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Anandamide-induced relaxation of sheep coronary arteries: the role of the vascular endothelium, arachidonic acid metabolites and potassium channels

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1 The effects of the endocannabinoid, anandamide, and its metabolically stable analogue, methanandamide, on induced tone were examined in sheep coronary artery rings *in vitro*.

2 In endothelium-intact rings precontracted to the thromboxane A_2 mimetic, U46619, anandamide (0.01–30 μM) induced slowly developing concentration-dependent relaxations (pEC_{50} [negative log of EC_{50}] = 6.1 ± 0.1; R_{max} [maximum response] = 81 ± 4%). Endothelium denudation caused a 10 fold rightward shift of the anandamide concentration-relaxation curve without modifying R_{max}. Methanandamide was without effect on U46619-induced tone.

3 The anandamide-induced relaxation was unaffected by the cannabinoid receptor antagonist, SR 141716A (3 μM), the vanilloid receptor antagonist, capsazepine (3 and 10 μM) or the nitric oxide synthase inhibitor, L-NAME (100 μM).

4 The cyclo-oxygenase inhibitor, indomethacin (3 and 10 μM) and the anandamide amidohydrolase inhibitor, PMSF (70 and 200 μM), markedly attenuated the anandamide response. The anandamide transport inhibitor, AM 404 (10 and 30 μM), shifted the anandamide concentration-response curve to the right.

5 Precontraction of endothelium-intact rings with 25 mM KCl attenuated the anandamide-induced relaxations (R_{max} = 7 ± 7%), as did K^+ channel blockade with tetraethylammonium (TEA; 3 μM) or iberiotoxin (100 nM). Blockade of small conductance, Ca^{2+}-activated K^+ channels, delayed rectifier K^+ channels, K_{ATP} channels or inward rectifier K^+ channels was without effect.

6 These data suggest that the relaxant effects of anandamide in sheep coronary arteries are mediated in part *via* the endothelium and result from the cellular uptake and conversion of anandamide to a vasodilatory prostanoid. This, in turn, causes vasorelaxation, in part, by opening potassium channels.

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Abbreviations: AM 404, N-(4-hydroxyphenyl) arachidonylamide; 4-AP, 4-aminopyridine; EDRF, endothelium-derived relaxing factor; GTN, glyceryl trinitrate; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; 17-ODYA, 17-octadecynoic acid; PMSF, phenylmethylsulphonyl fluoride; SR 141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; U46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$

Introduction

The endocannabinoid, anandamide, has been shown to exert potent dilator effects in a variety of vascular beds and preparations (Randall *et al*., 1996; Fulton & Quilley, 1998; Pratt *et al*., 1998; Zygmunt *et al*., 1999), and to induce hypotension in conscious and anaesthetized rats and guinea-pigs (Lake *et al*., 1997; Calignano *et al*., 1997). However, its precise mechanism(s) of vasorelaxation remains as yet unclear and controversial (Randall & Kendall, 1998a; Kunos *et al*., 2000).

In the rat mesenteric and coronary vaculatures, anandamide-induced relaxations were independent of the endothelium and endothelium-derived relaxing factors (Randall *et al*., 1996; Plane *et al*., 1997; White & Hiley, 1997). By contrast, the anandamide effect in rat renal arterioles, bovine coronary arteries and rabbit cerebral and mesenteric arteries was endothelium-dependent and/or susceptible to blockade by nitric oxide synthase (Deutsch *et al*., 1997) or cyclo-oxygenase inhibition (Ellis *et al*., 1995; Pratt *et al*., 1998; Fleming *et al*., 1999). Similarly, in several studies in rat coronary, mesenteric, renal and rabbit mesenteric arteries, the vasorelaxant effects of anandamide were antagonized by the selective cannabinoid (CB_1) receptor antagonist, SR 141716A, suggesting the involvement of CB_1 receptors (Randall *et al*., 1996; Deutsch *et al*., 1997; White & Hiley, 1997; Fulton & Quilley, 1998; Chaytor *et al*., 1999). In contrast, the relaxant effect of anandamide in other studies in rat mesenteric and bovine coronary arteries was SR 141716A-resistant (Plane *et al*., 1997; Pratt *et al*., 1998; Zygmunt *et al*., 1999). Indeed, in a recent study in guinea-pig basilar and rat hepatic and mesenteric arteries, anandamide-induced relaxation was unrelated to either the endothelium or cannabinoid receptors, but attributable to vanilloid VR1 receptors located on perivascular sensory nerves (Zygmunt *et al*., 1999).

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The precise cellular mechanisms underlying anandamide-induced vasorelaxations are also unclear. The ability of raised external K^+ concentrations (to 25–60 mM) to abolish or attenuate anandamide-induced relaxations is suggestive of the involvement of K^+ channel activation (Randall *et al*., 1996; Plane *et al*., 1997; White & Hiley, 1997; 1998b; Zygmunt *et al*., 1997). However, the identity of the K^+ channels involved remains unclear. Whereas several studies in rat mesenteric arteries suggested a role for the large conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channels (Plane *et al*., 1997; Ishioka & Bukoski, 1999), others did not (White & Hiley, 1997; Randall & Kendall, 1998b; Fulton & Quilley, 1998). By contrast, anandamide was found to inhibit delayed rectifier K^+ (K_V) currents in rat aortic and hepatic arterial myocytes (Zygmunt *et al*., 1997; Van den Bossche & Vanheel, 2000). Furthermore, anandamide-induced relaxation has also been attributed to the inhibition of L-type Ca^{2+} currents in cat cerebral arterioles (Gebremedhin *et al*., 1999) and to the inhibition of intracellular Ca^{2+} mobilization in rat hepatic arteries (Zygmunt *et al*., 1997).

The contribution of vascular tissue and/or species differences to this heterogeneity of vascular action of anandamide is not clear. The aim of this study, therefore, was to characterize the possible mechanism(s) underlying the relaxant effects of anandamide in the sheep coronary artery, by examining, firstly, the possible involvement of the vascular endothelium, endothelium-derived relaxing factors, cannabinoid and vanilloid receptors and, secondly, the putative role of potassium channels in its actions. Preliminary findings of the study have been previously reported (Grainger *et al*., 2000).

Methods

Tissue preparation

Sheep hearts were collected from a local abattoir and delivered to the laboratory in cold Krebs-Henseleit solution. The circumflex coronary artery was carefully dissected and cleared of fat and connective tissue. Two adjacent rings (~4 mm in length and 1.2–2.2 mm in outer diameter) were removed from each artery and mounted under 2 g resting tension, *via* two stainless steel hooks, in a 10 ml organ bath containing pre-warmed (37°C) and aerated (with 95%O_2 and CO_2) Krebs-Henseleit solution. The composition of the Krebs-Henseleit solution was as follows (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 11.1; pH adjusted to 7.4 with 1 M HCl. Some of the rings were deliberately endothelium denuded, by rubbing and rolling them around stainless steel forceps, before being mounted. Successful removal of the endothelium was confirmed by the lack of relaxant response (<10%) to the calcium ionophore, A23817 (0.3 μM) following precontraction to the thromboxane A_2-mimetic, U46619. Isometric tension was monitored *via* a force displacement transducer (FT03, Grass Instruments) connected to a Grass 79D polygraph. Tissues were allowed to equilibrate for 90 min and challenged with KCl (60 mM) to assess viability.

Experimental protocol

The effects of anandamide were always examined in paired rings derived from the same arteries. In the first series of experiments, the effects of anandamide and its metabolically stable analogue, methanandamide, on U46619-induced tone in endothelium intact arterial rings were examined. Rings were pre-contracted to U46619 and once the contractions had stabilized, they were randomized to cumulative addition of anandamide (0.01–30 μM), methanandamide (0.01–30 μM), or vehicle (ethanol or 1:1 Soya oil/water emulsion). The effect of endothelium denudation on the relaxant effect of anandamide was similarly examined by pre-contracting pairs of endothelium-intact and denuded rings to U46619, followed by cumulative additions of anandamide (0.01–30 μM) in half-log molar concentrations until the maximum possible relaxation was attained.

