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The actions of some cannabinoid receptor ligands in the rat isolated mesenteric artery

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1 The actions of a number of cannabinoid receptor ligands were investigated using the myograph-mounted rat isolated mesenteric artery. Anandamide, CP 55,940, HU-210, palmitoylethanolamide and WIN 55,212-2 all caused concentration-dependent relaxations of methoxamine-precontracted vessels which were not affected by removal of the endothelium.

2 Precontracting vessels with 60 mM KCl instead of methoxamine greatly reduced the vasorelaxant effects of anandamide and palmitoylethanolamide. High K^+ solution caused a modest decrease in the relaxant potency of CP 55,940 and HU-210, and had no effect on relaxations induced by WIN 55,212-2.

3 Relaxations of methoxamine-induced tone by anandamide, CP 55,940 and HU-210, but not palmitoylethanolamide and WIN 55,212-2, were attenuated by the cannabinoid receptor antagonist, SR 141716A. Relaxation of vessels contracted with 60 mM KCl by CP 55,940 was also sensitive to SR 141716A.

4 Anandamide and CP 55,940 caused small but concentration-dependent contractions in resting vessels in the absence of extracellular calcium. These were not sensitive to SR 141716A. Palmitoylethanolamide and WIN 55,212-2 produced smaller contractions only at higher concentrations.

5 Anandamide and CP 55,940, but not palmitoylethanolamide and WIN 55,212-2, caused concentration-dependent inhibition of the phasic contractions induced by methoxamine in calcium-free conditions, but only anandamide caused inhibition of contractions to caffeine under such conditions. These inhibitory effects were not antagonised by SR 141716A.

6 The present study provides the first detailed investigation of the actions of cannabinoid agonists on vascular smooth muscle. Our results show that these compounds exert both receptor-dependent and -independent effects on agonist-induced calcium mobilization in the rat isolated mesenteric artery.

Keywords: Cannabinoids; rat small mesenteric artery; cannabinoid antagonists; SR 141716A; anandamide; palmitoylethanolamide; caffeine

Introduction

The central and peripheral actions of marijuana, mediated principally by Δ^9-tetrahydrocannabinol, have been shown relatively recently to be mediated by cannabinoid receptors, of which CB_1, CB_{1A} and CB_2 subtypes have been cloned (see Howlett, 1995, for review). The CB_1 and CB_{1A} subtypes are generally considered to be central receptors (Matsuda *et al*., 1990; Shire *et al*., 1995), whereas the CB_2 receptor has been localized principally to peripheral tissues, and in particular immune tissue (Munro *et al*., 1993; Facci *et al*., 1995).

There has recently been considerable interest in the actions of cannabinoids on the cardiovascular system, especially since Randall *et al*. (1996) proposed that arachidonylethanolamide (anandamide), the putative endogenous ligand for the CB_1 receptor (Di Marzo *et al*., 1994), might represent an endothelium-derived hyperpolarizing factor (EDHF). Subsequent studies have cast doubt on this hypothesis, however, in particular, relaxation to anandamide is not affected by a combination of K^+ channel blockers (apamin and charybdotoxin) that is known to abolish the actions of EDHF (White & Hiley, 1997; Zygmunt *et al*., 1997). Furthermore, Zygmunt *et al*. (1997) showed that anandamide caused smooth muscle hyperpolarization only in the presence of a functional endothelium, which argues against it representing an EDHF. Their results also showed that anandamide caused inhibition of spontaneous transient outward currents (STOCs) and outward currents activated by caffeine, and they therefore concluded that anandamide might cause vasorelaxation by interfering with calcium mobilization in smooth muscle. Indeed, cannabinoids have been shown to cause release of calcium from intracellular stores through both receptor-dependent (Filipeanu *et al*., 1997) and receptor-independent (Felder *et al*., 1995) mechanisms. Furthermore, R-methanandamide, a stable cannabinoid derivative, causes depletion of such stores in astrocytes (Venance *et al*., 1997).

There is evidence that the actions of cannabinoids may vary between different preparations; whereas anandamide causes endothelium-independent relaxations sensitive to a cannabinoid receptor antagonist, (SR 141716A; Rinaldi-Carmona *et al*., 1994) in rat mesenteric vessels (Randall *et al*., 1996; White & Hiley, 1997), the relaxation induced in bovine coronary arteries is endothelium-dependent, insensitive to SR 141716A, and probably involves metabolism of anandamide to vasoactive arachidonic acid derivatives (Pratt *et al*., 1998). Establishing the mechanism of action of cannabinoid agents on vascular smooth muscle is of considerable significance, as there is growing evidence that endogenous cannabinoids may have important cardiovascular actions (see Randall & Kendall, 1998, for review); for example, Deutsch *et al*. (1997) showed that anandamide might represent an endothelium-derived vasodilator in the rat kidney. Furthermore, Wagner *et al*. (1997) have provided evidence that anandamide may be the mediator of haemorrhagic hypotension, whilst Lake *et al*. (1997) showed that anandamide-induced hypotension and bradycardia may be mediated through cannabinoid CB_1 receptors. Vidrio *et al*. (1996) showed that the stable

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cannabinoid agonist HU-210 caused bradycardia and hypotension in both conscious and anaesthetized rats, suggesting that metabolism of cannabinoids to other vasoactive compounds does not underlie their vascular actions *in vivo*.

The aim of the present study was to examine the actions of a number of cannabinoid agonists in the rat isolated mesenteric artery, and to investigate the mechanisms underlying these effects as far as possible in the myograph-mounted preparation. Cannabinoid receptor pharmacology is currently limited by a lack of availability of selective agonists and antagonists. However in the present study we have used anandamide as well as the putative endogenous CB_2 receptor ligand, palmitoylethanolamide (Facci *et al*., 1995) and the synthetic agonists CP 55,940, HU-210, WIN 55,212-2 and R(+)-methanandamide. SR 141716A was used to determine the role of cannabinoid receptors in the responses to these agonists. In order to define more precisely the mechanisms of action of the cannabinoids, we examined their actions under a number of conditions. Firstly, the agents were added to both resting and methoxamine-precontracted vessels. Secondly, phasic contractions to caffeine and methoxamine in calcium-free solution in the absence or presence of the cannabinoids were used to investigate possible effects on intracellular calcium stores. Finally, precontraction of vessels with depolarising K^+ solution (60 mM K^+) was used to reveal possible inhibition of voltage-operated calcium channels (VOCCs).

