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Cannabinoid activation of recombinant and endogenous vanilloid receptors

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Abstract

The effects of three structurally related cannabinoids on human and rat recombinant vanilloid VR1 receptors expressed in human embryonic kidney (HEK293) cells and at endogenous vanilloid receptors in the rat isolated mesenteric arterial bed were studied. In the recombinant cells, all three were full agonists, causing concentration-dependent increases in $[Ca^{2+}]_i$ (FLIPR™), with a rank order of potency relative to the vanilloids capsaicin and olvanil, of olvanil ≥ capsaicin > AM404 ((allZ)-*N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide) > anandamide > methanandamide. These responses were inhibited by the vanilloid VR1 receptor antagonist, capsazepine. In the mesenteric arterial bed, vasorelaxation was evoked by these ligands with a similar order of potency. The AM404-induced vasorelaxation was virtually abolished by capsaicin pretreatment. AM404 inhibition of capsaicin-sensitive sensory neurotransmission was blocked by ruthenium red, but not by cannabinoid CB_1 and CB_2 receptor antagonists. AM404 had no effect on relaxations to calcitonin gene-related peptide. These data demonstrate that the vasorelaxant and sensory neuromodulator properties of AM404 in the rat isolated mesenteric arterial bed are mediated by vanilloid VR1 receptors.

Keywords: Anandamide; Cannabinoid; Capsaicin; Mesenteric arterial bed; Sensory nerve; Vanilloid receptor

1. Introduction

In recent years, there has been renewed interest in the cardiovascular effects of cannabinoids (see Randall and Kendall, 1998). Vasomotor actions of cannabinoids mediated at both the smooth muscle and the endothelium have been described (Randall and Kendall, 1998; White and Hiley, 1998; Jarai et al., 1999; Wagner et al., 1999). In 1999, it was reported that anandamide, an endogenous cannabinoid, mediates vasorelaxation of isolated small arteries by actions at vanilloid receptors on sensory nerves and release of calcitonin gene-related peptide (Zygmunt et al., 1999). The vanilloid VR1 receptor, a ligand-gated cation channel sensitive to capsaicin and its analogues, had been cloned a few years earlier (Caterina et al., 1997) and, in patch clamp experiments, Zygmunt et al. (1999) additionally showed that anandamide is a partial agonist at the rat vanilloid VR1 receptor expressed in human embryonic kidney (HEK293) cells. Anandamide has also been shown to be a full agonist at the human vanilloid VR1 receptor (Smart et al., 1999). In addition, methanandamide, a stable analogue of anandamide, mediates vasorelaxation via activation of vanilloid receptors on sensory nerves in rat small mesenteric arteries and mesenteric arterial bed (Ralevic et al., 2000b). Most recently, AM404 ((all Z)-*N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide), a competitive inhibitor of carrier-mediated anandamide transport (Beltramo et al., 1997) and analogue of anandamide, was shown to activate the rat vanilloid VR1 receptor and endogenous vanilloid receptors in rat isolated hepatic arteries (Zygmunt et al., 2000). Notably, these cannabinoids display a high structural similarity to capsaicin.

There is, therefore, compelling evidence that certain cannabinoids can activate vanilloid receptors. Vanilloid receptors are expressed almost exclusively on sensory nerves, and an important implication of these findings is

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that cannabinoids may be endogenous ligands of vanilloid receptors and, thus, may participate in the afferent and efferent functions of capsaicin-sensitive sensory neurones (Zygmunt et al., 1999). There is, however, evidence for the expression of specific receptors for cannabinoids on sensory neurones. Cannabinoid CB_1 receptor mRNA is synthesized in dorsal root ganglion cells (Hohmann and Herkenham, 1999), and there is trafficking of cannabinoid CB_1 receptors from dorsal root ganglia to the periphery (Hohmann and Herkenham, 1998). Moreover, there is evidence that the synthetic cannabinoid HU210 ((−)-11-hydroxy-Δ^8-tetrahydrocannabinol-dimethylheptyl) can modulate capsaicin-sensitive sensory neurotransmission in the rat mesenteric arterial bed (Ralevic and Kendall, 2001).

The aim of the present study was to characterise the effects of the structurally related cannabinoids AM404, anandamide and methanandamide on rat and human vanilloid VR1 receptors expressed in HEK293 cells and at endogenous vanilloid receptors in the rat isolated mesenteric arterial bed. The relative potencies of the cannabinoids compared to the archetypal vanilloid capsaicin, and to olvanil, were evaluated. Responses at rat and human vanilloid VR1 receptors were measured using a fluorimetric imaging plate reader (FLIPR)-based Ca^{2+} influx assay. The possible involvement of endogenous vanilloid and/or cannabinoid receptors in cannabinoid (AM404) modulation of perivascular capsaicin-sensitive sensory neurotransmission in the mesenteric arterial bed was also investigated.