All subsequent experiments were performed using rings with intact endothelium. To examine the possible role of cannabinoid (CB) and vanilloid (VR) receptors in the anandamide effect, paired rings were pretreated for 30 min with either the selective CB_1 receptor antagonist, SR 141716A (3 μM; White & Hiley, 1998a), the VR1 receptor antagonist, capsazepine (3 and 10 μM; Szallasi & Blumberg, 1999), or vehicle (i.e., control). Similarly, the possible role of the EDRFs, nitric oxide and prostanoids, in the anandamide effect was examined by pretreating paired rings for 30 min with either the NO synthase inhibitor, L-NAME (100 μM), the cyclo-oxygenase inhibitor, indomethacin (3 and 10 μM), or vehicle. To test whether prior cellular uptake and metabolic conversion of anandamide to arachidonic acid is required for the anandamide effect, rings were pretreated for 30 min with either the anandamide transport inhibitor, AM 404 (10 and 30 μM; Beltramo *et al*., 1997), the amidohydrolase inhibitor, phenylmethylsulphonyl fluoride (PMSF; 70 and 200 μM; Pertwee *et al*., 1995), or vehicle. Likewise, the possible role of cytochrome P450 metabolites in the anandamide effect was examined by pretreating rings for 30 min with either 17-ODYA (5 and 10 μM), miconazole (1 and 10 μM; Zou *et al*., 1994), or vehicle. In each case, after the pretreatment period, both drug treated and control rings were precontracted to U46619 and relaxed to cumulative concentrations of anandamide, as before. The selectivity of action of AM 404, PMSF and miconazole was further examined by evaluating their effect on the actions of a variety of vasorelaxant agents. Following pretreatment for 30 min with of AM 404, PMSF, miconazole, or vehicle, paired rings were precontracted to U46619 and randomized to cumulative additions of adenosine (0.01–100 μM), glyceryl trinitrate (GTN; 0.1–1000 nM), levcromakalim (0.01–3 μM), or the calcium ionophore, A23187 (1–1000 nM), until the maximal relaxation was achieved.

Finally, the putative role of potassium channels in the vasorelaxant effects of anandamide was examined. To assess the possible involvement of K^+ channel activation, paired rings were precontracted, one to U46619 and the other following exposure to 25 mM K^+-containing Krebs-Henseleit solution (Adeagbo & Triggle, 1993), and maximally relaxed by cumulative addition of anandamide. The 25 mM K^+ Krebs-Henseleit solution was prepared by equimolar substitution of NaCl with KCl in the standard Krebs-Henseleit solution. To provide further insight into the identity of the K channel(s) involved, paired rings were randomized to 30 min pretreatment with either vehicle or one of the potassium channel blockers, TEA (1 and 3 mM), iberiotoxin (100 nM), 4-aminopyridine (1 mM), glibenclamide, (3 and 10 μM),

apamin (1 μM), or barium (100 μM; Nelson & Quayle, 1995). Thereafter, rings were precontracted to U46619 and maximally relaxed to anandamide, as before.

Data and statistical analysis

All relaxation responses were expressed as a percentage of the initial U46619 or KCl-evoked contraction and are reported as mean ± s.e. mean (n = number of coronary artery rings). pEC_{50} values for the relaxant agents refer to the negative log concentration of the agent that produced half (50%) the maximal relaxation. EC_{50} values were obtained from each concentration-relaxation curve by fitting the normalized data to the four-parameter logistic equation (GraphPad PRISM V3):

$$Y = \text{Bottom} + (\text{Top-Bottom})/1 + 10^{(\text{LogEC50-X}).\text{HillSlope}} \quad (1)$$

Where X is the logarithm of drug concentration and Y is the response; Bottom is the lower response plateau, Top is the upper response plateau, equivalent to the per cent maximal relaxation response (R_{max}), EC_{50} is the X value when Y is halfway between Bottom and Top and HillSlope is the slope factor that describes the steepness of the curve.

Mean pEC_{50} and R_{max} values for anandamide and all other relaxant agents in control and treated rings were compared using paired Student's t-test or analysis of variance (ANOVA), followed by Dunnett's *post-hoc* test, as appropriate. P values less than 0.05 were considered statistically significant.

Drugs

Anandamide was obtained from Sigma (Sigma Chemical Company, Poole, Dorset, U.K.) or Tocris Cookson (Bristol). Anandamide (Sigma) was supplied as a yellowish oil and was dissolved in 100% ethanol (10 mg.ml^{-1} stock) and stored at −20°C. Serial dilutions were prepared daily, first in 50% (v v^{-1}) ethanol and, subsequently, in Krebs-Henseleit solution. Anandamide (Tocris), used in some of the initial studies, was formulated in a 1 : 1 Soya oil/water emulsion and stored at +4°C. Serial dilutions were prepared daily in Krebs-Henseleit solution. No differences in relaxant potency or efficacy were observed between the two samples of anandamide, and neither vehicle exhibited any relaxant effects on their own. Accordingly, respective drug and vehicle-treated data were pooled for analysis. Stock solutions of R(+)-methanandamide (Tocris) were prepared, stored and diluted daily as described above for anandamide (Sigma). 4-Aminopyridine, tetraethylammonium bromide, apamin, adenosine (Sigma), barium chloride (BDH) and iberiotoxin (RBI) were dissolved in de-ionized water and serially diluted, where necessary, in Krebs-Henseleit solution. Indomethacin and GTN (Sigma) were dissolved in Krebs-Henseleit solution. All other drugs were dissolved in 100% ethanol. These included N^{G}-nitro-L-arginine methyl ester (L-NAME), phenylmethylsulphonyl fluoride (PMSF), 9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619), miconazole, A23187 (Sigma), capsazepine, 17-octadecynoic acid (17-ODYA), AM 404 (Tocris), glibenclamide (gifted by Smith-Kline Beecham, Surrey, U.K.) and SR 141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (gifted by Sanofi Recherche, Montpellier, France).

Results

Effects of anandamide and methanandamide on U46619-induced tone in endothelium-intact sheep coronary arteries

The effects of anandamide and its metabolically stable analogue, methanandamide, on U46619-contracted sheep coronary arteries are shown in Figure 1a. Anandamide (0.01 – 30 μM) caused concentration-dependent relaxation of U46619-induced tone in rings with intact endothelium, with a mean pEC_{50} of 6.08 ± 0.1 and R_{max} of 80.8 ± 3.6% (n = 17). The relaxant effects were slow in onset and took about 10 – 12 min to peak. By contrast, methanandamide (0.01 – 30 μM) did not affect U46619-induced tone in endothelium-intact rings. Methanandamide tended to increase arterial tone at higher concentrations. However, the increases were similar to those seen in vehicle (ethanol or Soya oil/water)-treated rings, and these, in turn, were not statistically different from the small time-dependent increases in tone observed in time-matched control rings (Figure 1a).

Effects of the cannabinoid and vanilloid receptor antagonists, SR 141716A and capsazepine, on anandamide-induced vasorelaxation

Pre-treatment of endothelium-intact rings with the selective CB_1 receptor antagonist, SR 141716A (3 μM), or the VR1 receptor antagonist, capsazepine (3 and 10 μM), did not modify anandamide-induced relaxations (Figure 1b, c). Mean pEC_{50} and R_{max} values for anandamide in SR 141716A (3 μM)-treated rings were 6.16 ± 0.12 and 85.9 ± 5.6%, compared with 5.96 ± 0.20 and 96.2 ± 3.7%, respectively, in control rings (n = 5). Similarly, the mean pEC_{50} and R_{max} values for anandamide in capsazepine (3 and 10 μM)-treated rings (5.86 ± 0.14, 87.5 ± 4.5% and 6.13 ± 0.13, 82.2 ± 7.0%, respectively) were not different from those in control rings (5.95 ± 0.11 and 86.5 ± 5.6%; n = 4 – 10).

Effect of endothelium denudation on anandamide-induced vasorelaxation

Removal of the endothelium resulted in a significant reduction in the vasorelaxant potency of anandamide (Figure 2a). Thus, in endothelium-denuded rings, the concentration-response curve to anandamide was shifted rightward by 10 fold (mean pEC_{50} reduced from 6.06 ± 0.10 in endothelium-intact rings to 5.06 ± 0.12; P < 0.01; n = 7). R_{max} for anandamide, however, remained unaffected (86.8 ± 4 and 95.6 ± 2.5% in endothelium-denuded and intact rings, respectively). In view of this, all subsequent experiments were performed in endothelium-intact vessels, unless otherwise indicated.