A preliminary account of some of these results was presented at the Harrogate meeting of the British Pharmacological Society in December 1997 (White & Hiley, 1998a).

Methods

Male Wistar rats (250–350 g; Tucks, Rayleigh, Essex) were anaesthetised 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; 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, JP Trading, Aarhus, Denmark) as described in White & Hiley (1997). 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 normalised 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 362 ± 3 μm ($n = 221$). 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 intimal surface 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 12.0 ± 0.2 mN ($n = 221$). 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 mean tension generated by methoxamine (10 μM) in endothelium-denuded vessels was 12.5 ± 1.1 mN ($n = 19$), which was not significantly different from intact vessels.

Experiments in the presence of extracellular calcium

After the test for endothelial integrity, vessels were left for 30 min before being precontracted with 10 μM methoxamine in order to obtain cumulative concentration-relaxation data to the appropriate cannabinoid agonist. Preliminary experiments showed that 10 μM methoxamine caused approximately 80% of the maximum contraction of vessels and was therefore a suitable submaximal concentration for precontraction of both endothelium-denuded and intact vessels. In experiments investigating the effects of cannabinoids on VOCCs, vessels denuded of endothelium were precontracted with high K^+ (60 mM) Krebs–Henseleit solution, which was prepared by equimolar substitution of NaCl for KCl in the standard Krebs–Henseleit buffer described previously. The mean tension generated by endothelium-denuded vessels in response to 60 mM KCl was 12.7 ± 0.7 mN ($n = 33$), which was not significantly different from the response to 10 μM methoxamine. The presence of an intact endothelium did not alter the response to 60 mM KCl (13.5 ± 1.0 mN; $n = 8$).

Where the role of cannabinoid receptors in relaxant responses was to be determined, the cannabinoid antagonist SR 141716A (1 or 3 μM) was added to the bath 30 min prior to construction of the concentration-response curve to the cannabinoid agonist under study. A higher concentration of SR 141716A (10 μM) alone caused 25.7 ± 4.6% relaxation ($n = 4$) of methoxamine-induced tone, and hence was not used in this study (see White & Hiley, 1998b); the two concentrations that were employed had no significant effect on contractions to methoxamine. As the effects of the cannabinoid agents under investigation were not reversed by washing, preparations were discarded after a single concentration-response curve had been constructed.

As both WIN 55,212-2 and palmitoylethanolamide only caused relaxation at relatively high concentrations, vehicle controls (for DMSO and ethanol respectively) were established by adding the appropriate volume of vehicle to methoxamine-precontracted arteries with intact endothelium.

Calcium-free experiments

Experiments in the absence of extracellular calcium were essentially carried out according to the methods used by Julou-Schaeffer & Freslon (1988). Briefly, intracellular stores were replenished by incubating vessels for 45 min in normal Krebs–Henseleit solution, with the bath solution being changed four times during this period. Extracellular calcium was then removed by washing vessels three times with calcium-free Krebs–Henseleit solution (composition the same as normal Krebs–Henseleit buffer but with calcium chloride omitted), and then incubating them for a further 45 min in calcium-free solution, with the bath solution being changed a further four times during this period. Release of intracellular stores was then determined by eliciting phasic contractions to either caffeine (50 mM) or methoxamine (10 μM). In order to examine the effects of cannabinoid agonists on such contractions, these agents were incubated with vessels for 10 min before addition of caffeine or methoxamine. In experiments examining the possible involvement of cannabinoid receptors, vessels were incubated with SR 141716A (3 μM) for 30 min prior to addition of the appropriate cannabinoid agonist.

Preliminary experiments revealed that up to three reproducible contractions to either caffeine or methoxamine could

be obtained from a single preparation. However, as the effects of the cannabinoid agents under investigation were not reversed by washing, a typical experiment involved eliciting a control contraction to either caffeine or methoxamine, then replenishing intracellular calcium and removing extracellular calcium in the manner detailed above, before obtaining a test contraction in the presence of the cannabinoid agonists under examination. Vessels were then discarded.

The effect of cannabinoid agents on resting vessels under calcium-free conditions was also investigated. In order to examine the possible role of cannabinoid receptors, vessels were incubated with SR 141716A (3 μM) for 30 min prior to addition of the appropriate cannabinoid agonist. The small contractions elicited by cannabinoid agonists in resting vessels under calcium-free conditions were not significantly different when experiments were carried out in the presence of extracellular calcium (data not shown).

Data and statistical analysis

All relaxation responses are expressed as the percentage relaxation of the tone induced by 10 μM methoxamine or 60 mM KCl. Data are given as the mean $\pm$ s.e.mean. EC_{50} values for vasorelaxant responses were obtained from individual concentration-response curves by fitting the data to the logistic equation:

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

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 using KaleidaGraph software (Synergy Software, Reading, PA, U.S.A) running on a Macintosh computer. Statistical analysis of the variables was carried out by two-way analysis of variance and an *F*-test.

Where concentration-response data could not be fitted to a logistic function, or for comparison of cannabinoid-induced-contractions, statistical comparison of the data was carried out by Mann-Whitney U test using InStat (GraphPad Software, San Diego, CA, U.S.A.). For comparison of contractions to caffeine or methoxamine, the paired *t*-test was used. *P* values less than 0.05 were considered to be statistically significant.

Drugs

All solutions were prepared on the day of the experiment. Methoxamine, arachidonic acid (sodium salt) and carbachol (Sigma Chemical Company, Poole, Dorset, U.K.) were dissolved in distilled water. Palmitoylethanolamide (Tocris Cookson, Bristol) and R-(+)-methanandamide (Research Biochemicals International, Natick, MA, U.S.A.) were dissolved in 100% ethanol. CP 55,940, HU-210 (both Tocris) and WIN 55,212-2 (RBI) were dissolved in 100% DMSO. Caffeine (Sigma) was dissolved in Krebs–Henseleit buffer. Anandamide was synthesized and dissolved in an inert oil/water emulsion by Dr E.A. Boyd (University of Nottingham). SR 141716A was provided by Research Biochemicals International as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract N01MH30003, and was dissolved in 100% ethanol. Dilutions were made in distilled water.

