. However, there is evidence against this possibility. Firstly, NA relaxed the precontracted vessel rings only in the presence of α_1-adrenoceptor blockade and NA had little relaxant effect even when the vascular tone was raised, whereas EFS was applied when the vessel rings were at resting tension. This implies that the weak α_2-adrenoceptor action of NA is profoundly masked by its α_1-adrenoceptor action. Secondly, a similar magnitude of contractile response to PE and $PGF_{2\alpha}$ was augmented by L-NMMA to the same extent as in vessels contracted with NA, although neither had any relaxant effect on the precontracted vessel rings. It is more likely that there is a continuous basal release of NO from vascular endothelium which gives a tonic inhibition of the contractile responses to NA and adrenergic nerve stimulation. Inhibition of this NO release by L-NMMA enhances contraction to adrenergic stimulation. This explanation is supported by our observation that L-NMMA increased basal smooth muscle tone in a concentration-related manner. Basal release of NO has previously been reported both in systemic (Rees *et al.*, 1989) and pulmonary (Wiklund *et al.*, 1990; Liu *et al.*, 1991a) circulations.

An increase in endothelial shear stress by changing the perfusate velocity and viscosity has been demonstrated to inhibit adrenergic contraction to electrical field stimulation in perfused carotid artery (Tesfamariam & Cohen, 1988). Whether smooth muscle contraction also imposes shear stress on endothelial cells and increases NO release remains to be determined.

In summary, our results demonstrate that endogenous nitric oxide inhibits adrenergic neurogenic vasoconstriction via a postjunctional mechanism. Basal release of nitric oxide from endothelial cells may account for this inhibition. Stimulation of α_2-adrenoceptors on endothelium is unlikely to contribute to this effect, although these receptors are present on endothelial cells of these vessels.

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