Effects of L-NAME and indomethacin on anandamide-induced vasorelaxation

As shown in Figure 2b, pretreatment with the nitric oxide synthase inhibitor, L-NAME (100 μM), did not significantly

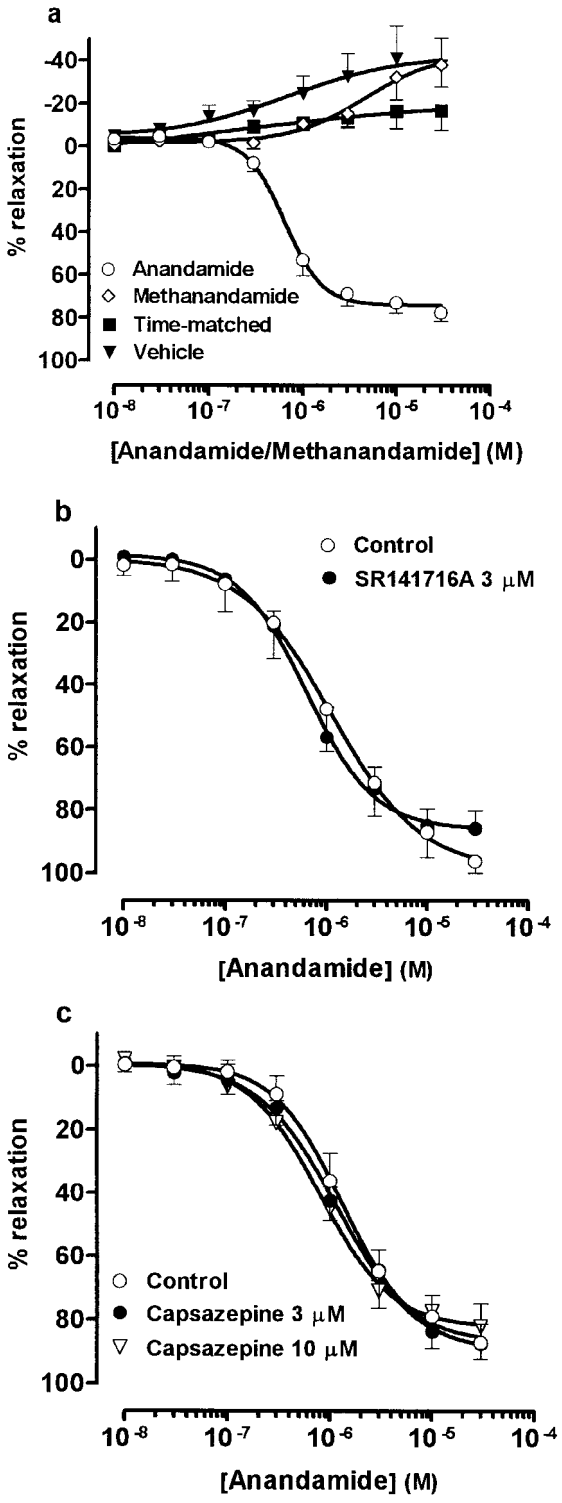

Figure 1 Concentration-response curves for anandamide and methanandamide in endothelium-intact sheep coronary arteries precontracted to U46619. (a) Comparison of the effects of anandamide ($n=17$), methanandamide ($n=7$) and vehicle (ethanol or Soya oil/water emulsion; $n=7$) on U46619-induced tone. (b) Effects of 30 min pretreatment with SR 141716A (3 μM) on relaxant responses to anandamide ($n=5$). (c) Effects of 30 min pretreatment with capsazepine (3 and 10 μM) on relaxant responses to anandamide ($n=4-10$). Data are presented as means and were fitted to curves according the procedure described in Methods. Vertical bars indicate s.e.mean.

affect the relaxant effects of anandamide (mean pEC_{50} and R_{max} values were 5.65 ± 0.20 and $94.3\pm6.9\%$ in treated rings, compared with 5.70 ± 0.20 and $90.9\pm4.3\%$ in control rings; $n=5$). By contrast, the cyclo-oxygenase inhibitor, indomethacin (3 and 10 μM), attenuated anandamide-induced relaxations, significantly reducing the R_{max} from $91.4\pm6.4\%$ in control rings to $32.5\pm15\%$ and $15.2\pm10.5\%$, respectively, in indomethacin-treated rings ($P<0.01$; $n=4-8$; Figure 2c). Indomethacin (10 μM) also markedly attenuated anandamide-induced relaxations in endothelium-denuded rings, reducing the R_{max} from $91.6\pm4.9\%$ in control rings to $18.6\pm10.0\%$ ($P<0.01$; $n=7$).

Effects of PMSF and AM 404 on anandamide-induced vasorelaxation

The effects of the anandamide amidohydrolase inhibitor (PMSF) and transport inhibitor (AM 404) on anandamide-induced vasorelaxation are shown in Figure 3. Pretreatment with PMSF (70 and 200 μM) resulted in a marked attenuation of the relaxant effects of anandamide, the R_{max} being significantly reduced from $95.1\pm3.1\%$ in control rings to 17.5 ± 11.2 and $7.9\pm16.7\%$, respectively, in treated rings ($P<0.01$; $n=5-11$; Figure 3a). By contrast, PMSF (200 μM) did not modify relaxant responses to A23187, adenosine or GTN (Table 1).

Figure 3b shows that pretreatment with AM 404 (10 and 30 μM) also resulted in a concentration-dependent reduction in the vasorelaxant potency of anandamide. Thus, in the presence of AM 404 (10 and 30 μM), the concentration-response curve to anandamide was shifted rightward by 4.4 and 7.9 fold, respectively (pEC_{50} reduced from 6.07 ± 0.16 in control rings to 5.42 ± 0.15 and 5.17 ± 0.20, respectively, in treated rings; $P<0.01$; $n=4-13$). The R_{max} for anandamide, however, was only significantly reduced at the higher dose of AM 404, from $95.2\pm2.4\%$ in control rings to $82.7\pm1.9\%$ ($P<0.05$). As shown in Table 1, AM 404 (30 μM) did not affect the relaxant effects of adenosine or GTN, although it did induce a significant rightward shift in the concentration-relaxation curve to A23187.

Table 1 Effects of AM 404 and PMSF on relaxation induced by a variety of vasorelaxant agents in sheep coronary arteries precontracted to U46619

Treatment	pEC_{50}	R_{max} (%)	n
GTN control	7.74 ± 0.12	91.6 ± 5.6	8
PMSF (200 μM)	7.76 ± 0.15	95.6 ± 3.9	4
AM 404 (30 μM)	8.37 ± 0.27	98.4 ± 4.0	4
Adenosine control	5.39 ± 0.13	89.8 ± 5.0	6
PMSF (200 μM)	5.46 ± 0.29	102.6 ± 1.8	3
AM 404 (30 μM)	5.33 ± 0.35	97.6 ± 7.3	3
A23817 control	7.58 ± 0.11	98.4 ± 2.0	15
PMSF (200 μM)	7.82 ± 0.22	101.4 ± 2.9	4
AM 404 (10 μM)	7.40 ± 0.13	92.6 ± 4.6	5
AM 404 (30 μM)	7.09 ± 0.07*	93.6 ± 4.5	6

Data are presented as mean$\pm$s.e. mean. pEC_{50} and R_{max} were derived by curve-fitting as described in Methods. n values indicate the number of arterial rings tested, with each pair of rings (control and treated) being derived from one sheep heart. *$P<0.05$ compared to control values.

