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A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery

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1 Relaxation of the methoxamine-precontracted rat small mesenteric artery by endothelium-derived hyperpolarizing factor (EDHF) was compared with relaxation to the cannabinoid, anandamide (arachidonylethanolamide). EDHF was produced in a concentration- and endothelium-dependent fashion in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) by either carbachol (pEC_{50} [negative logarithm of the EC_{50}] = 6.19 ± 0.01, R_{max} [maximum response] = 93.2 ± 0.4%; n = 14) or calcium ionophore A23187 (pEC_{50} = 6.46 ± 0.02, R_{max} = 83.6 ± 3.6%; n = 8). Anandamide responses were independent of the presence of endothelium or L-NAME (control with endothelium: pEC_{50} = 6.31 ± 0.06, R_{max} = 94.7 ± 4.6%; n = 10; with L-NAME: pEC_{50} = 6.33 ± 0.04, R_{max} = 93.4 ± 6.0%; n = 4).

2 The selective cannabinoid receptor antagonist, SR 141716A (1 μM) caused rightward shifts of the concentration-response curves to both carbachol (2.5 fold) and A23187 (3.3 fold). It also antagonized anandamide relaxations in the presence or absence of endothelium giving a 2 fold shift in each case. SR 141716A (10 μM) greatly reduced the R_{max} values for EDHF-mediated relaxations to carbachol (control, 93.2 ± 0.4%; SR 141716A, 10.7 ± 2.5%; n = 5; P < 0.001) and A23187 (control, 84.8 ± 2.1%; SR 141716A, 3.5 ± 2.3%; n = 6; P < 0.001) but caused a 10 fold parallel shift in the concentration-relaxation curve for anandamide without affecting R_{max}.

3 Precontraction with 60 mM KCl significantly reduced (P < 0.01; n = 4 for all) relaxations to 1 μM carbachol (control 68.8 ± 5.6% versus 17.8 ± 7.1%), A23187 (control 71.4 ± 6.1% versus 3.9 ± 0.45%) and anandamide (control 71.1 ± 7.0% versus 5.2 ± 3.6%). Similar effects were seen in the presence of 25 mM K^+. Incubation of vessels with pertussis toxin (PTX; 400 ng ml^{-1}, 2 h) also reduced (P < 0.01; n = 4 for all) relaxations to 1 μM carbachol (control 63.5 ± 7.5% versus 9.0 ± 3.2%), A23187 (control 77.0 ± 5.8% versus 16.2 ± 7.1%) and anandamide (control 89.8 ± 2.2% versus 17.6 ± 8.7%).

4 Incubation of vessels with the protease inhibitor phenylmethylsulphonyl fluoride (PMSF; 200 μM) significantly potentiated (P < 0.01), to a similar extent (~2 fold), relaxation to A23187 (pEC_{50}: control, 6.45 ± 0.04; PMSF, 6.74 ± 0.10; n = 4) and anandamide (pEC_{50}: control, 6.31 ± 0.02; PMSF, 6.61 ± 0.08; n = 8). PMSF also potentiated carbachol responses both in the presence (pEC_{50}: control, 6.25 ± 0.01; PMSF, 7.00 ± 0.01; n = 4; P < 0.01) and absence (pEC_{50}: control, 6.41 ± 0.04; PMSF, 6.88 ± 0.04; n = 4; P < 0.001) of L-NAME. Responses to the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) were also potentiated by PMSF (pEC_{50}: control, 7.51 ± 0.06; PMSF, 8.00 ± 0.05, n = 4, P < 0.001).

5 EDHF-mediated relaxation to carbachol was significantly attenuated by the K^+ channel blocker tetraethylammonium (TEA; 1 mM) (pEC_{50}: control, 6.19 ± 0.01; TEA, 5.61 ± 0.01; n = 6; P < 0.01). In contrast, TEA (1 mM) had no effect on EDHF-mediated relaxation to A23187 (pEC_{50}: control, 6.47 ± 0.04; TEA, 6.41 ± 0.02, n = 4) or on anandamide (pEC_{50}: control, 6.28 ± 0.06; TEA, 6.09 ± 0.02; n = 5). TEA (10 mM) significantly (P < 0.01) reduced the R_{max} for anandamide (control, 94.3 ± 4.0%; 10 mM TEA, 60.7 ± 4.4%; n = 5) but had no effect on the R_{max} to carbachol or A23187.

6 $BaCl_2$ (100 μM), considered to be selective for blockade of inward rectifier K^+ channels, had no significant effect on relaxations to carbachol or A23187, but caused a small shift in the anandamide concentration-response curve (pEC_{50}: control, 6.39 ± 0.01; Ba^{2+}, 6.20 ± 0.01; n = 4; P < 0.01). $BaCl_2$ (1 mM; which causes non-selective block of K^+ channels) significantly (P < 0.01) attenuated relaxations to all three agents (pEC_{50} values: carbachol, 5.65 ± 0.02; A23187, 5.84 ± 0.04; anandamide, 5.95 ± 0.02; n = 4 for each).

7 Apamin (1 μM), a selective blocker of small conductance, Ca^{2+}-activated, K^+ channels (SK_{Ca}), 4-aminopyridine (1 mM), a blocker of delayed rectifier, voltage-dependent, K^+ channels (K_v), and ciclazindol (10 μM), an inhibitor of K_v and adenosine 5′-triphosphate (ATP)-sensitive K^+ channels (K_{ATP}), significantly reduced EDHF-mediated relaxations to carbachol, but had no significant effects on A23187 or anandamide responses.

8 Glibenclamide (10 μM), a K_{ATP} inhibitor and charybdotoxin (100 or 300 nM), a blocker of several K^+ channel subtypes, had no significant effect on relaxations to any of the agents. Iberiotoxin (50 nM), an inhibitor of large conductance, Ca^{2+}-activated, K^+ channels (BK_{Ca}), had no significant effect on the relaxation responses, either alone or in combination with apamin (1 μM). Also, a combination of apamin (1 μM) with either glibenclamide (10 μM) or 4-aminopyridine (1 mM) did not inhibit relaxation to carbachol significantly more than apamin alone. Neither combination had any significant effect on relaxation to A23187 or anandamide.

9 A combination of apamin (1 μM) with charybdotoxin (100 nM) abolished EDHF-mediated relaxation to carbachol, but had no significant effect on that to A23187. Apamin (1 μM) and charybdotoxin (300 nM) together consistently inhibited the response to A23187, while apamin (1 μM) and ciclazindol (10 μM) together inhibited relaxations to both carbachol and A23187. None of these toxin combinations had any significant effect on relaxation to anandamide.

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10 It was concluded that the differential sensitivity to K^+ channel blockers of EDHF-mediated responses to carbachol and A23187 might be due to actions on endothelial generation of EDHF, as well as its actions on the vascular smooth muscle, and suggests care must be taken in choosing the means of generating EDHF when making comparative studies. Also, the relaxations to EDHF and anandamide may involve activation of cannabinoid receptors, coupled via PTX-sensitive G-proteins to activation of K^+ conductances. The results support the hypothesis that EDHF is an endocannabinoid but relaxations to EDHF and anandamide show differential sensitivity to K^+ channel blockers, therefore it is likely that anandamide is not identical to EDHF in the small rat mesenteric artery.

Keywords: EDHF; mesenteric artery; anandamide; calcium ionophore A23187; carbachol; endothelium; cannabinoid receptors; K^+ channels; pertussis toxin; ciclazindol

Introduction

Vasorelaxation to muscarinic agonists is endothelium-dependent (Furchgott & Zawadzki, 1980) and, in part, it is mediated by the endothelium-derived relaxing factor (EDRF) which Palmer *et al*. (1987) identified as nitric oxide. However, it has become clear that nitric oxide does not account for all EDRF activity and that an additional factor, known as endothelium-derived hyperpolarizing factor (EDHF), contributes to such activity by hyperpolarizing the vascular smooth muscle through K^+ channel activation (see Garland *et al*., 1995, for review). Indeed, EDHF is now thought to assume greater importance than nitric oxide in resistance beds, since Shimokawa *et al*. (1996) have shown that the contribution of EDHF to endothelium-dependent relaxation in the rat is greater in smaller diameter vessels. Furthermore, EDHF activity may be increased under circumstances where nitric oxide activity is impaired (Kilpatrick & Cocks, 1994; Kemp *et al*., 1995; Bauersachs *et al*., 1996; McCulloch *et al*., 1997).

In comparison to nitric oxide, relatively little is known about EDHF. There is evidence that it may be derived from arachidonic acid (Cohen & Vanhoutte, 1995), possibly through the cytochrome P450 pathway (Campbell *et al*., 1996). However, Fukao *et al*. (1997) showed that the contribution of cytochrome P450-derived metabolites to EDHF-mediated hyperpolarization was minimal or absent in the rat isolated mesenteric artery and, recently, Randall *et al*. (1996) proposed that arachidonylethanolamide (anandamide), a cannabinoid derivative of arachidonic acid (Di Marzo *et al*., 1994), may represent an EDHF. This hypothesis was based on the finding that a selective cannabinoid receptor antagonist, SR 141716A (Rinaldi-Carmona *et al*., 1994), inhibited the actions of EDHF in rat isolated mesenteric bed and in the conscious rat. Anandamide has been proposed to be an endogenous ligand for cannabinoid receptors, of which CB_1, CB_{1A} and CB_2 subtypes have currently been identified; all three subtypes have been shown to activate pertussis toxin-(PTX) sensitive G-proteins and cause inhibition of adenylyl cyclase (Felder *et al*., 1995; Rinaldi-Carmona *et al*., 1996). The central CB_1 receptor, but not the peripheral CB_2 receptor, activates an inward rectifier K^+ channel and inhibits Q-type Ca^{2+} channels via PTX-sensitive G-proteins when expressed in AtT20 cells (Felder *et al*., 1995).