Results

Effect of endothelial removal on the actions of cannabinoid receptor ligands

Anandamide, CP 55,940, HU-210, palmitoylethanolamide and WIN 55,212-2 all caused concentration-dependent relaxations of methoxamine-precontracted vessels. Table 1 shows that removal of the endothelium had no significant effect on the EC_{50} or R_{max} values for relaxation to anandamide, CP 55,940 or HU-210 which were obtained from the curve fitting procedure. Relaxations to palmitoylethanolamide and WIN 55,212-2 generally occurred only at high concentrations such that solubility limitations prevented definition of a true maximum response. Therefore concentration-response data for intact and endothelium-denuded arteries are presented in Figure 1 along with vehicle controls for both palmitoylethanolamide (100% ethanol) and WIN 55,212-2 (100% DMSO); data were compared by Mann–Whitney U test. Figure 1 shows that removal of endothelium had no significant effect on relaxation to either palmitoylethanolamide or WIN 55,212-2.

Actions of cannabinoid receptor ligands in vessels precontracted with 60 mM KCl

Figure 2 shows that precontracting endothelium-denuded arteries with 60 mM KCl greatly reduced the maximal relaxant effect of anandamide (R_{max}: methoxamine-induced tone, $84.4 \pm 0.3\%$; tone induced by 60 mM KCl $= 37.1 \pm 6.7\%$; $n = 4$; $P < 0.001$; Figure 2a). Similarly, the relaxant effect of palmitoylethanolamide was almost abolished in vessels precontracted with high K^+ (relaxation of methoxamine-induced tone by 100 μM palmitoylethanolamide $= 63.8 \pm 8.3\%$; relaxation of KCl-induced tone $= 19.5 \pm 2.5\%$; $n = 4$; $P < 0.01$). Arachidonic acid (10 μM) induced only $7.8 \pm 2.6\%$ relaxation of methoxamine-induced tone ($n = 4$; Figure 2a), whilst the stable anandamide derivative, R-(+)-methanandamide (Abadji *et al*., 1994) not only caused complete relaxation of endothelium-denuded vessels but was more potent than anandamide ($EC_{50} = 55 \pm 3$ nM; $R_{max} = 99.5 \pm 1.6\%$; $n = 6$; Figure 2a).

Table 1 Effect of endothelial removal on relaxation of methoxamine-precontracted rat isolated mesenteric artery by anandamide, CP 55,940 and HU-210

	Endothelium intact			*Endothelium denuded*		
	EC_{50} (μM)	R_{max} (%)	n	EC_{50} (μM)	R_{max} (%)	n
Anandamide	0.31 ± 0.07	95.9 ± 9.0	3	0.39 ± 0.06	99.5 ± 7.2	3
CP 55,940	3.92 ± 0.44	85.9 ± 3.0	4	4.31 ± 0.25	85.1 ± 1.6	3
HU-210	3.00 ± 0.91	86.7 ± 5.0	4	4.72 ± 0.39	88.8 ± 1.6	4

Data are expressed as mean $\pm$ s.e.mean. EC_{50} and R_{max} values were obtained from the curve fitting procedure described in Methods. *n* values indicate the number of animals used. No significant differences from control values were found.

Contraction with high K^+ solution caused a 2.7-fold rightward shift ($P<0.01$) in the concentration-response curve to CP 55,940 relative to results with methoxamine without significantly altering the maximum relaxation (Figure 2b). A slightly larger shift (4.4-fold; $P<0.001$) was observed for responses to HU-210 and, again, there was no significant effect on the maximum response.

Figure 2c shows that the relaxant effect of WIN 55,212-2 was not significantly different between vessels contracted with methoxamine or high K^+.

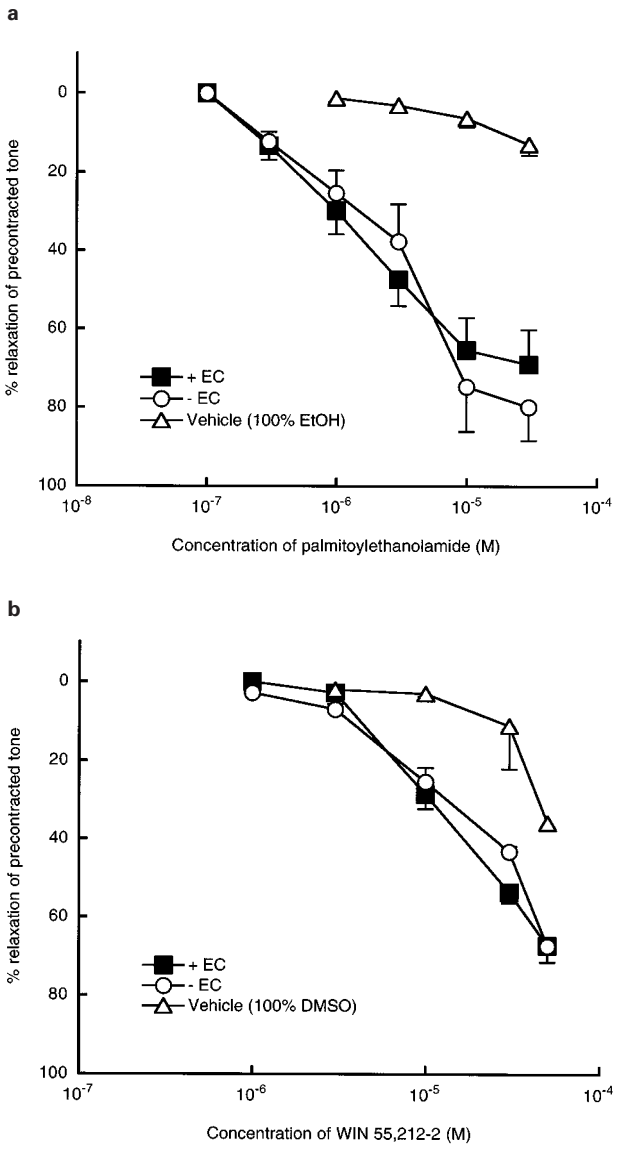

Figure 1 Concentration-response data for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery. Relaxation was induced by (a) palmitoylethanolamide or (b) WIN 55,212-2 and was determined in the presence (+EC) or absence (−EC) of a functional endothelium. Values are shown as mean and vertical lines indicate s.e.mean. Endothelium intact; (a) $n=6$; (b) $n=3$; endothelium denuded; (a) $n=6$; (b) $n=3$; vehicle control; (a) 100% ethanol, $n=4$; (b) 100% DMSO, $n=8$. Final vehicle concentrations in the myograph ranged from 0.01% (v/v) for addition of 1 μM relaxant to a maximum of 0.3% ethanol (for 30 μM palmitoylethanolamide) or 0.5% DMSO (for 50 μM WIN 55,212-2). Data were compared by Mann–Whitney U test at individual concentrations. No significant differences were found between relaxations in the absence and presence of endothelium; all relaxations to the agonists were found to be significantly ($P<0.05$) greater than the effect of the respective vehicle.