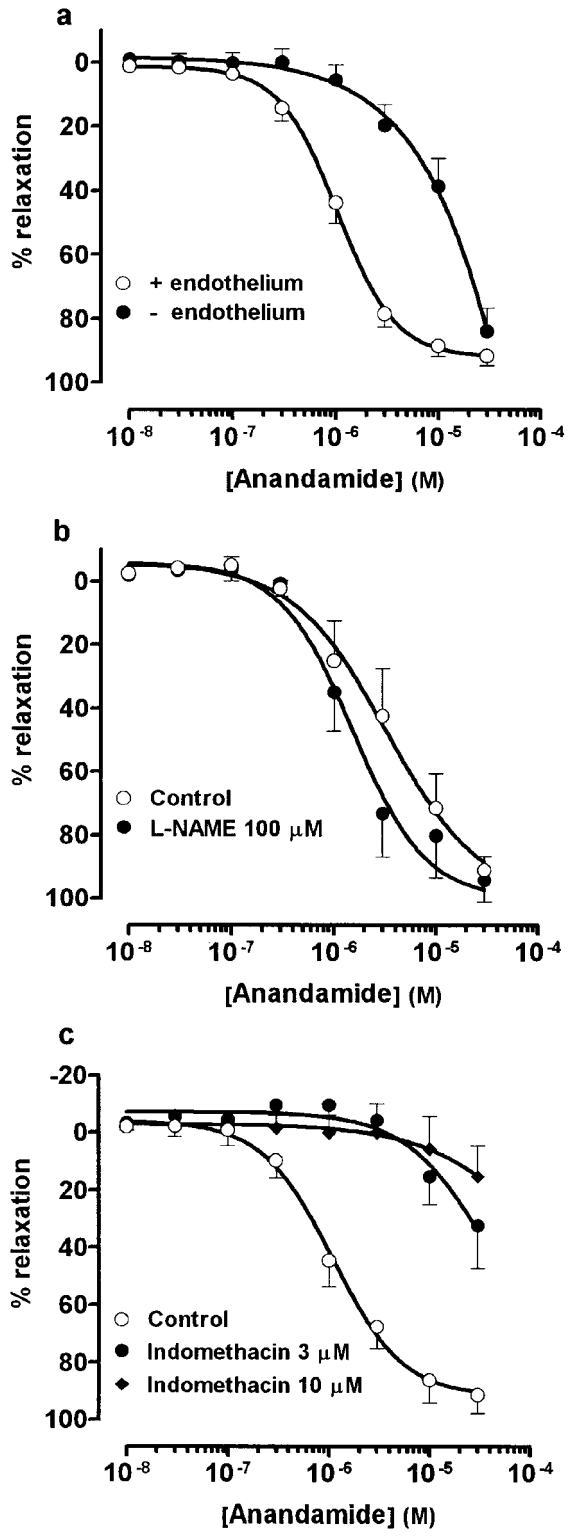

Figure 2 Effect of endothelium denudation or pretreatment with L-NAME or indomethacin on anandamide-induced relaxation in sheep coronary arteries precontracted to U46619. (a) Effects of endothelium denudation on relaxant responses to anandamide ($n=7$). (b) Effects of 30 min pretreatment with L-NAME (100 μM) on relaxant responses to anandamide ($n=5$). (c) Effects of 30 min pretreatment with indomethacin (3 and 10 μM) on relaxant responses to anandamide ($n=4-8$). Data are presented as means and were fitted to curves according the procedure described in Methods. Vertical bars indicate s.e.mean.

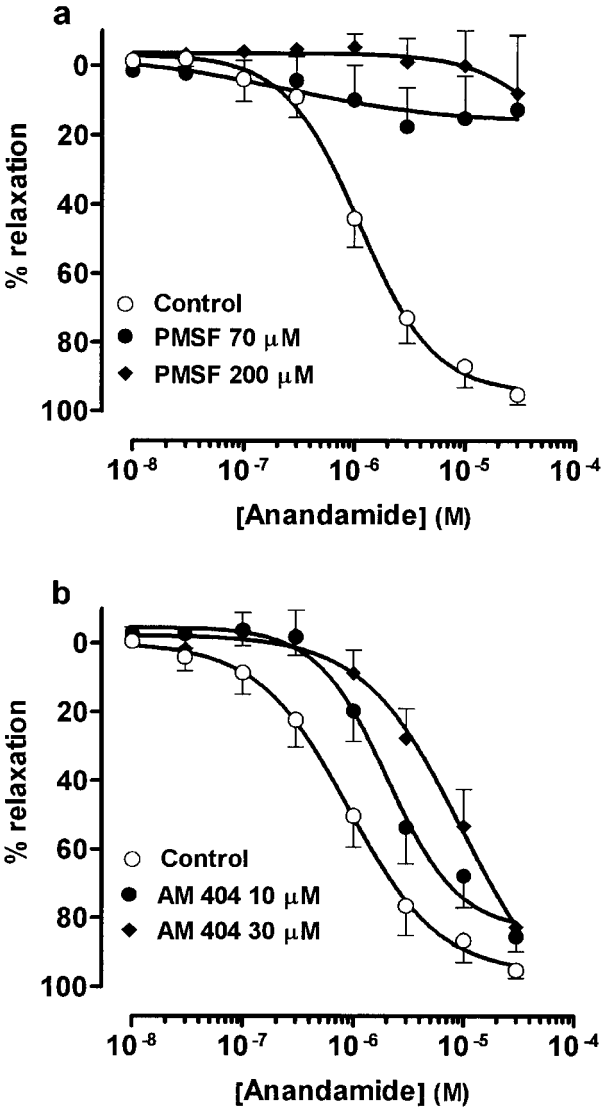

Figure 3 Effect of PMSF and AM 404 on anandamide-induced relaxation in endothelium-intact sheep coronary arteries precontracted to U46619. (a) Effects of 30 min pretreatment with PMSF (70 and 200 μM) on relaxant responses to anandamide ($n=5-11$). (b) Effects of 30 min pretreatment with AM404 (10 and 30 μM) on relaxant responses to anandamide ($n=4-13$). Data are presented as means and were fitted to curves according the procedure described in Methods. Vertical bars indicate s.e.mean.

Effects of cytochrome P450 inhibitors, 17-ODYA and miconazole, on anandamide-induced vasorelaxation

Figure 4 depicts the effects of the cytochrome P450 inhibitors, 17-ODYA and miconazole, on the vasorelaxant actions of anandamide. Exposure of rings to 17-ODYA (5 and 10 μM) failed to modify the relaxant effects of anandamide. Mean pEC_{50} values for anandamide in 5 and 10 μM 17-ODYA-treated rings were 6.17 ± 0.08 and 6.10 ± 0.10, which were not different from that in control rings (6.22 ± 0.14; $n=4-10$; Figure 4a). R_{max} of anandamide was also unaffected (90.4 ± 5.4 and 91.7 ± 8.3% in treated rings, compared with 88.8 ± 3.7% in control rings; $n=4-10$).

Pretreatment with miconazole (1 μM) also failed to modify anandamide-induced relaxation (mean pEC_{50} and R_{max}

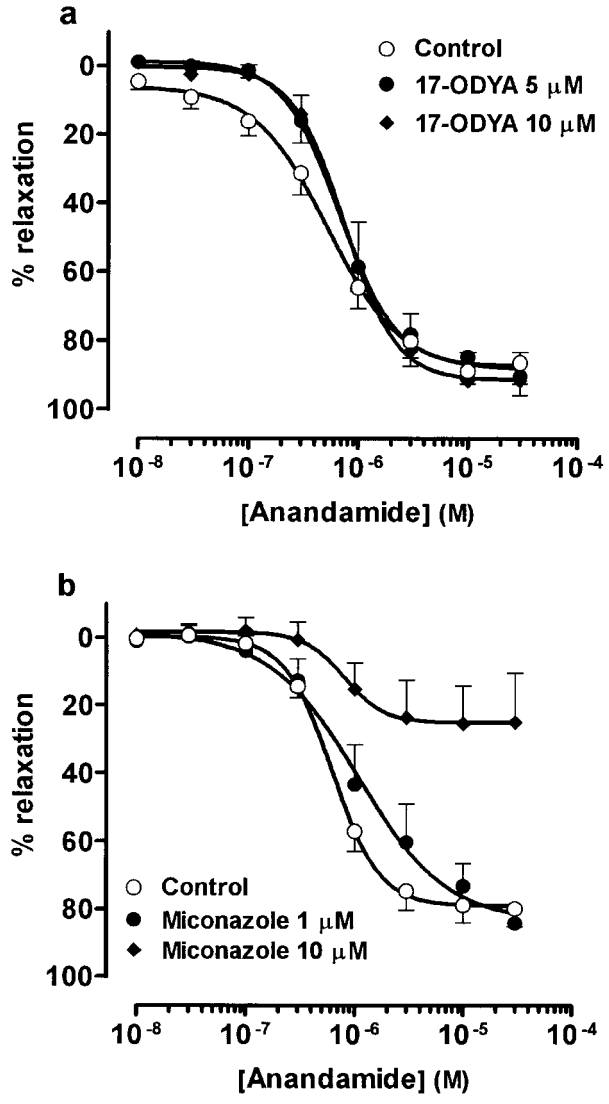

Figure 4 Effects of 17-ODYA and miconazole on anandamide-induced relaxation in endothelium-intact sheep coronary arteries precontracted to U46619. (a) Effects of 30 min pretreatment with 17-ODYA (5 and 10 μM) on relaxant responses to anandamide ($n=4-10$). (b) Effects of 30 min pretreatment with miconazole (1 and 10 μM) on relaxant responses to anandamide ($n=6-12$). Data are presented as means and were fitted to curves according the procedure described in Methods. Vertical bars indicate s.e.mean.