EDHF relaxes vessels by increasing K^+ conductance, with the subsequent hyperpolarization leading to decreased Ca^{2+} influx due to reduced opening of voltage-gated Ca^{2+} channels. Indeed, Adeagbo & Triggle (1993) showed that raising extracellular K^+ to 60 mM inhibited the relaxant effect of EDHF, but not of nitric oxide, in the rat isolated perfused mesenteric bed. Waldron & Garland (1994a) similarly showed that 25 mM extracellular K^+ inhibited relaxation and hyperpolarization to acetylcholine obtained in the presence of a nitric oxide synthase (NOS) inhibitor in the rat isolated mesenteric artery. The transduction system for EDHF may involve PTX-sensitive G-proteins, as PTX can inhibit the actions of EDHF in bovine coronary artery, although it has no effect in porcine coronary arteries (Graier *et al*., 1996).

Waldron & Garland (1994b) showed that NOS inhibitor-resistant hyperpolarization to acetylcholine in the rat isolated mesenteric artery was attenuated by apamin, and this has recently been substantiated by the findings of Hutri-Kähönen *et al*. (1997). The effect of EDHF was completely inhibited by a combination of apamin and charybdotoxin. Olesen & Hansen (1995), in the same preparation, have shown that the effect of EDHF was inhibited by iberiotoxin, a selective blocker of large conductance, Ca^{2+}-activated K^+ channels (BK_{Ca}). 4-Aminopyridine, a blocker of the voltage-gated, delayed rectifier K^+ channel (K_v) has also been found to attenuate (Olesen & Hansen, 1995) or abolish (Zakharenko, 1996) EDHF-mediated hyperpolarizations to acetylcholine in the rat isolated mesenteric artery.

However, there is evidence that K^+ channels may be present on endothelial cells (Groschner *et al*., 1992) and may have an important role in hyperpolarizing the endothelium and thus facilitating entry of Ca^{2+} ions (Chen & Cheung, 1992; Groschner *et al*., 1992), which is the stimulus for synthesis of nitric oxide and possibly of arachidonate via phospholipase A_2 (Bauersachs *et al*., 1996). None of the studies hitherto carried out in the rat isolated mesenteric artery have taken into account the possible effects of K^+ channel blockers on the endothelial cells which could inhibit EDHF synthesis, rather than its effects.

The aim of the present study was to compare the characteristics of relaxations to anandamide with those to EDHF by use of a variety of pharmacological tools, including K^+ channel blockers, a cannabinoid antagonist (SR 141716A), PTX and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF). EDHF was generated during nitric oxide synthase inhibition with N^G-nitro-L-arginine methyl ester (L-NAME) by both muscarinic receptor-dependent (carbachol) and receptor-independent (the calcium ionophore A23187) pathways. The latter should be relatively resistant to the effects of K^+ channel blockers on the endothelium.

A preliminary account of some of this work was presented to the Bristol Meeting of the British Pharmacological Society in July 1997 (White & Hiley, 1997).

Methods

Male Wistar rats (250–350 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p., Sagatal, Rhone Merieux, Harlow, Essex). The mesentery was removed and placed in ice-cold, gassed (95% O_2/5% CO_2) Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose, 10. Segments (2 mm in length) of third order branches of the superior mesenteric artery were removed and mounted in a Mulvany-Halpern myograph (Model 500A, J.P. Trading, Aarhus, Denmark) as described by Garland & McPherson (1992). Vessels were maintained at 37°C in Krebs-Henseleit solution, containing indomethacin (10 μM) and bubbled with 95% O_2/5% CO_2, and were allowed to equilibrate under zero tension for 60 min. After equilibration, vessels were normalized to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany & Halpern, 1977). The

mean vessel diameter under these conditions was 341 ± 3 μm ($n = 352$). The vessels were left for another 30 min before experiments commenced. In experiments for which the endothelium was not required, it was removed by rubbing the intima with a human forearm hair.

Experimental protocol

After the equilibration period, the integrity of the endothelium was assessed by precontracting the vessels with methoxamine (10 μM) and then adding carbachol (10 μM). The mean tension generated by vessels in response to methoxamine was 13.4 ± 0.2 mN ($n = 352$). Tissues which relaxed to carbachol by greater than 90% were designated as endothelium-intact and those in which carbachol caused less than 10% relaxation were designated as endothelium-denuded.

The actions of EDHF were examined by assessing the vasorelaxation to carbachol and A23187 in the presence of L-NAME, which was added 30 min before and was then present throughout, construction of the agonist concentration-response curves. Although preincubation of tissues with L-NAME (100 μM, 30 min) had no effect on resting tension, the vasoconstrictor effect of methoxamine was greatly augmented. In view of this, the concentration of methoxamine used to precontract vessels ($1 - 5$ μM) was such that an equivalent level of tone was induced as that obtained in response to 10 μM methoxamine in the same tissue in the absence of L-NAME.

The effect of L-NAME on the vasorelaxant effects of anandamide was investigated in the same manner. Although there was no significant difference between curves constructed in the absence or presence of L-NAME, it was routinely included in experiments involving anandamide, as it has recently been shown that nitric oxide may, under certain conditions, activate K^+ channels (Plane *et al*., 1996). It has been suggested that the presence of more than one NOS inhibitor may be necessary to inhibit nitric oxide synthesis fully (e.g. Plane *et al*., 1996). However, pilot experiments revealed no difference between experiments in which L-NAME, or a combination of L-NAME and N^G-nitro-L-arginine were present (data not shown). Therefore L-NAME alone was used in this study.

The involvement of cannabinoid receptors in the relaxations mediated by each agent was assessed by constructing concentration-response curves in the absence and presence of SR 141716A (100 nM, 1 μM or 10 μM, 30 min). To test for non-specific effects of SR 141716A at a concentration of 10 μM, the agent was used against relaxations induced by the nitric oxide donor, SNAP and the K^+ channel activator levcromakalim.

The contribution of K^+ conductances to the vasorelaxation responses to each agent was assessed by obtaining concentration-response curves after precontracting vessels with high K^+ (25 or 60 mM K^+) Krebs-Henseleit solution (these were prepared by substituting equimolar concentrations of NaCl with KCl in the standard Krebs-Henseleit solution). K^+ (60 mM) gave an increase in tone of 13.3 ± 1.0 mN ($n = 13$) which was not significantly different from that induced by methoxamine (see above). The identity of the K^+ channel, or channels, activated was investigated by preincubating vessels with known K^+ channel blocking agents, alone or in combination. The agents and concentrations used were iberiotoxin (50 nM), apamin (1 μM), charybdotoxin (100 or 300 nM), 4-aminopyridine (50 μM or 1 mM), glibenclamide (10 μM), ciclazindol (10 μM), $BaCl_2$ (100 μM or 1 mM) and TEA (1 mM or 10 mM). Each was incubated with the tissue for 30 min before its effects on the vasorelaxation to a given agonist were assessed.

The role of PTX-sensitive G-proteins in the vasorelaxations to carbachol, A23187 and anandamide was investigated by pre-incubating vessels with PTX (400 ng ml^{-1}) for 2 h before concentration-response data were obtained.

The level of enzymatic degradation of EDHF and anandamide was assessed by pre-incubating vessels with the serine protease inhibitor, PMSF (200 μM), which is known to potentiate the actions of anandamide via inhibition of anandamide amidase (Pertwee *et al*., 1995). In all experiments, the concentration of methoxamine used to precontract vessels was adjusted such that the level of tone induced was equivalent to that obtained in the initial test of endothelial integrity.

Data and statistical analysis

All relaxation responses are expressed as the percentage relaxation of the tone induced by methoxamine or K^+. Data are given as the mean $\pm$ s.e.mean and were compared, as appropriate, by Student's unpaired t test or analysis of variance with statistical significance being determined by Dunnett's *post-hoc* test.

EC_{50} values for vasorelaxant responses were obtained from individual concentration-response curves by fitting the data to the logistic equation:

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

where R is reduction in tone, A the concentration of the agonist, R_{max} the maximum reduction of established tone, n_H the slope function and EC_{50} the concentration of relaxant giving half the maximal relaxation. The curve fitting was carried out by use of KaleidaGraph software (Synergy Software, Reading, PA, U.S.A.) running on a Macintosh computer. The EC_{50} values were converted to the pEC_{50} (the negative logarithm of the EC_{50}) for statistical analysis. P values less than 0.05 were considered to be statistically significant.