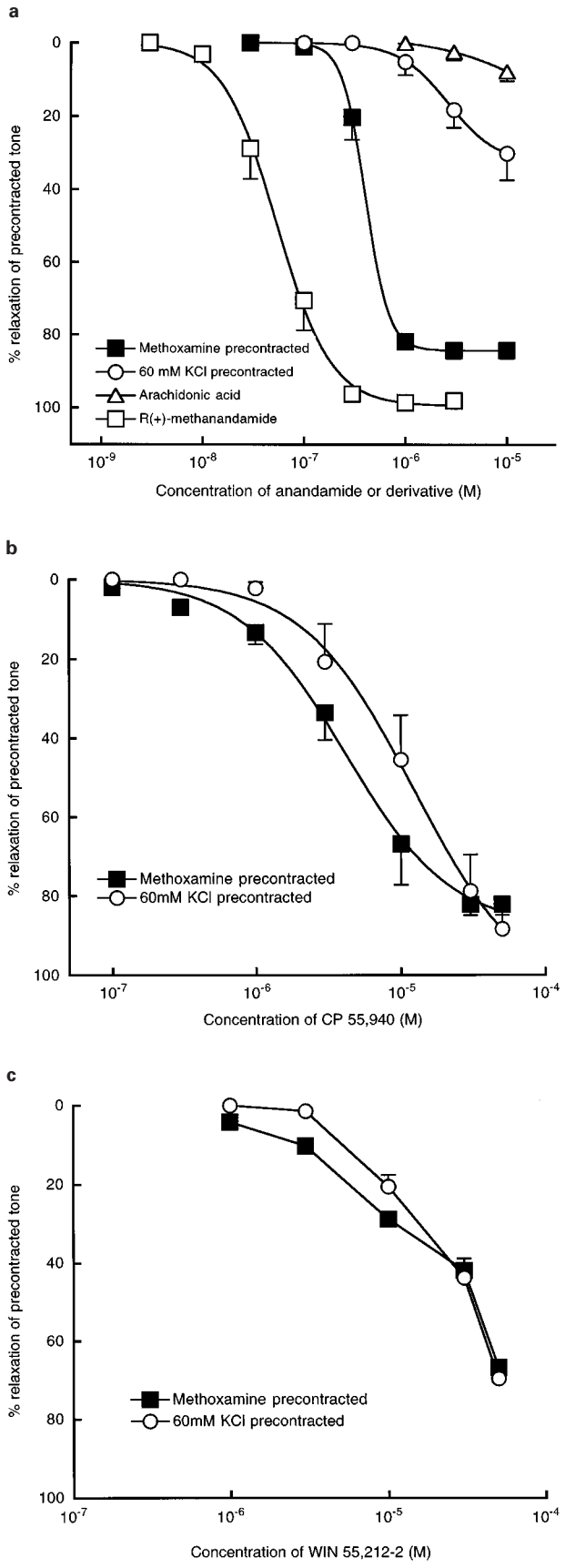

Figure 2 Concentration-response data for relaxation of precontracted rat isolated mesenteric artery by (a) anandamide, (b) CP 55,940 or (c) WIN 55,212-2. Vessels were precontracted with either methoxamine or 60 mM KCl. Values are shown as mean and vertical lines indicate s.e.mean, $n=4$ for all data. For (a) and (b), the curves drawn are those obtained from the curve fitting procedure. In (c), data were compared by the Mann–Whitney U test at individual concentrations; no significant differences were found.

Effect of the cannabinoid receptor antagonist, SR 141716A on relaxations of methoxamine-induced tone by cannabinoid receptor ligands

SR 141716A caused rightward shifts in the concentration-response curves to both anandamide and CP 55,940 in mesenteric arteries without endothelium. The curve for relaxations induced by anandamide was shifted rightwards 1.6-fold ($P<0.01$) by 1 μM SR 141716A with no change in the maximum relaxation. SR 141716A (3 μM) caused a larger 2.8-fold rightward shift ($P<0.01$) and also reduced the maximum response ($P<0.001$; Figure 3a).

SR 141716A (1 μM) caused a 2-fold rightward shift in the concentration-response curve to CP 55,940 ($P<0.01$) and also reduced the maximum response ($P<0.05$; Table 2). SR 141716A (3 μM) caused a 3.6-fold rightward shift in the CP 55,940 concentration-response curve ($P<0.01$) and a significant decrease in maximum relaxation ($P<0.05$; Table 2).