values in treated rings were 5.92 ± 0.22 and $84.2\pm4.7\%$, compared to 6.11 ± 0.10 and $80.0\pm5.4\%$, respectively, in control rings; $n=6-12$; Figure 4b). By contrast, miconazole (10 μM) markedly attenuated the vasorelaxant effects of anandamide, reducing R_{max} from $80.0\pm5.4\%$ in control rings to $25.1\pm14.5\%$ ($P<0.01$; $n=6-12$; Figure 4b). Ten μM miconazole also attenuated relaxant responses to the selective K_{ATP} channel opener, levcromakalim (mean pEC_{50} and R_{max} values in treated rings were 6.44 ± 0.10 and $60.8\pm10.8\%$, compared to 6.97 ± 0.09 and $94.4\pm3.6\%$, respectively, in control rings; $P<0.01$; $n=3-6$), whereas 1 μM miconazole had no effect (mean pEC_{50} and R_{max} values in treated rings of 6.97 ± 0.06 and $95.8\pm4.3\%$ were not different from those in control rings; $n=3-6$). Similarly, 10 μM miconazole (but not 1 μM) caused a 2.8 fold rightward shift in the concentration-relaxation curve for adenosine (mean pEC_{50} value reduced from 5.63 ± 0.12 in control rings to 5.18 ± 0.20; $P<0.05$; $n=5$), but did not affect the R_{max} (103.1 ± 5.2 and $97.0\pm15.7\%$ in control and treated rings, respectively).

Effect of precontraction of rings with high K^+ on anandamide-induced vasorelaxation

Precontraction of the arterial rings with 25 mM K^+, instead of U46619, almost completely abolished relaxations by anandamide. R_{max} induced by anandamide in U46619-contracted vessels was $83.4\pm4.4\%$, compared with R_{max} of $7.0\pm6.9\%$ in corresponding rings precontracted to 25 mM K^+ ($P<0.001$; $n=8$; Figure 5a). Mean tone induced by 25 mM K^+ and U46619 was 5.4 and 3.7 g, respectively ($P<0.01$; $n=8$).

Effects of K^+ channel blockade with TEA, iberiotoxin, apamin, 4-aminopyridine, glibenclamide and barium on anandamide-induced vasorelaxation

TEA (1 mM), a selective blocker of large conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channels, failed to modify anandamide-induced relaxation (pEC_{50} and R_{max} were 6.36 ± 0.22 and $98.2\pm1.6\%$ in treated rings compared to 6.20 ± 0.12 and $91.1\pm3.9\%$ in controls; $n=4-10$). At a higher concentration (3 mM), however, TEA markedly attenuated the vasorelaxant effects of anandamide, decreasing the pEC_{50} from 6.20 ± 0.12 in control rings to 5.10 ± 0.12 and reducing the R_{max} from 91.1 ± 3.9 to $65.0\pm9.6\%$ ($P<0.01$; $n=6-10$; Figure 5b). Similarly, iberiotoxin (100 nM), a selective blocker of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, significantly reduced the vasorelaxant potency of anandamide (pEC_{50} decreased from 6.30 ± 0.16 in control rings to 5.60 ± 0.15; $P<0.01$; $n=8$; Figure 5c), but was without effect on its efficacy (R_{max} was 97.3 ± 1.9 and $87.3\pm4.4\%$ in treated and control rings, respectively; $n=8$).

By contrast, pretreatment of rings with the small conductance, Ca^{2+}-activated K^+ (SK_{Ca}) channel blocker, apamin (1 μM) or the selective blocker of ATP-sensitive (K_{ATP}) K^+ channels, glibenclamide (3 and 10 μM), failed to modify anandamide-induced relaxations (Table 2). Ba^{2+} (100 μM), a reputed selective inhibitor of inward rectifier K^+ channels, also failed to modify anandamide-induced relaxation (Table 2). Likewise, 4-aminopyridine (1 mM), a concentration believed to block delayed rectifier K^+ channels selectively, failed to affect the vasorelaxant potency of anandamide, although it did reduce the R_{max} (Table 2).

Discussion

The aim of the present study was to attempt an elucidation of the vasorelaxant mechanisms of anandamide in sheep coronary arteries. Anandamide induced a concentration-dependent relaxation of U46619-contracted, endothelium-intact sheep coronary artery rings *in vitro*. This is consistent with previous findings in rat and bovine coronary arteries (Randall & Kendall, 1997; Fulton & Quilley, 1998; Pratt *et al*., 1998) and in a variety of vascular tissues from the rat, rabbit and guinea-pig (Ellis *et al*., 1995; Deutsch *et al*., 1997; Fleming *et al*., 1999; Zygmunt *et al*., 1999). The slowly

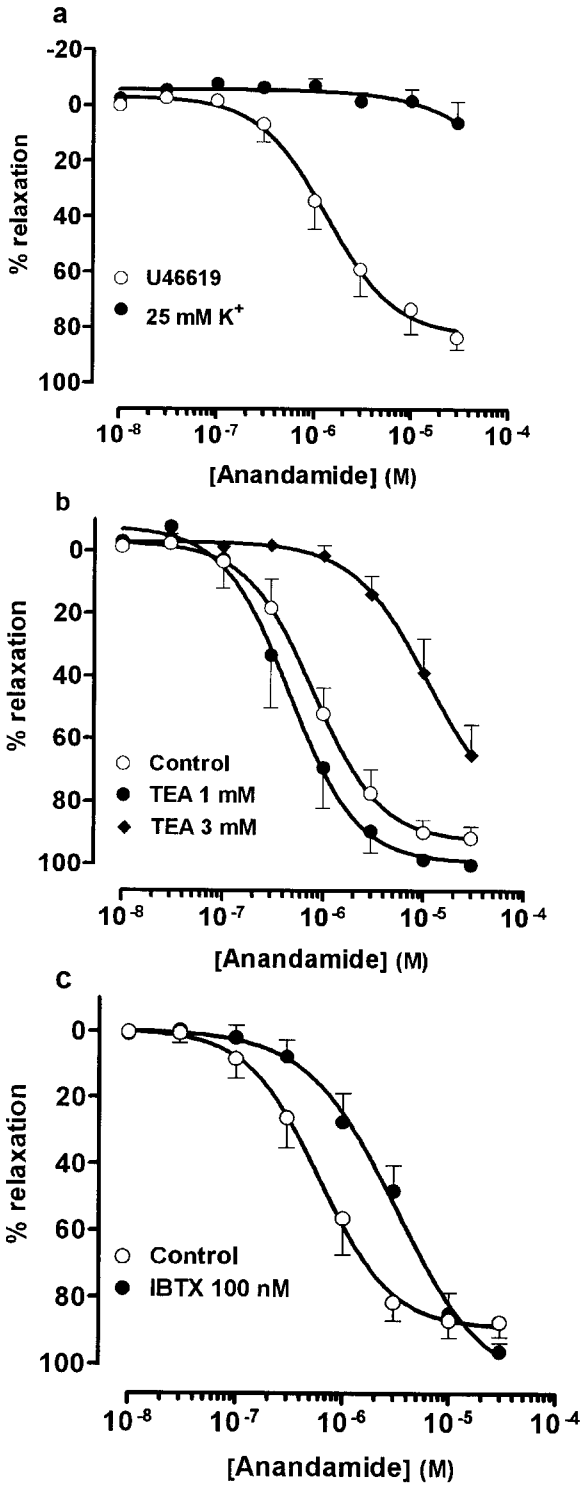

Figure 5 Effects of high K^+ (25 mM) and K^+ channel blockade with TEA and IBTX on anandamide-induced relaxation in endothelium-intact sheep coronary arteries. (a) Comparison of the relaxant responses to anandamide in arterial rings precontracted to 25 mM K^+ and U46619, respectively ($n=8$). (b) Effects of 30 min pretreatment with TEA (1 and 3 mM) on relaxant responses to anandamide in arterial rings precontracted to U46619 ($n=4-10$). (c) Effects of 30 min pretreatment with IBTX (100 nM) on relaxant responses to anandamide in arterial rings precontracted to U46619 ($n=8$). Data are presented as means and were fitted to curves according the procedure described in Methods. Vertical bars indicate s.e.mean.