Drugs

All solutions were prepared on the day of the experiment. SR 141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride) was purchased from Tocris Cookson (Bristol) or provided by Research Biochemicals International (RBI) (Natick, MA, U.S.A.), as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract N01MH30003. Methoxamine hydrochloride, carbachol, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), N^G-nitro-L-arginine, tetraethylammonium chloride (TEA), charybdotoxin (Sigma Chemical Company, Poole, Dorset), apamin, pertussis toxin (PTX; Calbiochem, Nottingham), iberiotoxin (RBI) and $BaCl_2$ (Fisher Scientific, Loughborough, Leicestershire) were dissolved in distilled water. 4-Aminopyridine (Sigma) was dissolved in 1 M HCl. Glibenclamide (Hoechst UK Ltd, Hounslow, Middlesex) was dissolved in dimethylsulphoxide (Sigma). A23187 (Sigma), phenylmethylsulphonyl fluoride (PMSF; Calbiochem), S-nitroso-N-acetylpenicillamine (SNAP; Calbiochem) and SR 141716A were dissolved in 100% ethanol. Arachidonylethanolamide (anandamide) was synthesized and dissolved in an inert oil/water emulsion by Dr E.A. Boyd (Department of Pharmaceutical Sciences, University of Nottingham). Ciclazindol (Professor M.L.J. Ashford, Department of Biomedical Sciences, University of Aberdeen) was dissolved in distilled water. Levcromakalim (SmithKline Beecham, Betchworth, Surrey) was dissolved in 70% (v/v) ethanol to give a 10 mM stock solution.

Results

Effects of L-NAME and endothelial destruction on relaxation

Carbachol caused concentration-dependent relaxations of methoxamine-induced tone ($pEC_{50} = 6.41 \pm 0.02$, $R_{max} = 91.8 \pm 2.0\%$; $n = 16$) and the presence of 100 μM L-NAME significantly ($P < 0.01$) reduced the potency of carbachol ($pEC_{50} = 6.19 \pm 0.01$), but did not affect the maximal response ($R_{max} = 93.2 \pm 0.4\%$; $n = 14$).

In contrast, the concentration-dependent relaxations of methoxamine-induced tone caused by anandamide

($pEC_{50} = 6.31 \pm 0.06$, $R_{max} = 94.7 \pm 4.6\%$; $n = 10$) were unaffected by the presence of 100 μM L-NAME ($pEC_{50} = 6.33 \pm 0.04$, $R_{max} = 93.4 \pm 6.0\%$; $n = 4$).

A concentration-response curve for A23187 was only determined in the presence of 100 μM L-NAME. It caused concentration-dependent relaxations of methoxamine-induced tone ($pEC_{50} = 6.46 \pm 0.02$, $R_{max} = 83.6 \pm 3.6\%$; $n = 8$) with an R_{max} which was not significantly different from those for carbachol, in the presence of L-NAME or anandamide.

Removal of the vascular endothelium, by rubbing the intimal layer of vessels, abolished the relaxations to carbachol and A23187 in the presence or absence of L-NAME, but had no effect upon relaxation to anandamide (data not shown).

Except where stated, all subsequent experiments with carbachol, A23187 and anandamide were carried out in the presence of an intact endothelium and 100 μM L-NAME.

Effects of the cannabinoid receptor antagonist, SR 141716A

At a concentration of 100 nM, SR 141716A did not affect the relaxations to any of the three agents tested (data not shown). However, SR 141716A (1 μM) caused a significant ($P < 0.01$) 2.5 fold rightward shift in the concentration-response curve for carbachol (pEC_{50}: control, 6.19 ± 0.01; SR 141716A, 5.79 ± 0.01; $n = 8$; Figure 1a), and also caused a significant ($P < 0.05$) 3.3 fold rightward shift in the response to A23187 (pEC_{50}: control, 6.50 ± 0.03; SR 141716A, 5.98 ± 0.15; $n = 4$; Figure 1b). SR 141716A (1 μM) also caused a 2 fold rightward shift of the response to anandamide (pEC_{50}: control, 6.37 ± 0.05; SR 141716A, 6.07 ± 0.01; $P < 0.05$; $n = 5$; Figure 1c). SR 141716A (1 μM) did not significantly affect the maximal relaxation to any of the three agents tested.

SR 141716A (1 μM) also caused a significant ($P < 0.01$) 2 fold shift in the concentration-relaxation curve for anandamide in endothelium-denuded vessels (pEC_{50}: control, 6.57 ± 0.02, $n = 6$; with antagonist, 6.27 ± 0.01, $n = 6$) without affecting the maximal response (control, $86.0 \pm 1.6\%$; with antagonist, $90.6 \pm 2.0\%$).

At a concentration of 10 μM, SR 141716A almost completely abolished relaxations to both carbachol (control R_{max} $93.2 \pm 0.4\%$ versus $10.7 \pm 2.5\%$ in the presence of 10 μM SR 141716A; $n = 5$; Figure 1a) and A23187 (control R_{max} $84.8 \pm 2.1\%$ versus $3.5 \pm 2.3\%$; $n = 6$; Figure 1b). The relaxation obtained at the highest concentration of anandamide used in the presence of 10 μM SR 141716A was not significantly different from that seen in the control but the curve was further shifted rightwards by approximately one order of magnitude compared with 1 μM SR 141716A ($n = 6$; Figure 1c).

In order to test for possible non-specific inhibition of vasorelaxation by 10 μM SR 141716A, this agent was tested in vessels denuded of endothelium against submaximal concentrations of SNAP and levcromakalim. SNAP (100 nM) gave relaxations of $86.8 \pm 3.9\%$ and $82.1 \pm 7.1\%$, which were not statistically different, in the presence and absence of 10 μM SR 141716A ($n = 6$ for both). In contrast, 10 μM SR 141716A reduced the relaxation to 1 μM levcromakalim from $75.5 \pm 5.8\%$ to $1.9 \pm 1.1\%$ ($n = 6$ for both), although 1 μM SR 141716A had no significant effect (control, $51.8 \pm 8.9\%$; 1 μM SR 141716A, $49.9 \pm 9.0\%$; $n = 6$ for both).

Effects of preconstriction with K^+

Figure 2 shows that preconstriction of the vessels with 60 mM K^+ instead of methoxamine reduced the ability of carbachol, A23187 and anandamide (all used at a submaximal concentration of 1 μM) to relax the vessels. It can also be seen from the figure that precontraction of the vessels with 25 mM K^+ (in the presence of 1–3 μM methoxamine to give the same degree of precontraction as 60 mM K^+ alone) also significantly reduced the relaxation to all three vasodilator agents.

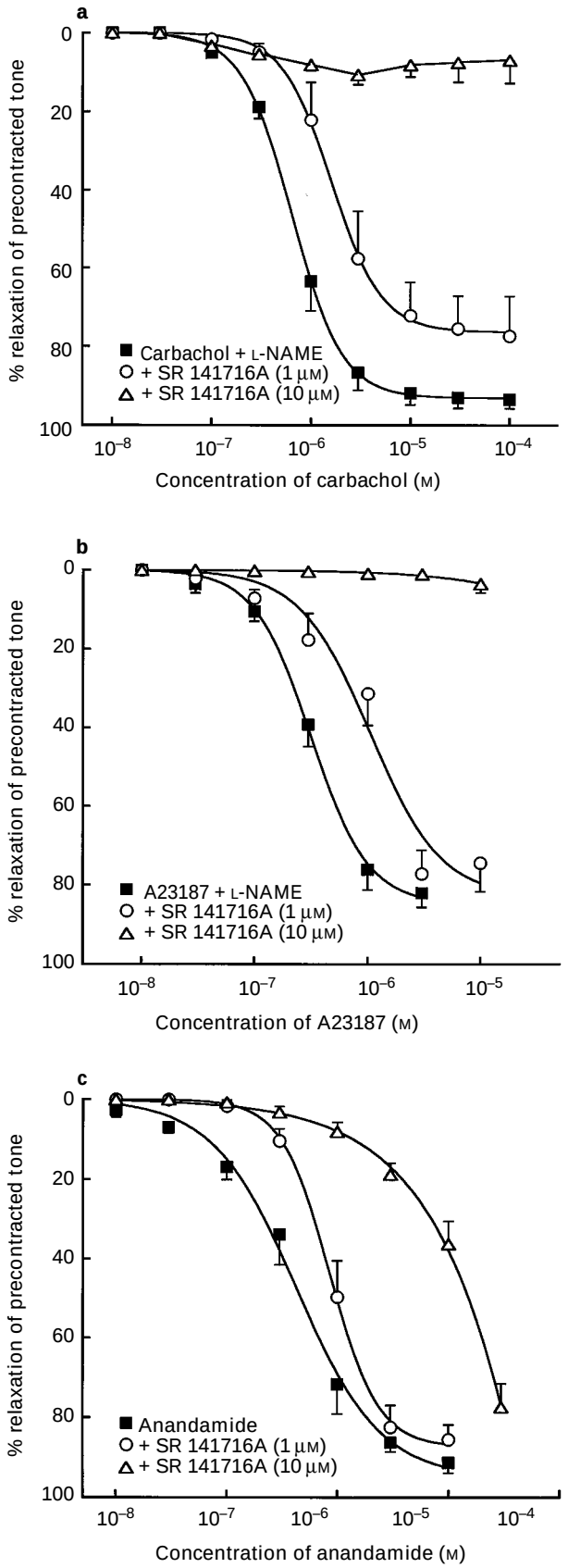

Figure 1 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery in the presence of 100 μM L-NAME and 10 μM indomethacin. Relaxation was induced by (a) carbachol, (b) A23187 or (c) anandamide and was determined in the presence and absence of the cannabinoid antagonist SR 141716A. Values are shown as mean and vertical lines indicate s.e.mean, $n = 4-8$. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text.