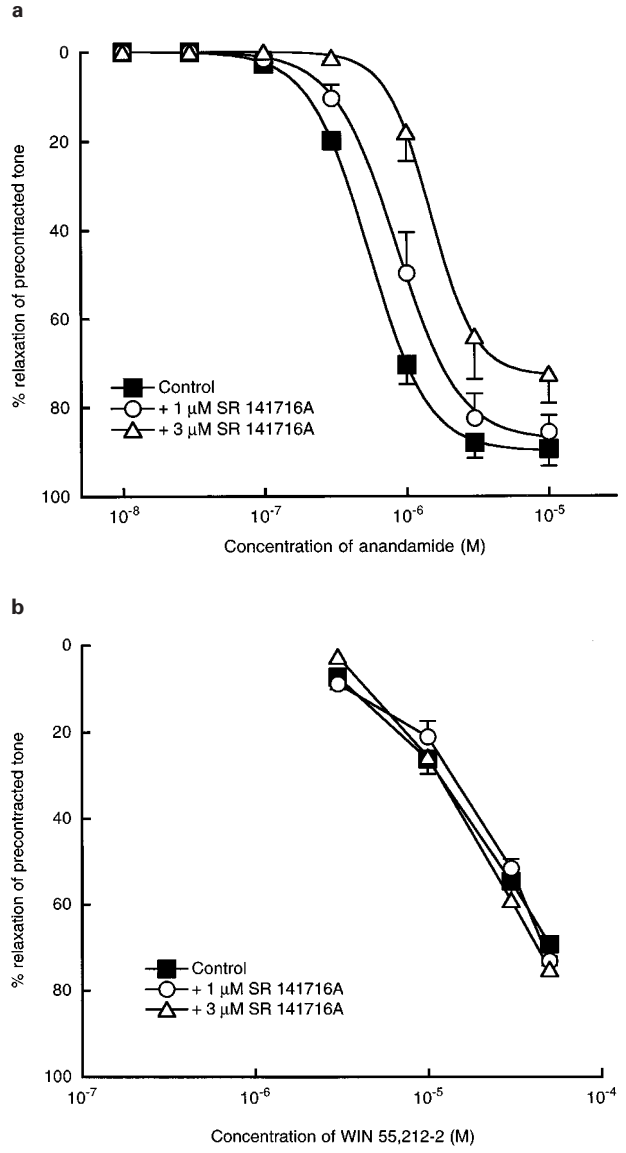

Figure 3 Concentration-response data for relaxation of methoxamine-precontracted rat isolated mesenteric artery by (a) anandamide or (b) WIN 55,212-2. Relaxations were determined in either the absence or the presence of either 1 μM or 3 μM SR 141716A. Values are shown as mean and vertical lines indicate s.e.mean, $n=4$-8. For (a), the curves drawn are those obtained from the curve fitting procedure. In (b), data were compared by Mann–Whitney U test at individual concentrations; no significant differences were found.

Although complete concentration-response curves could not be constructed due to solubility limitations, the relaxations to palmitoylethanolamide (data not shown) and WIN 55,212-2 were not affected by SR 141716A (1 or 3 μM; Figure 3b).

Table 2 also shows that SR 141716A (1 μM) caused a rightward shift ($P<0.01$) in the concentration-response curve to HU-210 but had no effect on the maximum response.

Effect of the cannabinoid receptor antagonist, SR 141716A on relaxation by CP 55,940 of tone induced by 60 mM KCl

CP 55,940 caused concentration-dependent relaxation of mesenteric arteries without endothelium precontracted with 60 mM KCl (Figure 4). The concentration-response curve was shifted rightwards 1.9-fold ($P<0.01$) by 1 μM SR 141716A. SR 141716A (3 μM) caused a further rightwards shift ($P<0.01$); although solubility limitations prevented definition of a true maximum response, the shift at the EC_{50} was 4.9-fold ($n=4$). Control data in Figure 4 are pooled for clarity.

Effect of cannabinoid receptor ligands on resting tone

Figure 5 shows that both anandamide and CP 55,940 caused small, but concentration-dependent contractions in endothelium-denuded resting vessels in the absence of extracellular calcium. These were not inhibited by preincubation of preparations with 3 μM SR 141716A. This figure also shows that palmitoylethanolamide and WIN 55,212-2 caused small contractions at high concentrations. Anandamide and CP 55,940 also caused small contractions of resting mesenteric arteries in the presence of extracellular calcium that were not significantly different from those seen under calcium-free conditions (data not shown).

Effect of cannabinoid receptor ligands on contractions to caffeine in the absence of extracellular calcium

Caffeine (50 mM) caused transient contractions of denuded resting vessels in the absence of extracellular calcium that were significantly reduced in a concentration-dependent manner by prior incubation of preparations with anandamide and were also reduced by R(+)-methanandamide (Figure 6). The inhibitory effects of anandamide were not reversed by

Table 2 Effect of the cannabinoid receptor antagonist, SR 141716A, on relaxation of methoxamine-precontracted, endothelium-denuded rat isolated mesenteric artery by CP 55,940 and HU-210

	EC_{50} (μM)	R_{max} (%)	n
CP 55,940			
Control	3.92±0.44	85.9±3.0	4
+SR 141716A (1 μM)	7.73±0.28**	78.6±1.0*	6
Control	2.40±0.55	73.3±5.8	4
+SR 141716A (3 μM)	8.67±0.55**	62.5±0.3*	8
HU-210			
Control	1.72±0.33	63.6±3.0	4
+SR 141716A (1 μM)	5.41±0.33**	56.8±8.9	5

Data are expressed as mean±s.e.mean. EC_{50} and R_{max} values were obtained from the curve fitting procedure desribed in Methods. n values indicate the number of animals used. *$P<0.05$, **$P<0.01$ indicate significant differences from control values.

SR 141716A (3 μM). The cannabinoid receptor antagonist alone had no effect on contractions to caffeine (control contraction to 50 mM caffeine = 2.90 ± 0.51 mN; in the presence of 3 μM SR 141716A = 2.69 ± 0.41; $n = 3$).

Figure 6 also shows that neither CP 55,940 nor palmitoylethanolamide had a significant effect on caffeine-induced contractions.

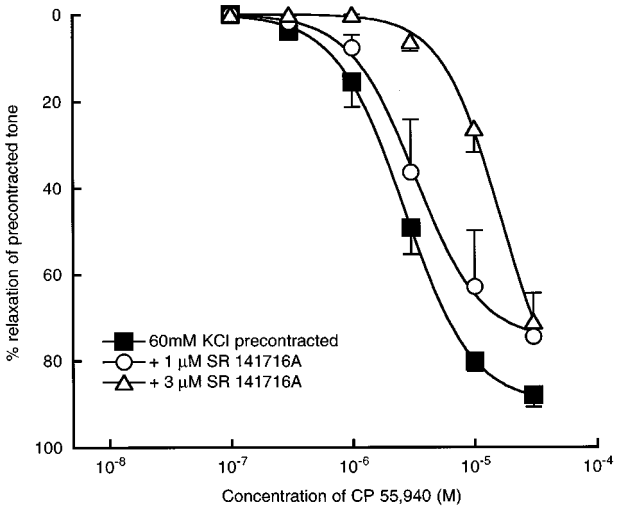

Figure 4 Concentration-response curves for relaxation by CP 55,940 of rat mesenteric arteries precontracted with 60 mM KCl. Relaxations were determined in either the absence or the presence of either 1 μM or 3 μM SR 141716A. Values are shown as mean and vertical lines indicate s.e.mean, $n = 4$-8. The curves drawn are those obtained from the curve fitting procedure.