Table 2 Effects of selected K^+ channel blockers on anandamide-induced relaxation in endothelium-intact sheep coronary arteries precontracted to U46619

Treatment	pEC_{50}	R_{max} (%)	n
Control	5.94±0.13	86.9±4.0	10
Glibenclamide (3 μM)	5.97±0.12	70.0±9.4	5
Glibenclamide (10 μM)	6.20±0.10	68.4±10.0	5
Control	6.27±0.21	91.6±5.4	7
4-Aminopyridine (1 mM)	6.21±0.13	65.6±9.1*	7
Control	6.00±0.31	94.5±4.8	4
Apamin (1 μM)	5.80±0.21	86.7±8.7	4
Control	6.10±0.18	89.0±5.9	3
$BaCl_2$ (100 μM)	6.40±0.20	99.6±2.4	3

Data are presented as mean±s.e.mean. pEC_{50} and R_{max} were derived by curve-fitting as described in Methods. *n* values indicate the number of arterial rings tested, with each pair of rings (control and treated) being derived from one sheep heart. $^*P<0.05$ compared to control value.

developing nature of the anandamide-induced relaxation is also similar to that reported in bovine coronary and rabbit mesenteric arteries (Pratt *et al*., 1998; Fleming *et al*., 1999). By contrast, the metabolically stable analogue of anandamide, methanandamide, had no effect on induced tone in sheep coronary arteries. Methanandamide, a potent CB_1 receptor agonist, exhibits relaxant activity in rabbit mesenteric and rat hepatic and mesenteric arteries (White & Hiley, 1998b; Chaytor *et al*., 1999; Zygmunt *et al*., 1999). Therefore, its failure to mimic anandamide-induced relaxation in sheep coronary arteries suggests that the anandamide effect is neither due to anandamide *per se* nor mediated *via* CB_1 receptors.

Consistent with this, the selective CB_1 receptor antagonist, SR 141716A, did not modify anandamide-induced relaxation in sheep coronary arteries. Both SR 141716A-sensitive (Randall *et al*., 1996; White & Hiley, 1997; Deutsch *et al*., 1997; Randall & Kendall, 1997; Fulton & Quilley, 1998) and SR 141716A-resistant (Pratt *et al*., 1998; Zygmunt *et al*., 1999) relaxant responses to anandamide have been reported in a variety of vascular beds. However, the micromolar concentrations of SR 141716A required to antagonize anandamide-induced relaxation in most of the former studies have also been shown to exert additional vascular effects that are unrelated to cannabinoid receptors (White & Hiley, 1997, 1998a; Pratt *et al*., 1998; Zygmunt *et al*., 1999; Mombouli *et al*., 1999). In the study by White & Hiley (1998a) in rat mesenteric arteries, SR 141716A concentrations above that used in the present study (3 μM) were associated with marked inhibitory effects on both Ca^{2+} entry and K^+ channel activity. Thus, the role of CB_1 receptors in anandamide-induced relaxation remains uncertain.

Like SR 141716A, pretreatment with the selective VR1 receptor antagonist, capsazepine (Szallasi & Blumberg, 1999), also failed to modify anandamide-induced relaxation in sheep coronary arteries. This contrasts with the findings in guinea-pig basilar and rat hepatic and mesenteric arteries (Zygmunt *et al*., 1999; Ralevic *et al*., 2000) implicating VR1 receptors, located on sensory nerves, in anandamide (and methanandamide)-induced relaxations. Anandamide has, indeed, been shown to bind rat and human recombinant VR1 vanilloid receptors with

a consequent capsazepine-sensitive activation of inward currents (Zygmunt *et al*., 1999; Smart *et al*., 2000). Therefore, the apparent lack of involvement of vanilloid receptors in the anandamide effect in sheep coronary arteries may be attributable to species and/or vascular tissue differences.

Although the vasorelaxant potency of anandamide was reduced by endothelium denudation, its maximal relaxation was unaffected. This suggests that the endothelium does not play an obligatory role in anandamide-induced relaxation in sheep coronary arteries. Both endothelium-dependent (Pratt *et al*., 1998; Fleming *et al*., 1999; Chaytor *et al*., 1999; Wagner *et al*., 1999) and endothelium-independent (Randall *et al*., 1996; White & Hiley, 1997; Zygmunt *et al*., 1997, 1999) anandamide effects have been reported in a variety of vascular tissues. Although functional CB_1 cannabinoid receptors have been described in vascular endothelial cells (Sugiura *et al*., 1998; Liu *et al*., 2000), the lack of effect of SR 141716A in the present study rules out their possible involvement in mediating the endothelium-dependent component of the anandamide effect in sheep coronary arteries. The potential involvement of an as-yet-unidentified endothelial anandamide receptor (Kunos *et al*., 2000) cannot be ruled out.

Rapid carrier-mediated uptake and enzymatic hydrolysis of anandamide to arachidonic acid is known to occur in vascular endothelial cells (Deutsch *et al*., 1997; Di Marzo, 1998; Maccarrone *et al*., 2000). Anandamide has also been shown to cause intracellular Ca^{2+} release in endothelial cells, with a concomitant release of nitric oxide (Mombouli *et al*., 1999; Fimiani *et al*., 1999). The endothelium-dependent component of anandamide-induced relaxation in sheep coronary arteries, therefore, could be mediated *via* either (i) an anandamide-evoked release of endothelium-derived relaxant factors, such as NO or prostanoids, or (ii) its uptake into endothelial cells and subsequent conversion to a vasodilatory metabolite. Consistent with the latter hypothesis, Chaytor *et al*. (1999) have shown that the endothelium-dependent component of anandamide-induced relaxation in the rabbit superior mesenteric artery results from the prior endothelial transport of anandamide, although, in this case, there was no evidence of a subsequent conversion into an active metabolite.

The nitric oxide synthase inhibitor, L-NAME, failed to modify anandamide-induced vasorelaxation, indicating that the anandamide effect in sheep coronary arteries is not mediated *via* release of NO from the endothelium. This is in agreement with previous findings in a variety of vascular tissues (Ellis *et al*., 1995; Randall *et al*., 1996; White & Hiley, 1997; Zygmunt *et al*., 1999; but see Deutsch *et al*., 1997). Conversely, the almost complete abolition of anandamide-induced vasorelaxation by the cyclo-oxygenase inhibitor, indomethacin, suggests that the vasodilatory effects of anandamide in sheep coronary arteries are mediated *via* prostanoids. Consistent with this hypothesis, similar cyclo-oxygenase-dependent, anandamide-induced relaxations have also been reported in bovine coronary and rabbit mesenteric and cerebral arteries (Ellis *et al*., 1995; Pratt *et al*., 1998; Fleming *et al*., 1999). Indeed, in bovine coronary and rabbit cerebral arterioles, the anandamide effect was associated with increased release of arachidonic acid-derived vasodilatory eicosanoids (Ellis *et al*., 1995; Pratt *et al*., 1998).

Significantly, indomethacin markedly attenuated anandamide-induced relaxations in both endothelium-intact and denuded arteries in the present study. This suggests that the cellular uptake and metabolic conversion of anandamide to vasodilatory prostanoids occurs in both the endothelial and vascular smooth muscle cells of sheep coronary arteries. Similar findings have been reported by Fleming *et al*. (1999) in rabbit mesenteric arteries, although in another study in rabbit superior mesenteric arteries, neither the endothelium-dependent nor endothelium-independent relaxant effect of anandamide was susceptible to inhibition by indomethacin (Chaytor *et al*., 1999). The cyclo-oxygenase-dependence of the relaxant actions of anandamide appears be a species and/vascular bed specific phenomenon, because anandamide-induced relaxation has been shown to be cyclo-oxygenase-independent in rat coronary, hepatic and mesenteric arteries (Randall *et al*., 1996; White & Hiley, 1997; Randall & Kendall, 1997; Zygmunt *et al*., 1997; Fulton & Quilley, 1998) and guinea-pig basilar arteries (Zygmunt *et al*., 1999).