Effects of pertussis toxin

Figure 3 shows that incubation of mesenteric arteries with PTX (400 ng ml^{-1}; 2 h) resulted in a significant reduction in the maximal relaxations to carbachol, A23187 or anandamide (all used at a submaximal concentration of 1 μM). In each case the relaxant response was reduced to less than 20%.

Effects of PMSF

In the presence of 100 μM L-NAME, the addition of PMSF (200 μM) significantly ($P<0.01$) enhanced the vasorelaxant effect of carbachol by 5.5 fold (pEC$_{50}$: control, 6.25 ± 0.01; PMSF, 7.00 ± 0.01; $n=4$). However, the relaxant effect of carbachol in the absence of L-NAME was also significantly ($P<0.001$) enhanced by PMSF by 2.9 fold (pEC$_{50}$: control, 6.41 ± 0.04; PMSF, 6.88 ± 0.04; $n=4$), and it should also be noted that PMSF significantly potentiated the relaxant effects of SNAP by 3 fold (pEC$_{50}$: control, 7.51 ± 0.06; PMSF, 8.00 ± 0.05, $n=4$, $P<0.001$) without affecting the maximum responses (control, 94.2 ± 1.6%; PMSF, 95.9 ± 2.3%).

The presence of PMSF also significantly ($P<0.01$) enhanced the L-NAME-resistant relaxation to A23187 by 2 fold (pEC$_{50}$: control, 6.45 ± 0.04; PMSF, 6.74 ± 0.10; $n=4$; Figure 4a). The potency of anandamide was enhanced by 1.9 fold by the presence of the protease inhibitor (pEC$_{50}$: control, 6.31 ± 0.02; PMSF, 6.61 ± 0.08; $n=8$; $P<0.01$; Figure 4b).

Effects of K$^+$ channel blockade with TEA

TEA (1 mM) significantly ($P<0.01$) reduced the potency of carbachol (pEC$_{50}$: control, 6.19 ± 0.01; TEA, 5.61 ± 0.01; $n=6$; Figure 5a), but had no significant effects on vasorelaxation

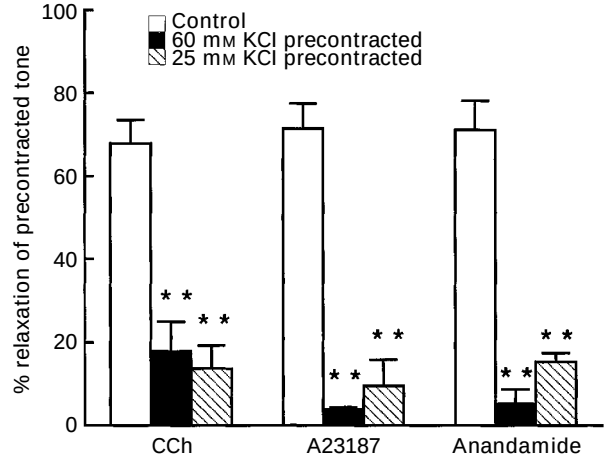

Figure 2 Relaxations of the rat isolated mesenteric artery precontracted with methoxamine, 60 mM K$^+$ or 25 mM K$^+$, in the presence of 100 μM L-NAME and 10 μM indomethacin. Relaxations were induced by carbachol, A23187 or anandamide (all at 1 μM). Values are shown as mean and vertical lines indicate s.e.mean ($n=4$ in each case). $^{**}P<0.01$ versus relaxations of methoxamine-precontracted tone.

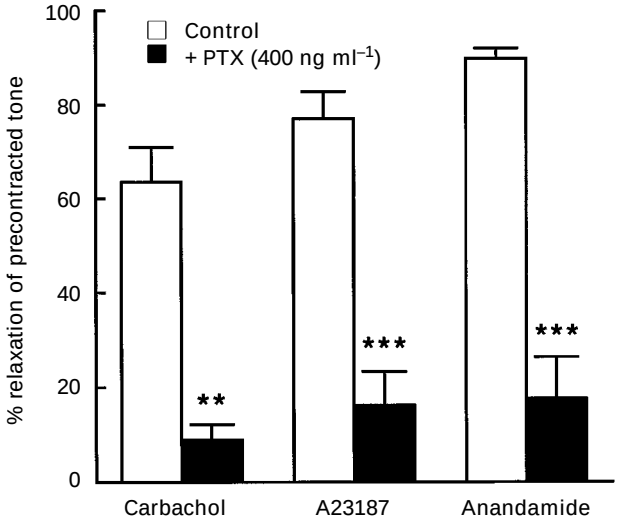

Figure 3 Effect of pertussis toxin (PTX; incubation at 37°C with 400 ng ml^{-1} for 2 h) on relaxations of the methoxamine-precontracted isolated mesenteric artery in the presence of 100 μM L-NAME and 10 μM indomethacin. Relaxations were induced by carbachol, A23187 or anandamide (all at 1 μM). Values are shown as mean and vertical lines indicate s.e.mean ($n=4$ in each case). $^{**}P<0.01$, $^{***}P<0.001$ versus control relaxations in the absence of pertussis toxin.

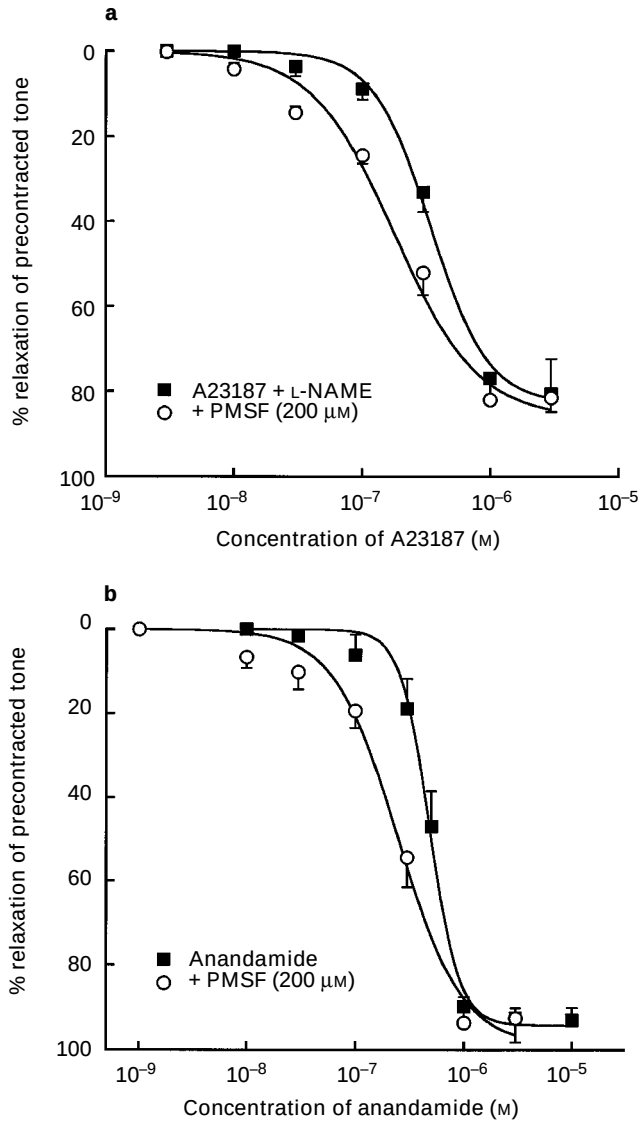

Figure 4 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery in the presence of 100 μM L-NAME and 10 μM indomethacin. Relaxations were induced by (a) A23187 or (b) anandamide and were determined in the presence and absence of the serine protease inhibitor PMSF. Values are shown as mean and vertical lines indicate s.e.mean ($n=4$ in each case). The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text.

produced by A23187 (pEC_{50}: control, 6.47 ± 0.04; TEA, 6.41 ± 0.02; $n=4$; Figure 5b) or anandamide (pEC_{50}: control, 6.28 ± 0.06; TEA, 6.09 ± 0.02; $n=5$; Figure 4c). TEA (1 mM) did not significantly affect the maximal relaxations to any of the three agents tested.

The presence of a higher concentration of TEA (10 mM) further reduced the potency of carbachol ($pEC_{50}=5.39 \pm 0.03$ in the presence of 10 mM TEA; $n=7$; $P<0.001$; Figure 5a). It also significantly ($P<0.05$) reduced the potencies of A23187 ($pEC_{50}=6.17 \pm 0.10$ in the presence of 10 mM TEA; $n=4$; Figure 5b) and anandamide ($pEC_{50}=5.95 \pm 0.09$ in the presence of 10 mM TEA; $n=6$; Figure 5c). TEA (10 mM) had no significant effect on the maximal relaxation caused by carbachol or A23187, but that to anandamide was significantly reduced (control, $94.3 \pm 4.0\%$ versus $60.7 \pm 4.4\%$ in the presence of 10 mM TEA; $n=5$; $P<0.01$).