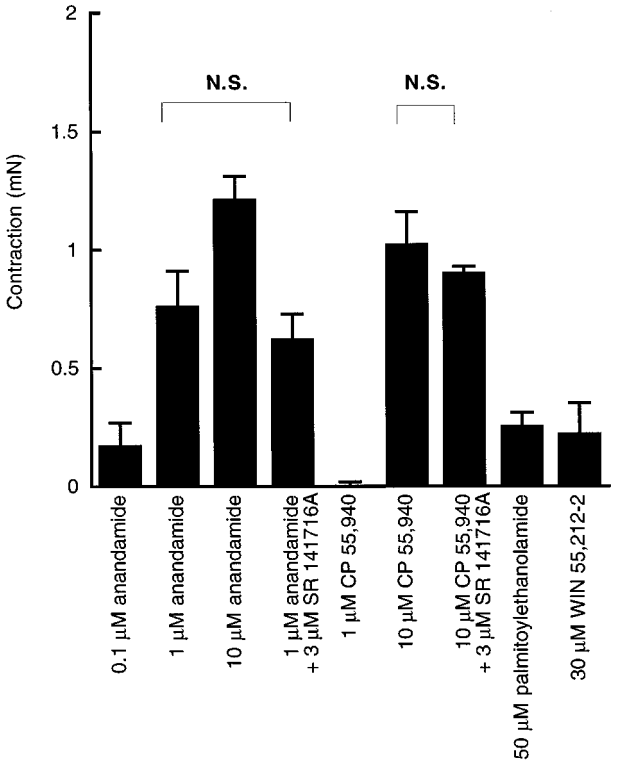

Figure 5 Peak contractions of resting rat mesenteric arteries induced by cannabinoid receptor ligands in the absence of extracellular calcium. Values are shown as mean and vertical lines indicate s.e.mean, $n = 4$–12. Data for contractions in the presence or absence of 3 μM SR 141716A were compared by paired t-test as indicated; no significant differences (NS) were found.

Effect of cannabinoid receptor ligands on contractions to methoxamine in the absence of extracellular calcium

Figure 7 shows that contractions of resting denuded vessels in the absence of extracellular calcium by methoxamine (10 μM) were reduced by prior incubation of preparations with anandamide or CP 55,940. The inhibitory effects of anand-

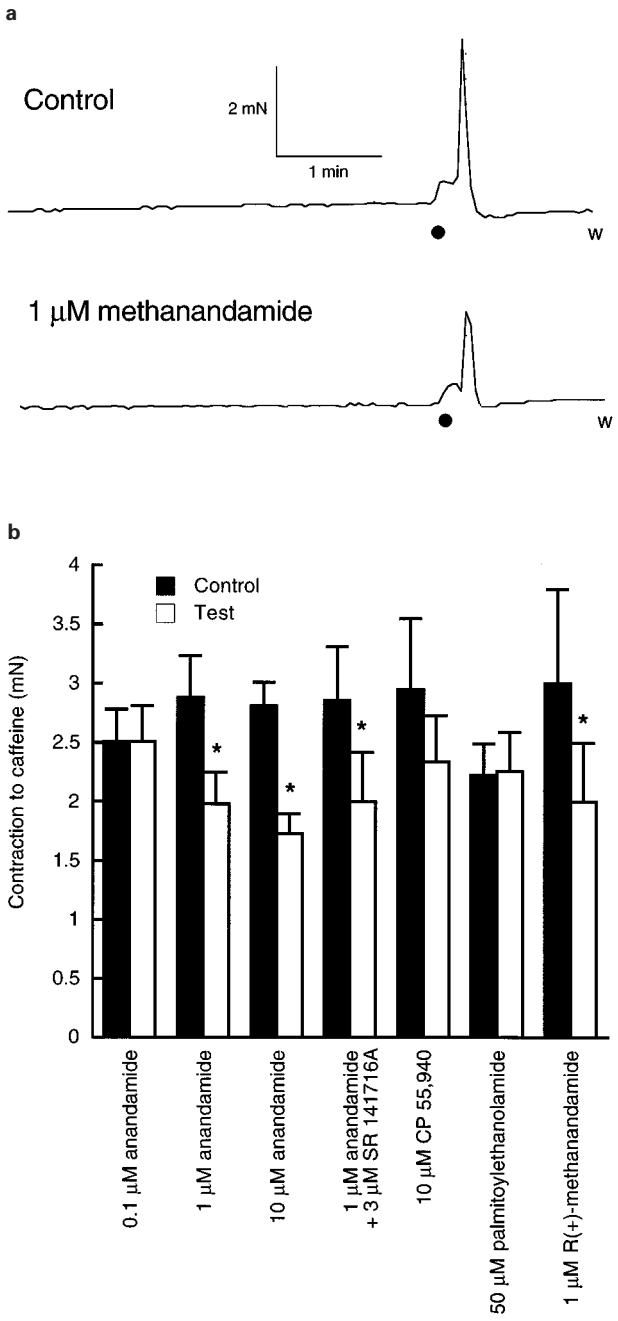

Figure 6 (a) Representative trace showing the phasic contractions induced by caffeine (50 mM) in endothelium-denuded rat mesenteric arteries in the absence of extracellular calcium. Contractions were obtained from a single vessel in the absence or presence of 1 μM methanandamide. Symbols denote addition of caffeine (filled circles) and washing (w) at the times indicated. (b) Peak contractions of resting rat isolated mesenteric artery induced by caffeine (50 mM) in the absence (filled columns) or presence (open columns) of cannabinoid receptor ligands in the absence of extracellular calcium. Values are shown as mean and vertical lines indicate s.e.mean, $n = 4$–6. Control and test data were compared by paired t-test as indicated; * indicates $P < 0.05$, and ** $P < 0.01$ compared to control values.

amide and CP 55,940 were not significantly altered by SR 141716A (3 μM), which alone had no effect on contractions to methoxamine (control contraction to 10 μM methoxamine = 3.52 ± 0.88 mN; in the presence of 3 μM SR 141716A = 3.71 ± 0.37; $n = 3$).

Figure 7 also shows that neither WIN 55,212-2 nor palmitoylethanolamide had any significant effect on methoxamine-induced contractions under calcium-free conditions.