The suggestion that anandamide is taken up by the coronary vasculature and converted to a vasodilatory prostanoid is confirmed by the additional finding that anandamide-induced relaxations were abolished or attenuated by both the anandamide transport inhibitor, AM 404 (Beltramo *et al*., 1997) and amidohydrolase inhibitor, PMSF (Di Marzo, 1998). The inhibitory effect of PMSF is consistent with that of another amidohydrolase inhibitor, diazomethylarachidonyl ketone (DAK), in bovine coronary arteries (Pratt *et al*., 1998). In this study, incubation of bovine coronary arteries and cultured coronary endothelial cells with radiolabelled anandamide resulted in the release of arachidonic acid and its metabolites. Inhibition of amidohydrolase activity with DAK blocked the hydrolysis of anandamide to arachidonic acid, with a concomitant abolition of the relaxant responses to anandamide (Pratt *et al*., 1998). Although the selectivity of action of PMSF in vascular tissues has been recently questioned (White & Hiley, 1997), PMSF (70–200 μM) did not modify relaxant responses of sheep coronary arteries to A23187, adenosine or GTN. This suggests that, at least in these tissues, PMSF must be acting selectively to inhibit anandamide amidohydrolase-catalysed hydrolysis of anandamide to arachidonic acid.

The inhibitory effect of AM 404 on anandamide-induced relaxations in sheep coronary arteries is also consistent with previous findings in rabbit and rat mesenteric arteries (Chaytor *et al*., 1999; Kunos *et al*., 2000). Although this finding is indicative of the inhibition of the cellular uptake of anandamide, it should be interpreted with caution, because the selectivity of action of AM404 in vascular tissues has not been evaluated. Although AM 404 (10–30 μM) had no effect on the relaxant responses to adenosine or GTN, it did attenuate the relaxant effects of A23187. It has also been shown to activate vanilloid receptors and to relax rat hepatic arteries (Zygmunt *et al*., 2000). Notwithstanding, the inhibitory effect of AM 404 effect on anandamide-induced relaxations is fully consistent with those of PMSF and indomethacin, and provides further suggestive evidence that the anandamide effect is consequent upon its uptake into the coronary vasculature. However, since the effect of AM 404 was only studied in endothelium-intact arteries, it is not possible to establish whether the observed inhibitory effect reflects its inhibition of anandamide transport into the endothelium or vascular smooth muscle or both. In rabbit superior mesenteric arteries, only the endothelium-dependent component of the anandamide-

induced relaxation was susceptible to inhibition by AM 404 (Chaytor *et al*., 1999).

The failure of effective cytochrome P450 inhibitory concentrations of 17-ODYA (5–10 μM) and miconazole (1 μM; Zou *et al*., 1994) to inhibit anandamide-induced relaxation in the present study argues against the involvement of cytochrome P450 metabolites. This is in agreement with previous findings in rat mesenteric branch arteries (Ishioka & Bukoski, 1999), but in contrast with those in rat and bovine coronary arteries (Fulton & Quilley, 1998; Pratt *et al*., 1998). Although, miconazole (10 μM) did inhibit anandamide-induced relaxation in the present study, our finding that it also significantly attenuated relaxant responses to adenosine and the K_{ATP} channel opener, levcromakalim, suggests that its effect might be unrelated to cytochrome P450 inhibition. A similar conclusion was drawn from studies using clotrimazole and proadifen in rat mesenteric arteries (Randall *et al*., 1997). Further studies with cytochrome P450 inhibitors that lack K^+ channel blocking actions will be required to resolve this question. However, the almost complete abolition of the anandamide effect by indomethacin suggests that any involvement of cytochrome P450 metabolites is likely to be a minor one.

Raising the extracellular concentration of K^+ (to 25 mM) markedly attenuated anandamide-induced relaxation in sheep coronary arteries, consistent with previous findings in rat hepatic and mesenteric arteries (Randall *et al*., 1996; Zygmunt *et al*., 1997; White & Hiley, 1997). This suggests that the anandamide effect in sheep coronary arteries may be mediated *via* an activation of a membrane K^+ conductance. This is confirmed by the inhibitory effect of TEA (3 mM), which is also in agreement with the reported inhibitory effect of TEA (10 mM) on anandamide-induced relaxations in rat mesenteric arteries (White & Hiley, 1997; Randall *et al*., 1997; Ishioka & Bukoski, 1999). The failure of TEA (1 mM) to modify the relaxant responses to anandamide, however, appears to rule out the involvement of large conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channels (Nelson & Quayle, 1995). However, consistent with previous findings in rat mesenteric arteries (Plane *et al*., 1997; Ishioka & Bukoski, 1999), the highly selective blocker of BK_{Ca} channels, iberiotoxin (100 nM), did significantly reduce the vasorelaxant potency of anandamide in the present study. These findings are difficult to reconcile, unless one postulates that TEA (1 mM) was ineffective in blocking BK_{Ca} channels in sheep coronary arteries. The inhibitory effect of iberiotoxin (100 nM) was quite small, when compared with those of 25 mM K^+ and TEA (3 mM). This suggests that BK_{Ca} channel activation may not be the sole or main mechanism underlying the relaxant effects of anandamide in sheep coronary arteries. The role of BK_{Ca} channel activation in anandamide-induced relaxation in other vascular beds remains controversial (Plane *et al*., 1997; White & Hiley, 1997; Randall & Kendall, 1998b; Fulton & Quilley, 1998).

Apamin (1 μM), 4-aminopyridine (1 mM), glibenclamide (3 and 10 μM) and barium (100 μM), selective blockers of small conductance, Ca^{2+}-activated K^+ (SK_{Ca}) channels, delayed rectifier K^+ (K_v) channels, K_{ATP} channels and inward rectifier K^+ channels (Nelson & Quayle, 1995), respectively, all failed to modify anandamide-induced relaxations in sheep coronary arteries. These findings are largely in agreement with various findings in rat mesenteric arteries (White & Hiley, 1997; Plane *et al*., 1997; Randall & Kendall, 1998b), although White & Hiley (1997) reported a small but significant inhibitory effect with barium (100 μM). They also suggest a lack of involvement of SK_{Ca}, K_v, K_{ATP} and inward rectifier K^+ channel activation in the anandamide effect in sheep coronary arteries. Therefore, the identity of the K^+ channel(s) that, together with BK_{Ca} channels, may be responsible for anandamide-induced relaxation in sheep coronary arteries remains unresolved. Combination studies with these K^+ channel blockers may be required to resolve this issue (Randall & Kendall, 1998b; but see Zygmunt *et al*., 1997; White & Hiley, 1997). However, the above findings do not rule out the possibility that part of the relaxant effect of anandamide in sheep coronary arteries is mediated *via* mechanisms other than K^+ channel activation.

In conclusion, the results of the present study show that the vasorelaxant effects of the endocannabinoid, anandamide, in sheep coronary arteries (i) are mediated in part *via* the vascular endothelium; (ii) require the prior cellular uptake and conversion of anandamide to arachidonic acid and, subsequently, to a vasodilatory prostanoid; and (iii) may be mediated in part *via* the activation of K^+ channels.

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References

ADEAGBO, A.S.O. & TRIGGLE, C.R. (1993). Varying extracellular [K^+]: A functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J. Cardiovasc. Pharmacol.,* **21,** 423–429.

BELTRAMO, M., STELLA, N., CALIGNANO, A., LIN, S.Y., MAKRIYANNIS, A. & PIOMELLI, D. (1997). Functional role of high affinity anandamide transport, as revealed by selective inhibition. *Science,* **277,** 1094–1097.

CALIGNANO, A., LA RANA, G, BELTRAMO, M., MAKRIYANNIS, A. & PIOMELLI, D. (1997). Potentiation of anandamide hypotension by the transport inhibitor AM404. *Eur. J. Pharmacol.,* **337,** R1–R2.

CHAYTOR, A.T., MARTIN, P.E.M., EVANS, W.H., RANDALL, M.D. & GRIFFITH, T.M. (1999). The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication. *J. Physiol (Lond),* **520,** 539–550.

DEUTSCH, D.G., GOLIGORSKY, M.S., SCHMID, P.C., KREBSBACH, R.J., SCHMID, H.H.O., DAS, S.K., DEY, S.K., ARREAZA, G., THORUP, C., STEFANO, G. & MOORE, L.C. (1997). Production and physiological actions of anandamide in the vasculature of the rat kidney. *J. Clin. Invest.,* **100,** 1538–1546.

DI MARZO, V. (1998). 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. *Biochim. Biophys. Acta,* **1392,** 153–175.

ELLIS, E.F., MOORE, S. & WILLOUGHBY, K.A. (1995). Anandamide and Δ^9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am. J. Physiol.,* **269,** H1859–H1864.

FIMIANI, C., MATTOCKS, D., CAVANI, F., SALZET, M., DEUTSCH, D.G., PRYOR, S., BILFINGER INGER, T.V. & STEFANO, G.B. (1999). Morphine and anandamide stimulate intracellular calcium transients in human arterial endothelial cells: coupling to nitric oxide release. *Cell Signal.,* **11,** 189–193.