Effects of Ba^{2+}

$BaCl_2$ at a concentration of 100 μM, where it is considered to be a relatively selective inhibitor of inward-rectifier K^+ channels, had no significant effect on relaxations to carbachol (pEC_{50}: control, 6.30 ± 0.02; Ba^{2+}, 6.29 ± 0.01; $n=4$; Figure 6a) or A23187 (pEC_{50}: control, 6.50 ± 0.04; Ba^{2+}, 6.39 ± 0.09; $n=4$; Figure 6b) but caused a small, but significant ($P<0.01$) shift in the concentration-response curve for anandamide (pEC_{50}: control, 6.39 ± 0.01; Ba^{2+}, 6.20 ± 0.01; $n=4$; Figure 6c). $BaCl_2$ (100 μM) also had no significant effect on the R_{max} for carbachol (control, $94.9 \pm 0.8\%$ versus $97.7 \pm 1.1\%$ in the presence of Ba^{2+}; $n=4$) or A23187 (control $87.7 \pm 3.7\%$ versus $96.6 \pm 0.9\%$, Ba^{2+}; $n=4$) or the maximal relaxation to anandamide (control $91.7 \pm 1.1\%$ versus $94.0 \pm 0.2\%$, Ba^{2+}; $n=4$).

At a concentration of 1 mM, where it is considered to be a non-selective inhibitor of K^+ channels, $BaCl_2$ significantly ($P<0.01$) reduced the potency of carbachol (to a pEC_{50} of 5.65 ± 0.02; $n=4$; Figure 6a), A23187 (to a pEC_{50} of 5.84 ± 0.04; $n=4$; Figure 6b) and anandamide (to a pEC_{50} of 5.95 ± 0.02; $n=4$; Figure 6c). $BaCl_2$ (1 mM) had no significant effect on the maximal relaxation to A23187 ($91.7 \pm 2.4\%$ in the presence of Ba^{2+}), but significantly ($P<0.01$) reduced the R_{max} to carbachol ($82.7 \pm 2.0\%$) and anandamide ($59.2 \pm 1.4\%$).

Effects of K^+ channel blockade with glibenclamide, 4-aminopyridine, apamin, iberiotoxin, charybdotoxin, ciclazindol and their combinations

A range of agents were tested for their abilities to affect the relaxant response to the agents under study. The results for carbachol, A23187 and anandamide are shown in Tables 1, 2 and 3, respectively. Tables 2 and 3 show that none of the agents tested (4-aminopyridine, 50 μM or 1 mM; apamin, 1 μM; iberiotoxin, 50 nM; charybdotoxin, 100 nM; glibenclamide, 10 μM and ciclazindol, 10 μM), when used alone, significantly affected the potency or maximal responses to A23187 or anandamide. Also without effect were combinations of apamin with glibenclamide or iberiotoxin, or of apamin with glibenclamide and 4-aminopyridine.

In contrast, Table 1 shows that apamin, 4-aminopyridine and ciclazindol significantly reduced the potency of carbachol by 2.5, 3.4 and 2.5 fold, respectively. Apamin also reduced the maximal response to carbachol, although no significant change was noted when apamin was combined with glibenclamide. It should be noted that the combination of glibenclamide or 4-aminopyridine, or both, with apamin caused no further effect on the potency of carbachol over that seen with apamin alone.

On the other hand, it can be seen from Figure 7a that a combination of apamin (1 μM) and charybdotoxin (100 nM) almost completely abolished the relaxation to carbachol. This toxin combination had no significant effect on relaxation to A23187 (Figure 7b) but a combination of apamin (1 μM) with a higher concentration of charybdotoxin (300 nM) significantly reduced the relaxant response to A23187 (Figure 7b). The combination of apamin with the higher concentration of

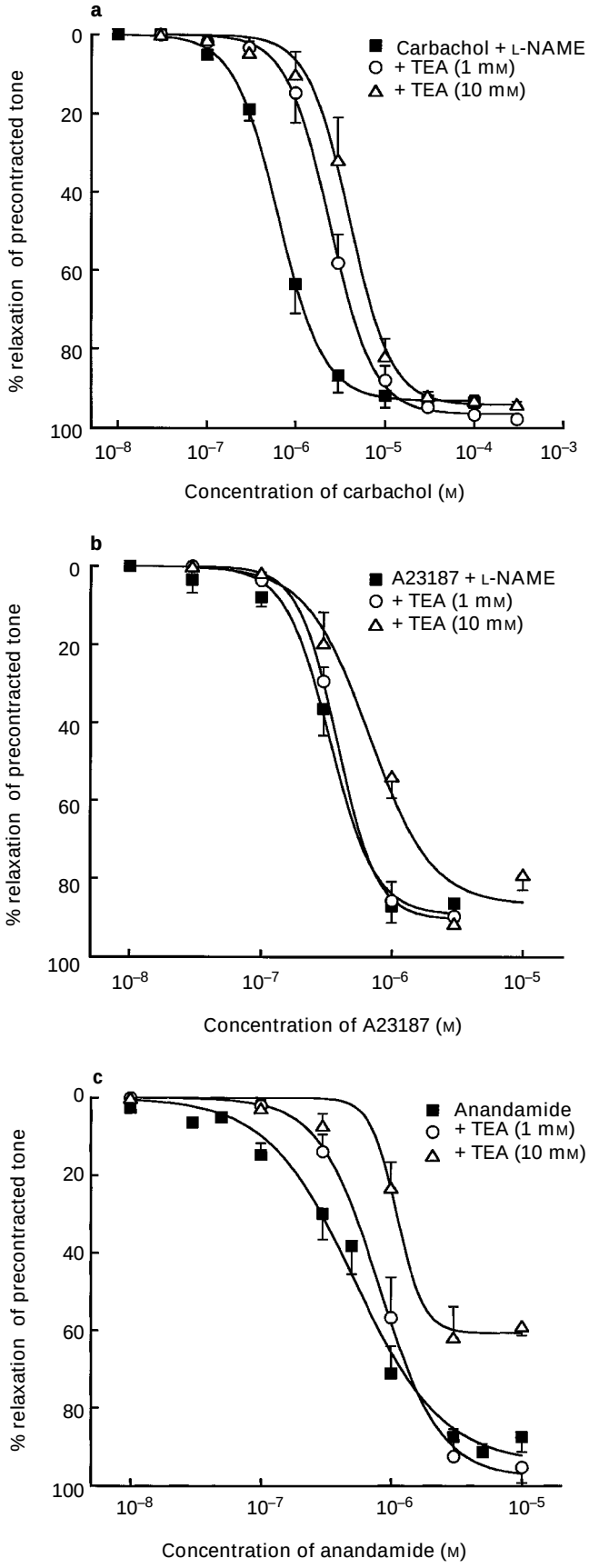

Figure 5 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery in the presence of 100 μM L-NAME and 10 μM indomethacin. Relaxations were induced by (a) carbachol, (b) A23187 or (c) anandamide in the presence and absence of TEA. Values are shown as mean and vertical lines indicate s.e.mean, $n=4-7$. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text.

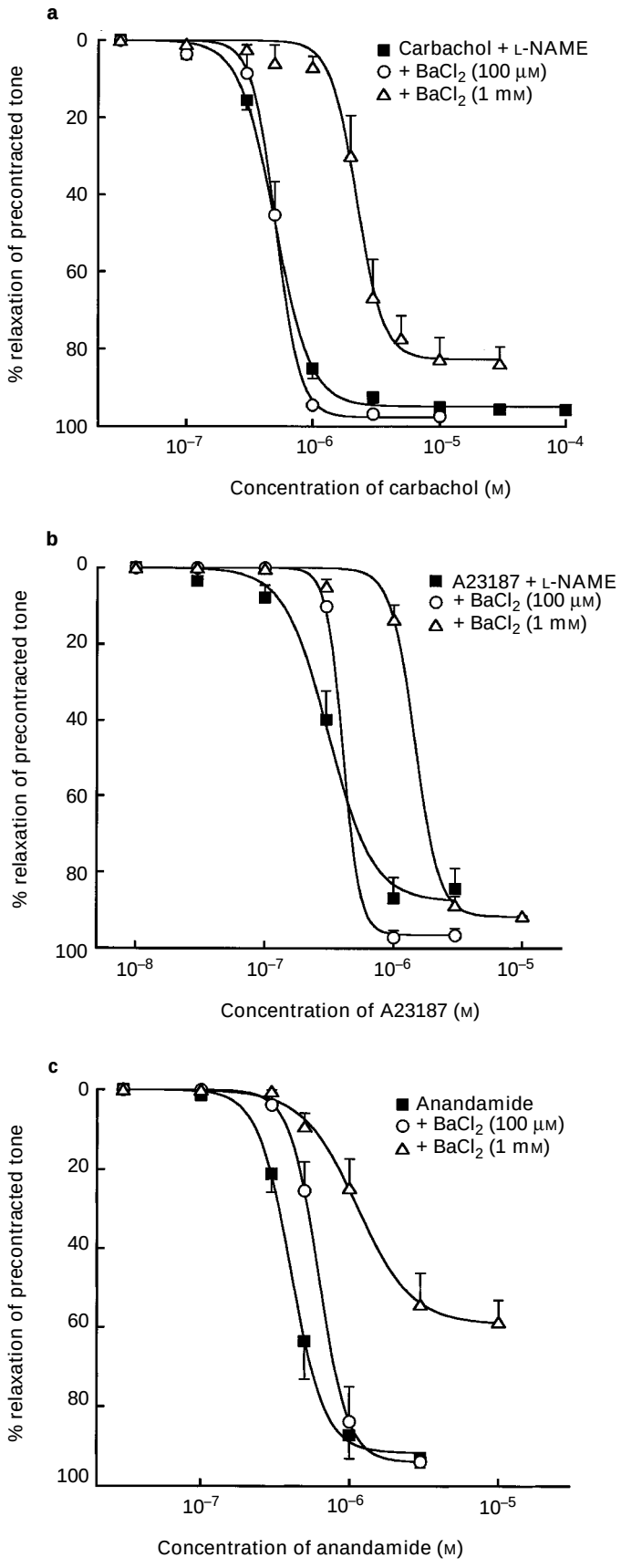

Figure 6 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery in the presence of 100 μM L-NAME and 10 μM indomethacin. Relaxations were induced by (a) carbachol, (b) A23187 or (c) anandamide in the presence and absence of barium chloride. Values are shown as mean and vertical lines indicate s.e.mean, $n=4$. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text.