Discussion

The present study represents the first extensive study of the actions of cannabinoid receptor ligands on vascular smooth muscle. Our results indicate that the cannabinoids show a range of both receptor-dependent and receptor-independent actions that interfere with the mobilization of calcium in vascular smooth muscle cells and hence cause the endothelium-independent relaxations that were common to all the agents tested.

Receptor-dependent mechanisms

Anandamide, CP 55,940 and HU-210 caused endothelium-independent relaxations of methoxamine-precontracted rat mesenteric arteries that were antagonised by the cannabinoid receptor antagonist SR 141716A. Palmitoylethanolamide and WIN 55,212-2 also caused endothelium-independent relaxations, but these were not sensitive to SR 141716A and are therefore unlikely to be mediated through cannabinoid receptors. Arachidonic acid had only a very slight relaxant effect on methoxamine-precontracted vessels with intact endothelium, which suggests that degradation of anandamide into vasoactive metabolites is unlikely to occur under the conditions of the present study, unlike in bovine coronary arteries (Pratt *et al*., 1998). Further evidence that degradation of anandamide to vasoactive metabolites does not contribute to its relaxant effects comes from our finding that the stable anandamide analogue, R-(+)-methanandamide, also caused endothelium-independent relaxation of mesenteric arteries. R-(+)-methanandamide was a more potent relaxant than anandamide which, taken together with our previous observation that anandamide-induced relaxation is potentiated by the anandamide aminohydrolase inhibitor, phenylmethylsulphonyl fluoride (White & Hiley, 1997), suggests that, although anandamide may undergo some degradation, the metabolites do not contribute to its relaxant effects.

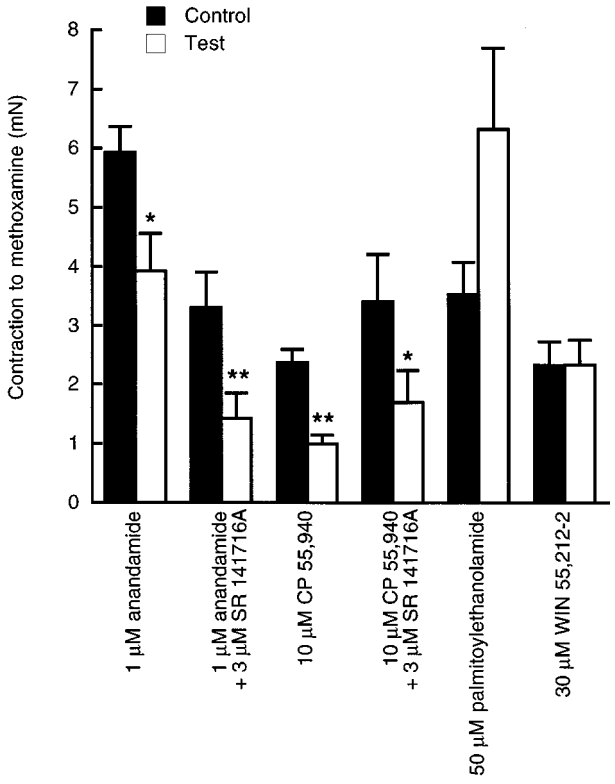

Figure 7 Peak contractions of resting rat isolated mesenteric artery induced in the absence of extracellular calcium by methoxamine (10 μM) either in the absence (filled columns) or presence (open columns) of cannabinoid receptor ligands. Values are shown as mean and vertical lines indicate s.e.mean, $n = 4-6$. Control and test data were compared by paired t-test as indicated; * indicates $P < 0.05$, and ** $P < 0.01$ compared to control values.

Our results appear to indicate the presence of two SR 141716A-sensitive relaxant mechanisms. The first might be inhibition of VOCCs, as both CP 55,940 and HU-210 caused relaxation of vessels precontracted with depolarizing K^+ solution; tone in such vessels is mediated almost entirely by VOCC activation (see review by Karaki *et al*., 1997). CP 55,940-induced relaxation of high K^+ precontracted preparations was inhibited by SR 141716A in a concentration-dependent fashion, with rightward shifts in the concentration-response curves of 1.9-fold (1 μM SR 141716A) and 4.9-fold (3 μM SR 141716A), indicating that inhibition of VOCCs may be mediated through cannabinoid receptors. Indeed, cannabinoid agonists have been shown to inhibit L-type calcium currents via a pertussis toxin-sensitive mechanism in a neuroblastoma cell line (Caulfield & Brown, 1992).

Relaxations to anandamide, however, are unlikely to involve such a mechanism, as precontracting arteries with high K^+ almost abolished relaxation to this agent. However concentration-response curves for relaxation of methoxamine-induced tone by anandamide were shifted rightwards by 1.6-fold and 2.8-fold in the presence of 1 μM and 3 μM SR 141716A respectively. These data suggest that anandamide causes relaxation via a cannabinoid receptor. However the magnitudes of the shifts caused by SR 141716A are consistent with this antagonist having a K_D of around 1.6 μM for such a site, which does not correspond to its affinity for either CB_1 (11.8 nM; Felder *et al*., 1995), CB_{1A} (43.3 nM; Rinaldi-Carmona *et al*., 1996) or CB_2 receptors (702 nM; Showalter *et al*., 1996). This is also true for the antagonism by SR 141716A of relaxations to CP 55,940 and HU-210. Hence the identity of the cannabinoid receptor(s) mediating these effects is not clear. Although the calculated dissociation constants for SR 141716A are closer to published data for CB_2 receptors, the relative lack of effect of WIN 55,212-2 and palmitoylethanolamide, which are more active at CB_2 than CB_1 receptors (Felder *et al*., 1995; Facci *et al*., 1995) does not support the involvement of this subtype.

The results show that anandamide causes concentration-dependent inhibition of caffeine-and methoxamine-induced contractions in calcium free solution; these were not sensitive to 3 μM SR 141716A. The small contractions produced by anandamide and CP 55,940 in resting vessels in the absence of extracellular calcium were also unaffected by the cannabinoid antagonist. Therefore, we are unable to identify directly the SR 141716A-sensitive mechanism by which anandamide causes relaxation, though the above observations suggest that it is unlikely to involve an effect on intracellular calcium stores, either directly or by, for example, increasing re-uptake of calcium. We can also rule out a reduction in the calcium sensitivity of the contractile apparatus as the basis for such a

mechanism, as if this were the case anandamide would be expected to cause relaxation of high K^+-precontracted vessels, which it does not. Recent work has shown that anandamide does not cause relaxation through activation of K^+ channels (White & Hiley, 1997; Zygmunt *et al*., 1997) and our previous observation that anandamide does not relax methoxamine-precontracted vessels in the presence of 25 mM KCl (White & Hiley, 1997) shows that anandamide does not act as an adrenergic antagonist. We therefore hypothesize that the SR 141716A-sensitive mechanism by which anandamide causes relaxation involves inhibition of the intermediate stage between release of intracellular calcium and activation of VOCCs, which is thought to involve activation of a calcium-activated chloride conductance (CAC; Criddle *et al*., 1996).