FLEMING, I., SCHERMER, B., POPP, R. & BUSSE, R. (1999). Inhibition of the production of endothelium-derived hyperpolarizing factor by cannabinoid receptor agonists. *Br. J. Pharmacol.,* **126,** 949–960.

FULTON, D. & QUILLEY, J. (1998). Evidence against anandamide as the hyperpolarizing factor mediating the nitric oxide-independent coronary vasodilator effect of bradykinin in the rat. *J. Pharmacol. Exp. Ther.,* **286,** 1146–1151.

GEBREMEDHIN, D., LANGE, A.R., CAMPBELL, W.B., HILLARD, C.J. & HARDER, D.R. (1999). Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca^{2+} channel current. *Am. J. Physiol.,* **276,** H2085–H2093.

GRAINGER, J., SENARATNA, R.N. & BOACHIE-ANSAH, G. (2000). The role of the endothelium and arachidonic acid metabolites in the vasorelaxant actions of anandamide in sheep coronary arteries. *Br. J. Pharmacol.,* **131,** 75P.

ISHIOKA, N. & BUKOSKI, R.D. (1999). A role for N-arachidonoylethanolamine (anandamide) as the mediator of sensory nerve-dependent Ca^{2+}-induced relaxation. *J. Pharmacol. Exp. Ther.,* **289,** 245–250.

KUNOS, G., JÁRAI, Z., VARGA, K., LIU, J., WANG, L. & WAGNER, J.A. (2000). Cardiovascular effects of endocannabinoids-the plot thickens. *Prostag. Lipid Med.,* **61,** 71–84.

LAKE, K.D., COMPTON, D.R., VARGA, K., MARTIN, B.R. & KUNOS, G. (1997). Cannabinoid-induced hypotension and bradycardia in rats is mediated by CB_1-like cannabinoid receptors. *J. Pharmacol. Exp. Ther.,* **281,** 1030–1037.

LIU, J., GAO, B., MIRSHAHI, F., SANYAL, A.J., KHANOLKAR, A.D., MAKRIYANNIS, A. & KUNOS, G. (2000). Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem. J.,* **346,** 835–840.

MACCARRONE, M., BARI, M., LORENZON, T., BISOGNO, T., DI MARZO, V. & FINAZZI-AGRO, A. (2000). Anandamide uptake by human endothelial cells and its regulation by nitric oxide. *J. Biol. Chem.,* **275,** 13484–13492.

MOMBOULI, J.V., SCHAEFFER, G., HOLZMANN, S., KOSTNER, G.M. & GRAIER, W.F. (1999). Anandamide-induced mobilization of cytosolic Ca^{2+} in endothelial cells. *Br. J. Pharmacol.,* **126,** 1593–1600.

NELSON, M.T. & QUAYLE, J.M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol.,* **268,** C799–C822.

PERTWEE, R.G., FERNANDO, S.R., GRIFFIN, G., ABADJI, V. & MAKRIYANNIS, A. (1995). Effect of phenylmethylsulphonyl fluoride on the potency of anandamide as an inhibitor of electrically evoked contractions in two isolated tissue preparations. *Eur. J. Pharmacol.,* **272,** 73–78.

PLANE, F., HOLLAND, M., WALDRON, G.J., GARLAND, C.J. & BOYLE, J.P. (1997). Evidence that anandamide and EDHF act *via* different mechanisms in rat isolated mesenteric arteries. *Br. J. Pharmacol.,* **121,** 1509–1511.

PRATT, P.F., HILLARD, C.J., EDGEMOND, W.S. & CAMPBELL, W.B. (1998). N-arachidonylethanolamide relaxation of bovine coronary artery is not mediated by CB1 cannabinoid receptor. *Am. J. Physiol.,* **274,** H375–H381.

RALEVIC, V., KENDALL, D.A., RANDALL, M.D., ZYGMUNT, P.M., MOHAVED, P. & HÖGESTÄTT, E.D. (2000). Vanilloid receptors on capsaicin-sensitive sensory nerves mediate relaxation to methanandamide in the rat isolated mesenteric arterial bed and small mesenteric arteries. *Br. J. Pharmacol.,* **130,** 1483–1488.

RANDALL, M.D., ALEXANDER, S.P.H., BENNETT, T., BOYD, E.A., FRY, J.R., GARDINER, S.M., KEMP, P.A., MCCULLOCH, A.I. & KENDALL, D.A. (1996). An endogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem. Biophys. Res. Commun.,* **229,** 114–120.

RANDALL, M.D. & KENDALL, D.A. (1997). Involvement of an endogenous cannabinoid in EDHF-mediated vasorelaxation in the rat coronary vasculature. *Eur. J. Pharmacol.,* **335,** 205–209.

RANDALL, M.D. & KENDALL, D.A. (1998a). Endocannabinoids: a new class of vasoactive substances. *Trends Pharmacol. Sci.,* **19,** 55–58.

RANDALL, M.D. & KENDALL, D.A. (1998b). Anandamide and endothelium-derived hyperpolarizing factor act via a common relaxant mechanism in rat mesentery. *Eur. J. Pharmacol.,* **346,** 51–53.

RANDALL, M.D., MCCULLOCH, A.I. & KENDALL, D.A. (1997). Comparative pharmacology of endothelium-derived hyperpolarizing factor and anandamide in rat isolated mesentery. *Eur. J. Pharmacol.,* **333,** 191–197.

SMART, D., GUNTHORPE, M.J., JERMAN, J.C., NASIR, S., GRAY, J., MUIR, A.I., CHAMBERS, J.K., RANDALL, A.D. & DAVIS, J.B. (2000). The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.,* **129,** 227–230.

SUGIURA, T., KODAKA, T., NAKANE, S., KISHIMOTO, S., KONDO, S. & WAKU, K. (1998). Detection of an endogenous cannabinoid molecule, 2-arachidonoylglycerol, and cannabinoid CB1 receptor mRNA in human vascular cells: is 2-arachidonoylglycerol a possible vasomodulator? *Biochem. Biophys. Res. Commun.,* **243,** 838–843.

SZALLASI, A. & BLUMBERG, M. (1999). Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol. Rev.,* **51,** 159–212.

VAN DEN BOSSCHE, I. & VANHEEL, B. (2000). Influence of cannabinoids on the delayed rectifier in freshly dissociated smooth muscle cells of rat aorta. *Br. J. Pharmacol.,* **131,** 85–93.

WAGNER, J.A., VARGA, K., JARAI, Z. & KUNOS, G. (1999). Mesenteric vasodilation mediated by endothelial anandamide receptors. *Hypertension,* **33,** 429–434.

WHITE, R. & HILEY, C.R. (1997). A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery. *Br. J. Pharmacol.,* **122,** 1573–1584.

WHITE, R. & HILEY, C.R. (1998a). The actions of the cannabinoid receptor antagonist, SR 141716A, in the rat isolated mesenteric artery. *Br. J. Pharmacol.,* **125,** 689–696.

WHITE, R. & HILEY, C.R. (1998b). The actions of some cannabinoid receptor ligands in the rat isolated mesenteric artery. *Br. J. Pharmacol.,* **125,** 533–541.

ZOU, A.P., MA, Y.H., SUI, Z.H., ORTIZ DE MONTELLANO, P.R., CLARK, J.E., MASTERS, B.S. & ROMAN, R.J. (1994). Effects of 17-octadecynoic acid, a suicide-substrate inhibitor of cytochrome P450 fatty acid ω-hydrolase, on renal function in rats. *J. Pharmacol. Exp. Ther.,* **268,** 474–481.

ZYGMUNT, P.M., CHUANG, H., JULIUS, D., MOHAVED, P. & HÖGESTÄTT, E.D. (2000). The anandamide transport inhibitor AM404 activates vanilloid receptors. *Eur. J. Pharmacol.,* **396,** 39–42.

ZYGMUNT, P.M., HÖGESTÄTT, E.D., WALDECK, K., EDWARDS, G., KIRKUP, A.J. & WESTON, A.H. (1997). Studies on the effects of anandamide in rat hepatic artery. *Br. J. Pharmacol.,* **122,** 1679–1686.

ZYGMUNT, P.M., PETERSSON, J., ANDERSSON, D.A., CHUANG, H., SØRGÅRD, M., DI MARZO, V., JULIUS, D. & HÖGESTÄTT, E.D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature,* **400,** 452–457.

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