charybdotoxin caused no further inhibition of relaxation to carbachol (data not shown). Neither combination of apamin (1 μM) with 100 nM (data not shown) or 300 nM charybdotoxin had any significant effect on relaxations to anandamide (Figure 7c).

A combination of apamin (1 μM) and ciclazindol (10 μM) caused almost complete inhibition of L-NAME-resistant relaxations to carbachol (Figure 7a) and A23187 (Figure 7b), but had no significant effect on relaxation to anandamide (Figure 7c).

Discussion

The aim of the present study was to compare the relaxations to anandamide with EDHF released by both receptor-dependent and -independent mechanisms. EDHF released by stimulating muscarinic receptors with carbachol in the presence of L-NAME produces relaxations which will be sensitive to blockade both at the level of the endothelium and smooth muscle cell. On the other hand, responses to EDHF released by the calcium ionophore A23187, also in the presence of L-NAME, will be sensitive to K^+ channel blockade at the level of the smooth muscle cell, but relatively insensitive to block at the endothelium. This is because the increase in intracellular Ca^{2+} caused by this compound is thought to occur via electroneutral exchange of one Ca^{2+} for two H^+ ions (Reed & Lardy, 1972), which would not be sensitive to endothelial membrane potential.

It is not feasible to use the effects of K^+ channel blocking agents on carbachol in the absence of L-NAME, as a test for the effects of these blockers on endothelial cells, for two reasons. Firstly, nitric oxide activates K^+ channels in some tissues (Plane *et al.*, 1996), and thus its actions may be affected by K^+ channel blockers. Secondly, it is possible that the attenuating effect of such agents on increases in cytoplasmic Ca^{2+} in endothelial cells may not be the same for nitric oxide and EDHF synthesis. Indeed, the threshold of intracellular Ca^{2+} concentration for prostacyclin formation in endothelial cells is markedly greater than that for nitric oxide synthesis (Parsaee *et al.*, 1992).

Effects of nitric oxide synthase inhibition

Addition of 100 μM L-NAME to mesenteric arteries caused a rightward shift of approximately two fold in the concentration-response curve to carbachol, but had no effect on the maximal relaxation. In contrast, anandamide caused concentration-dependent relaxations of the rat isolated mesenteric artery that were not affected either by removal of the endothelium, or by the presence of L-NAME, which is consistent with the action of exogenous anandamide as an endothelium-independent vasorelaxant. Bauersachs *et al.* (1996) showed that nitric oxide might inhibit EDHF release by decreasing cytoplasmic Ca^{2+} in endothelial cells, as this would inhibit the production of arachidonic acid by phospholipase A_2. Although their conclusions were made with respect to EDHF being a cytochrome P450 metabolite of arachidonic acid, they are just as valid if EDHF were anandamide, or a related cannabinoid agent, as these are also derivatives of arachidonic acid. If nitric oxide does inhibit EDHF activity at the level of synthesis, rather than at its site of action on vascular smooth muscle, inhibition of nitric oxide synthesis by L-NAME would be expected to have no effect on exogenous addition of EDHF.

Effects of the cannabinoid receptor antagonist, SR 141716A

The cannabinoid receptor antagonist SR 141716A (1 μM) caused a significant rightward shift of the concentration-response curves to anandamide, and to carbachol and A23187 in the presence of L-NAME. The shift was approximately the same in each case (carbachol, 2.5 fold; A23187, 3.3 fold; anandamide, 2 fold) and a 2 fold shift was observed with

Table 1 Effects of K^+ channel blockers on L-NAME-resistant relaxation to carbachol in rat isolated mesenteric arteries with intact endothelium and precontracted with methoxamine

Treatment	pEC_{50}	R_{max} (%)	n_H	n
Control	6.19±0.01	93.2±0.4	1.7±0.1	10
Glibenclamide (10 μM)	6.09±0.05	94.7±2.7	1.3±0.2	8
4-Aminopyridine (50 μM)	6.04±0.03	95.9±1.9	1.4±0.1	8
4-Aminopyridine (1 mM)	5.66±0.01**	91.6±1.0	2.7±0.2**	5
Control	6.19±0.01	91.4±0.6	1.7±0.1	7
Apamin (1 μM)	5.80±0.03*	82.1±2.2**	1.2±0.1	7
Apamin (1 μM)+glibenclamide (10 μM)	5.88±0.04*	91.5±2.7	2.4±0.4	6
Apamin (1 μM)+glibenclamide (10 μM)+4-aminopyridine (1 mM)	5.63±0.04*	85.8±2.0**	1.1±0.1	4
Control	6.30±0.02	94.9±0.8	3.1±0.2	8
Iberiotoxin (50 nM)	6.46±0.03	96.9±2.4	2.3±0.4	5
Apamin (1 μM)+iberiotoxin (50 nM)	6.34±0.05	93.4±3.4	2.3±0.4	4
Charybdotoxin (100 nM)	6.05±0.03	96.5±0.3	5.6±0.3**	8
Ciclazindol (10 μM)	5.90±0.01**	97.8±0.9	7.9±1.2**	4

Data are presented as mean±s.e.mean. pEC_{50}, R_{max} and n_H values were derived by the curve-fitting procedure described in Methods. *n* values denote the number of animals tested. $^*P<0.05$, $^{**}P<0.01$ indicate significant differences from control values as calculated by analysis of variance followed by Dunnett's *post-hoc* test.

Table 2 Effects of K^+ channel blockers on L-NAME-resistant relaxation to A23187 in rat isolated mesenteric arteries with intact endothelium and precontracted with methoxamine

Treatment	pEC_{50}	R_{max} (%)	n_H	n
Control	6.46±0.04	83.6±3.6	2.0±0.4	8
Glibenclamide (10 μM)	6.44±0.03	89.4±2.7	2.5±0.4	4
4-Aminopyridine (1 mM)	6.40±0.06	99.7±5.7	3.0±1.0	4
Apamin (1 μM)	6.34±0.03	77.3±2.2	2.0±0.2	4
Apamin (1 μM)+glibenclamide (10 μM)+4-aminopyridine (1 mM)	6.40±0.05	101.2±14.7	1.7±0.8	4
Control	6.47±0.04	89.3±3.9	2.5±0.7	6
Iberiotoxin (50 nM)	6.27±0.03	90.8±1.8	4.0±0.4	4
Apamin (1 μM)+iberiotoxin (50 nM)	6.39±0.04	95.5±3.3	3.4±0.9	4
Charybdotoxin (100 nM)	6.34±0.01	97.0±0.2	5.9±0.3*	4
Ciclazindol (10 μM)	6.38±0.04	97.0±3.0	3.8±1.0	4

Data are presented as mean±s.e. mean. pEC_{50}, R_{max} and n_H values were derived by the curve-fitting procedure described in Methods. *n* values denote the number of animals tested. $^*P<0.05$ indicates significant difference from control values as calculated by analysis of variance followed by Dunnett's *post-hoc* test.