Indeed, Plane *et al*. (1997) showed that anandamide caused repolarization as well as relaxation of phenylephrine-induced tone. This is not consistent with simple inhibition of VOCCs, as this would block agonist-induced contraction, but not depolarization (Clark & Garland, 1993). Furthermore, inhibition of store release is unlikely to be the basis for the relaxant effects of anandamide, as agents that are known to deplete intracellular calcium stores, such as ryanodine and caffeine, have little effect on agonist-induced contractions in the presence of extracellular calcium (Julou-Schaeffer & Freslon, 1988; Garcha & Hughes, 1995). Finally, Zygmunt *et al*. (1997) showed that anandamide abolished spontaneous transient outward currents (STOCs) triggered by release of intracellular calcium stores, but had no direct effect on the BK_{Ca} channel that carries such currents. This is very similar to the actions of niflumic acid, a known inhibitor of CAC channels (Kirkup *et al*., 1996). Taken together, these observations provide strong evidence that anandamide inhibits the CAC channel signal which couples release of intracellular stores to VOCC activation.

In summary, there would appear to be two cannabinoid receptor-mediated vasorelaxant mechanisms in the rat mesenteric artery. Firstly, inhibition of VOCCs, which enables relaxation of high K^+-induced tone by CP 55,940 and HU-210 but not by anandamide. Secondly, possible inhibition of the CAC channel is likely to represent the mechanism of anandamide relaxation. This mechanism may also contribute to relaxation by CP 55,940 and HU-210.

Receptor-independent mechanisms

Caffeine caused transient contractions in calcium-free solution that may be attributed to release of calcium from intracellular stores. Methoxamine also caused phasic contractions under such conditions; these were abolished by prior stimulation of vessels with caffeine, indicating that both agonists act to release a common calcium store in rat mesenteric arteries (Baro & Eisner, 1995). Anandamide and CP 55,940 caused small, concentration-dependent, contractions of resting vessels under conditions of low external calcium concentration at similar concentrations to their relaxant effects. These were not sensitive to SR 141716A, which suggests that cannabinoid receptors are not involved in this effect. Palmitoylethanolamide and WIN 55,212-2 caused very small contractions at high concentrations, but these were similar to those produced by their respective solvents. It is notable that the contractions induced by cannabinoid agonists were not significantly different in the presence of extracellular calcium, which shows that the low level of calcium released from intracellular stores by these agents is not sufficient to stimulate extracellular calcium entry in these vessels.

Anandamide and CP 55,940, but not palmitoylethanolamide and WIN 55,212-2, caused inhibition of contractions to methoxamine in calcium free solution, and this was not sensitive to 3 μM SR 141716A. Anandamide and R(+)-methanandamide, but not CP 55,940, inhibited caffeine-induced contractions, and this also was not sensitive to SR 141716A. These results show that anandamide and CP 55,940 may inhibit agonist-induced release of calcium from intracellular stores, possibly by themselves causing release of such stores (Felder *et al*., 1995; Filipeanu *et al*., 1997). However the observation that relaxation of methoxamine-induced tone by anandamide and CP 55,940 in the presence of extracellular calcium was antagonized by SR 141716A is evidence that the receptor-independent actions of these agents on intracellular stores may make only a minor contribution to their relaxant effects, as was noted previously. Furthermore, the observation that R(+)-methanandamide had the same effect on caffeine-induced contractions as anandamide shows that this effect is not due to breakdown of anandamide to, for example, arachidonic acid.

Although palmitoylethanolamide caused relaxation of methoxamine-precontracted arteries, it had no effect on vessels stimulated with high K^+ solution, which shows that this cannabinoid does not inhibit VOCCs. It also had no inhibitory effect on agonist-induced store release under calcium free conditions. We therefore suggest that palmitoylethanolamide inhibits coupling of intracellular store release to VOCC opening. Evidence for this comes from the study of Gamberucci *et al*. (1997), who showed that fatty acids, similar in structure to palmitoylethanolamide, inhibited calcium-induced calcium release through a direct action on cell membranes. This would explain the receptor-independent actions of the cannabinoid observed in the present study, and might also explain why the relaxant potency of this agent varied so widely between vessels.

WIN 55,212-2 caused relaxations of methoxamine- and high K^+-induced tone at high concentrations. These were not sensitive to 3 μM SR 141716A, hence it seems possible that the actions of WIN 55,212-2 are receptor-independent. It should be noted that both WIN 55,212-2 and palmitoylethanolamide may act on a receptor with a very low affinity for SR 141716A. However, we found that higher concentrations of SR 141716A (10 μM or greater) than those used in the present investigation caused marked relaxant effects, and it therefore seems likely that this drug has cannabinoid receptor-independent actions at high concentrations (White & Hiley, 1998b);. As was noted previously. However, published values for the affinity of SR 141716A for cloned cannabinoid receptors suggest that 3 μM SR 141716A should be sufficient to cause antagonism of these receptors. Therefore if WIN 55,212-2 and palmitoylethanolamide do act through a cannabinoid receptor, it is unlikely to correspond to any of the currently characterized subtypes.

In conclusion, this study has provided the first detailed investigation of the actions of cannabinoid agonists on vascular smooth muscle. Our results suggest that cannabinoids exert both receptor-dependent and -independent effects on agonist-induced calcium mobilization in the rat isolated mesenteric artery. The receptor-mediated actions are likely to involve both inhibition of VOCCs and of the intermediate step coupling intracellular store release and VOCC activation, which is probably the CAC channel. The cannabinoids studied also showed receptor-independent effects on store release, VOCC activation and calcium-induced calcium mobilization, although these are likely to

play only a minor role in the vasorelaxant effects of this family of compounds.

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