Table 3 Effects of K^+ channel blockers on relaxation to anandamide in rat isolated mesenteric arteries with intact endothelium and precontracted with methoxamine in the presence of 100 μM L-NAME

Treatment	pEC_{50}	R_{max} (%)	n_H	n
Control	6.44±0.04	90.6±4.3	2.4±0.6	10
Glibenclamide (10 μM)	6.34±0.05	91.2±3.5	1.5±0.2	6
4-Aminopyridine (50 μM)	6.34±0.01	92.4±0.9	1.7±0.1	6
4-Aminopyridine (1 mM)	6.43±0.04	92.1±2.5	3.9±1.6	4
Apamin (1 μM)	6.36±0.01	96.5±1.1	2.7±0.1	6
Apamin (1 μM)+glibenclamide (10 μM)	6.38±0.05	93.3±3.9	2.5±0.6	5
Apamin (1 μM)+glibenclamide (10 μM)+4-aminopyridine (1 mM)	6.47±0.01	95.7±1.1	2.2±0.2	4
Control	6.39±0.01	91.7±1.1	3.8±0.2	8
Iberiotoxin (50 nM)	6.36±0.04	97.3±3.1	3.7±0.8	5
Apamin (1 μM)+iberiotoxin (50 nM)	6.41±0.03	96.6±2.3	3.5±0.7	4
Charybdotoxin (100 nM)	6.34±0.02	96.1±1.5	3.8±0.4	4
Ciclazindol (10 μM)	6.42±0.02	97.1±0.8	5.0±0.8	4

Data are presented as mean±s.e. mean. pEC_{50}, R_{max} and n_H values were derived by the curve-fitting procedure described in Methods. *n* values denote the number of animals tested. There were no significant differences ($P<0.05$) from control values, as calculated by analysis of variance followed by Dunnett's *post-hoc* test.

anandamide in the absence of endothelium, which shows that anandamide and SR 141716A were acting directly on vascular smooth muscle. These shifts are consistent with SR 141716A having an affinity for a receptor activated by all three agents in the range 400 nM–1 μM. This is much lower than that for binding of SR 141716A to the central CB_1 receptor (11.8 nM, Felder *et al*., 1995; 12.3 nM, Showalter *et al*., 1996) or the CB_{1A} receptor (43.3 nM, Rinaldi-Carmona *et al*., 1996) and suggests that the receptor activated by EDHF and anandamide may be a CB_2-like receptor, as the effects of 1 μM SR 141716A are consistent with the affinity of this antagonist for this receptor (702 nM; Showalter *et al*., 1996).

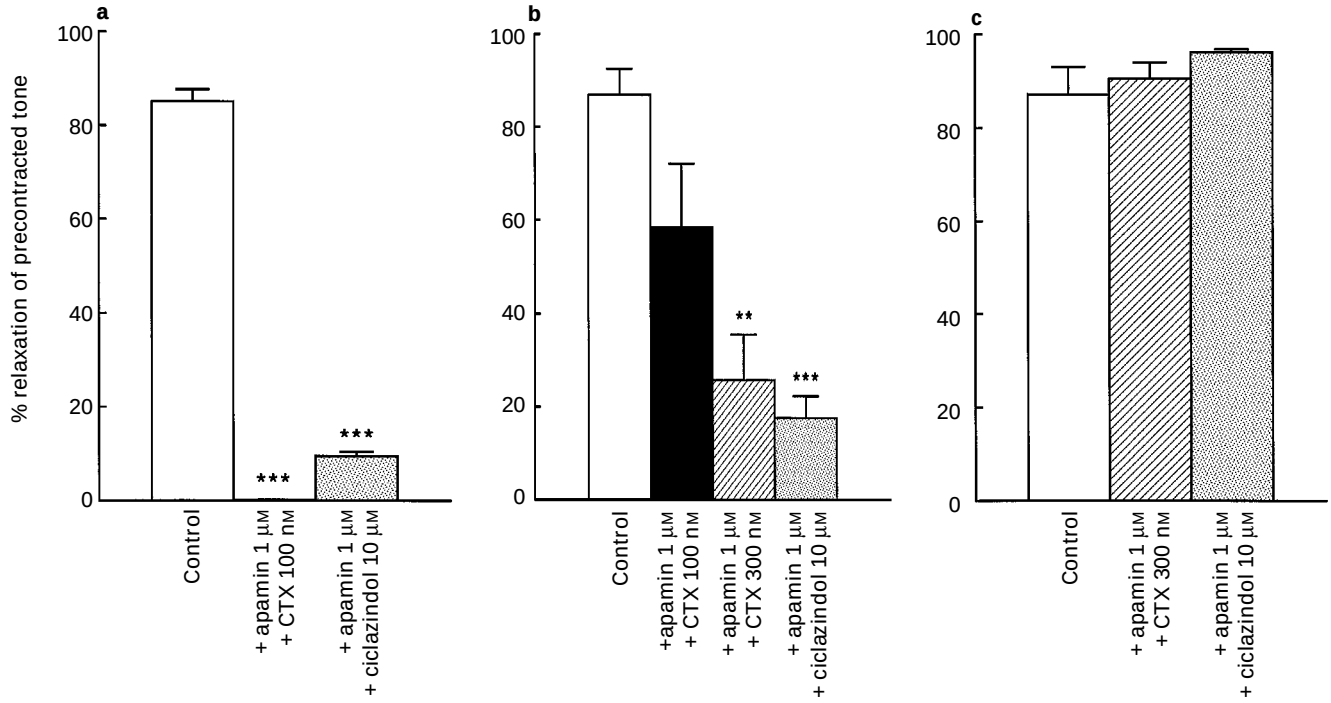

Figure 7 Effect of combinations of K^+ channel blocking agents on relaxation of methoxamine-induced tone by 1 μM concentrations of (a) carbachol, (b) A23187 and (c) anandamide in the rat isolated mesenteric artery in the presence of 100 μM L-NAME and 10 μM indomethacin. Values are shown as mean and vertical lines indicate s.e.mean, $n=4$. $^{**}P<0.01$, $^{***}P<0.001$ versus control relaxations in the absence of K^+ channel blockers.

The central CB_1, CB_{1A} and peripheral CB_2 receptors are all coupled to PTX-sensitive G-proteins and inhibit adenylyl cyclase (Felder *et al.*, 1995; Rinaldi-Carmona *et al.*, 1996), although the CB_1 receptor also activates an inward rectifier K^+ channel and inhibits Q-type Ca^{2+} channels when expressed in AtT20 cells, whereas the CB_2 receptor does not (Felder *et al.*, 1995). Although 10 μM SR 141716A abolished EDHF-mediated relaxation to carbachol and to A23187, this concentration only caused a further rightward shift, to approximately ten fold, in the concentration-response curve to anandamide, consistent with the action of SR 141716A as a competitive cannabinoid receptor antagonist. It is possible that 10 μM SR 141716A has additional inhibitory effects; although, in this study, it did antagonize levcromakalim in mesenteric vessels denuded of endothelium, it did not affect relaxations induced by the nitric oxide donor, SNAP. It should be noted that, at 1 μM, SR 141716A had no effect on the vasorelaxation to levcromakalim.

Randall *et al.* (1996) were the first to show that SR 141716A antagonized the effects of both EDHF and anandamide in rat perfused mesentery and, while the present paper was in preparation, other groups have published, in abstract or short communication form, comparisons with this cannabinoid receptor antagonist in rat mesenteric artery. Plane *et al.* (1997) found that 5 μM SR 141716A did not antagonize the responses to either EDHF or anandamide. Similarly, Chataigneau *et al.* (1997) also showed that 1 μM SR 141716A had no effect on anandamide- or acetylcholine-induced hyperpolarization. On the basis of our results, the concentration of anandamide (30 μM) used by Chataigneau *et al.* (1997) was supramaximal, and so the small degree of antagonism that would be produced by 1–3 μM SR 141716A would not be seen. The cause of the discrepancies with Plane *et al.* (1997) is not clear, but is not likely to be due to them using two nitric oxide synthesis inhibitors together in their experiments. The different sources of anandamide might be relevant; whereas our material was synthesized and purified by Dr E.A. Boyd of the University of Nottingham, they used commercially available material from Calbiochem which does not have the same chromatographic profile (M.D. Randall, personal communication). Similarly, the sources of SR 141716A were different. Other evidence in the abstract of Chataigneau *et al.* (1997) suggests differing effects of SR 141716A on both anandamide and EDHF in different vessels. In addition, at the same meeting, Campbell *et al.* (1997) demonstrated that 1 μM SR 141716A did not antagonize anandamide (which only acted in the presence of endothelium, unlike in the present study) in the bovine coronary artery and showed that anandamide was probably acting by being converted to vasodilator eicosanoids. Therefore, there appears to be heterogeneity of cannabinoid drug action when examined in different vessels.

Involvement of pertussis toxin-sensitive G-proteins

Incubation of tissues with PTX inhibited EDHF-mediated relaxations to both carbachol and A23187 and to anandamide. This suggests that both anandamide and EDHF cause relaxation via activation of PTX-sensitive G-proteins. PTX also inhibits the actions of EDHF released by A23187 in bovine coronary artery (Graier *et al.*, 1996). It is possible that PTX may have a further effect on L-NAME-resistant relaxations to carbachol by inhibiting the transduction mechanism for muscarinic receptors on endothelial cells. However this seems unlikely as Adeagbo & Triggle (1993) showed that the muscarinic receptor activated in the rat mesenteric bed is of the M_3 subtype, which is coupled to $G_{q/11}$, a PTX-insensitive G-protein.

Effect of phenylmethylsulphonylfluoride (PMSF)

Pretreatment with PMSF potentiated the vasorelaxant effects of anandamide and A23187 in the presence of L-NAME by approximately 2 fold. Thus the processes mediating breakdown of anandamide and of EDHF released by A23187 in the presence of L-NAME might be similar, as it would appear unlikely that a serine protease would mediate breakdown of A23187 itself to any great extent. The potentiation of relaxa-

tions to anandamide is consistent with the results of Pertwee *et al.* (1995), who showed that it is broken down by a PMSF-sensitive process, probably the enzyme anandamide amidase. However, although PMSF potentiated the effects of carbachol in the presence or absence of L-NAME, the relaxations in the presence of L-NAME were potentiated to a greater extent than those of anandamide. Control experiments showed that carbachol alone was also potentiated by approximately 3 fold by PMSF, and the results with SNAP show that nitric oxide-mediated responses were also potentiated. Therefore potentiation by PMSF is not a suitably simple criterion for testing for the presence of an endocannabinoid.

Involvement of K^+ channels

The vasorelaxant effects of endogenous EDHF, released by either carbachol or A23187 in the presence of L-NAME, and that of anandamide, were greatly reduced by precontracting vessels with either 60 mM or 25 mM K^+ Krebs-Henseleit buffer. This is consistent with these agents causing vasorelaxation by increasing K^+ conductance, although it is also possible that the observed inhibition was caused by the depolarizing effects of high K^+. Although Plane *et al.* (1997) found that responses to anandamide and EDHF in the rat mesenteric artery were inhibited by 60 mM KCl, the response to anandamide was not affected by 25 mM KCl. It is not clear why these differences between the two studies occurred, though, as noted above, it may be that the different sources of the anandamide used are important.

Actions of K^+ channel blockers on EDHF-mediated relaxation to carbachol and A23187

TEA (1 mM) had no significant effect on EDHF-mediated relaxation to A23187, but significantly inhibited relaxation to carbachol. This could be due to blockade of BK_{Ca} on the endothelium, which may inhibit EDHF release. However, this seems unlikely as the highly selective BK_{Ca} blocker iberiotoxin (Galvez *et al.*, 1990) did not inhibit relaxations to carbachol or A23187 in the present study. It is more likely that the observed inhibition was due to the antimuscarinic properties of TEA (Balduni *et al.*, 1990), although BK_{Ca} might have a role as Olesen & Hansen (1995) showed that iberiotoxin completely inhibited EDHF-mediated relaxation in the rat mesenteric artery.

The effects of Ba^{2+} (1 mM) suggest that K^+ channel blockade can affect relaxation to carbachol since, although it attenuated relaxation to both carbachol and A23187, only the maximal relaxation to carbachol was reduced. Although apamin attenuated relaxation to carbachol, consistent with previous studies on the rat mesenteric artery (Waldron & Garland, 1994b; Hutri-Kähönen *et al.*, 1997), it did not affect relaxation to A23187. Similar effects were seen for 4-aminopyridine (at 1 mM, but not 50 μM) and for ciclazindol, an inhibitor of K_v (Noack *et al.*, 1992b) and of K_{ATP} (Noack *et al.*, 1992a). Zakharenko (1996) showed that EDHF-mediated hyperpolarizations of the rat mesenteric artery could be abolished by 5 mM 4-aminopyridine; the discrepancy with the present study could be due to the use of a higher concentration. Combinations of apamin and glibenclamide with 4-aminopyridine were no more effective at blocking relaxation to carbachol than apamin or 4-aminopyridine alone, and had no effect on relaxations to A23187.

Taken together, these data suggest that the effects of 4-aminopyridine and ciclazindol might be due to inhibition of endothelial K_v, and the effect of apamin to inhibition of endothelial small conductance Ca^{2+}-activated channels (SK_{Ca}). As elevation of cytoplasmic Ca^{2+} in endothelial cells is thought to be modulated by K^+ channel activation (Groschner *et al.*, 1992), with the resulting hyperpolarization increasing the electrochemical gradient for Ca^{2+} entry, blockade of endothelial K^+ channels may therefore reduce synthesis of EDHF. Indeed, Groschner *et al.* (1992) showed that apamin-sensitive SK_{Ca} regulate endothelial membrane potential and its responses to vasodilator agonists, whilst Chen & Cheung (1992) have demonstrated a similar role for 4-aminopyridine-sensitive K^+ channels.

The differential sensitivity to K^+ channel blocking agents of L-NAME-resistant relaxations to carbachol and A23187 could be explained otherwise. For example, if there were more than one EDHF, the extent of the release by carbachol and A23187 may not be identical. However, it is clear that caution must be exercised in using the effects of K^+ channel blockers on endothelium-dependent vasodilators to define the K^+ conductances activated by EDHF.

Effects of K^+ channel blocking agents on relaxation to A23187 and anandamide

The findings of the present study with regard to EDHF are generally consistent with those of Waldron & Garland (1994b) and suggest that the actions of EDHF in the rat isolated mesenteric artery are very similar to those of EDHF in the guinea-pig carotid (Corriu *et al.*, 1996) and basilar arteries (Petersson *et al.*, 1997) and rat hepatic artery (Zygmunt & Högestätt, 1996).

TEA (10 mM) attenuated relaxations to both A23187 and anandamide, consistent with each activating K^+ conductances. However, only the maximal response to anandamide was reduced. Also, Ba^{2+} (100 μM) had no effect on relaxation to A23187, but caused a small, statistically significant, rightward shift in the concentration-response curve to anandamide. At 1 mM, Ba^{2+} attenuated relaxations to both agents, although again only the maximal response to anandamide was reduced. Thus, the greater sensitivity of anandamide-induced relaxations to TEA and Ba^{2+} may reflect activation of different K^+ conductances to EDHF released by A23187.

Relaxations to anandamide and A23187 were not affected by apamin, glibenclamide or 4-aminopyridine, either alone or in combination. It is generally agreed that glibenclamide has no effect on EDHF (Waldron & Garland, 1994b) but Chataigneau *et al.* (1997) showed that glibenclamide inhibited hyperpolarization to anandamide. However, the present results show ciclazindol, another K_{ATP} inhibitor, has no effect on anandamide, which argues against a role for this channel in mediating relaxation to anandamide. The insensitivity to apamin of the A23187 response is evidence that the effector mechanism for EDHF might vary between tissues as data have been presented on activation of SK_{Ca} in rabbit mesenteric (Murphy & Brayden, 1995), porcine coronary (Hecker *et al.*, 1994), bovine coronary (Graier *et al.*, 1996) and bovine oviductal arteries (Garcia-Pascual *et al.*, 1995). The effector mechanism for EDHF may even vary between different sized vessels in the same preparation, since Adeagbo & Triggle (1993) observed that the actions of EDHF in the rat perfused mesenteric bed were totally inhibited by apamin which is contrary to the findings of the present study. However, the mesenteric vessels used here are of larger diameter than those that influence overall vascular resistance in the perfused mesenteric bed. It is possible that the variation in the apparent effector mechanisms for EDHF is due to differing distribution of K^+ channel subtypes between and within tissues. Indeed, Berman & Griffith (1997) have recently shown that the effects of charybdotoxin vary between successive generations of the rabbit ear artery, suggesting that there may be heterogeneity of K^+ channel distribution in arterial beds.

Neither ciclazindol, charybdotoxin or iberiotoxin had any effect on relaxations to anandamide or A23187. The former findings are in contrast to those of Plane *et al.* (1997), who showed that anandamide-induced relaxation of the rat isolated mesenteric artery could be inhibited by both charybdotoxin and iberiotoxin. It is again possible that the different sources of anandamide used in each study may be important and result in different chemical entities eliciting the observed responses. The lack of effect of charybdotoxin and iberiotoxin on relaxation to anandamide in the present study argues against activation of

BK_{Ca}, and this is supported by our observation that 1 mM TEA did not inhibit anandamide-induced relaxation.

EDHF released by A23187 was inhibited by a combination of apamin and charybdotoxin, or apamin and ciclazindol, but not by these agents alone. It is possible that EDHF activates two separate K^+ conductances, one sensitive to apamin which is probably SK_{Ca} and one sensitive to charybdotoxin and ciclazindol which is distinct from K^+ channels sensitive to blockade by iberiotoxin (BK_{Ca}), 4-aminopyridine (K_v) and glibenclamide (K_{ATP}), with each conductance capable of causing full relaxation alone. However, it is also possible that EDHF activates a single K^+ channel subtype, to which both apamin and charybdotoxin or ciclazindol must bind for inhibition to occur (see Zygmunt *et al*., 1997). Relaxation to anandamide was not affected by the combination of apamin with charybdotoxin or ciclazindol, which is evidence that anandamide may not be identical with the EDHF released in the rat isolated mesenteric artery.

Conclusions

These results show that the choice of agent to release EDHF may be important when comparing the actions of K^+ channel blockers on endogenous EDHF and putative exogenous EDHFs, as these blockers could affect endothelial K^+ channels and thus inhibit EDHF release as well as its action. Comparison of the relaxations induced by EDHF released by A23187 and the endocannabinoid anandamide showed similarity between their pharmacological sensitivity to SR 141716A, PMSF and disturbance of transmembrane K^+ gradients. The degradation processes for EDHF generated by A23187 and anandamide may be similar, since responses to both were potentiated to a similar extent by PMSF, but the results with the protease inhibitor and carbachol suggest that the effects of PMSF might be more complex than simply blocking EDHF breakdown. There are important differences in the sensitivity of each to K^+ channel blocking agents, suggesting that anandamide is not identical to EDHF in the rat small mesenteric artery. This overall conclusion agrees with those of Plane *et al*. (1997) and Chataigneau *et al*. (1997), despite the differences in the results presented in the three studies. The diversity of results, especially from different tissues, suggests that EDHF activity may be due to more than one chemical entity, and further investigation of the relationship to cannabinoid receptor activation awaits use of other cannabinoid antagonists.

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