2. Materials and methods

2.1. Cloning and expression of vanilloid VR1 receptors in HEK293 cells

A rat vanilloid VR1 receptor mammalian expression construct was prepared by amplifying cDNA from reverse transcribed adult rat dorsal root ganglia mRNA, using forward and reverse primers incorporating the restriction sites shown, RVR1F–*Hin*dIII (5′-CATAAGCTTGCCGCCATGGAACAACGGGCTAGCTTAGACTCAGAGG) and RVR1R–*Xba*I (5′-CATTCTAGACCATTATTTCTCCCCTGGGACCATGG). Reaction products were cloned into pBSSKII$^+$ (Stratagene), confirmed by sequencing and then subcloned into the *Hin*dIII–*Xba*I sites of pcDNA3.1 (Invitrogen). HEK 293 cells were transfected with rVR1.pcDNA3.1 using Lipofectamine Plus (Life Technologies, Paisley, UK), according to the manufacturer's instructions, and stably expressed in HEK293 cells.

The human vanilloid VR1 receptor was cloned and expressed as described previously (Smart et al., 1999). Briefly, the expressed sequence tag T48002 was identified and its sequence extended by rapid amplification of the cDNA ends. The full cDNA was then amplified from brain cDNA, inserted into the expression vector pcDNA3.1, double strand sequenced, and stably expressed in HEK293 cells.

2.2. Cell culture

Vanilloid VR1 receptor-HEK293 cells were grown as monolayers in minimum essential medium supplemented with nonessential amino acids, 10% foetal calf serum, and 0.2 mM L-glutamine, and maintained under 95%/5% O_2/CO_2 at 37 °C. Cells were passaged every 3–4 days and the highest passage number used was 20.

2.3. Measurement of $[Ca^{2+}]_i$ using the FLIPR

Rat or human vanilloid VR1 receptor-HEK293 cells were seeded into black walled clear-base 96-well plates (Costar UK) at a density of 25,000 cells/well in minimum essential medium, supplemented as above, and cultured overnight. The cells were then incubated with minimum essential medium containing the cytoplasmic Ca^{2+} indicator, Fluo-3AM (4 μM; Teflabs, Austin, TX) at 25 °C for 120 min. The cells were washed four times with, and finally cultured in, Tyrode's medium containing 0.2% bovine serum albumin, before being incubated for 30 min at 25 °C with either buffer alone (control) or buffer containing various antagonists. The plates were then placed into a FLIPR (Molecular Devices, Sunnyvale, CA) to monitor cell fluorescence (λ_{EX} = 488 nm, λ_{EM} = 540 nm) (Sullivan et al., 1999) before and after the addition of various agonists. Neither the addition of agonists or antagonists affected the pH of the assay buffer. In some studies, the pH was altered by the addition of HCl (1–2.4 mM).

2.4. Isolated perfused mesenteric arterial beds

Male Wistar rats (250–300 g) were killed by decapitation after exposure to CO_2. Mesenteric beds were isolated and perfused as described previously (Ralevic et al., 1996). In brief, the abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was cut, blood flushed from the preparation with 0.5 ml of Krebs' solution and the gut dissected carefully away from the mesenteric vasculature. The preparation was mounted on a stainless steel grid (7 × 5 cm) in a humid chamber and perfused at a constant flow rate of 5 ml min^{-1} using a peristaltic pump (model 7554-30, Cole-Parmer Instrument, Chicago, IL). The perfusate was Krebs' solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8, gassed with 95% O_2–5% CO_2 and maintained at 37 °C. After 10 min, guanethidine (5 μM) was added to the perfusate in order to block sympathetic neurotransmission, and after a further 20 min methoxamine (5–40 μM) was added in order to raise the tone of the preparations (by 30–80 mmHg) above baseline. Electrical field stimulation (2–12

Hz, 0.1 ms, 60 V, 30 s) (Grass S9D stimulator) was used to generate frequency–response curves. Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model P23XL, Viggo-Spectramed, Oxnard, CA) on a side arm of the perfusion cannula, and recorded on a polygraph (model 7D, Grass Instrument, Quincy, MA).

2.5. Experimental protocol

Two consecutive frequency-dependent relaxant response curves to electrical field stimulation (2–12 Hz) were generated in each mesenteric arterial bed preparation. These are reproducible when generated under control conditions. The first response curve acted as the control and the second was conducted in the presence of AM404 (0.1–3 μM), added 20 min before starting electrical field stimulation. Tone of the preparations was maintained when necessary by further addition of methoxamine (to 40–80 μM). In other groups of preparations, both the control frequency–response curve and the response curve in the presence of AM404 were conducted in the presence of either ruthenium red (1 μM), SR 144528 (1 μM; (*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide)), LY 320135 (1 μM; [6-methoxy-2-(4-methoxyphenyl)benzo[b]thien-3-yl][4-cyanophenyl]methanone)) or capsazepine (1 μM), after incubation with these drugs for at least 30 min. In another group of mesenteric arterial beds, vasorelaxant responses to acetylcholine (0.5 pmol–50 nmol), sodium nitroprusside (5 pmol–50 nmol), calcitonin gene-related peptide (0.5–500 pmol) and capsaicin (5 pmol–5 nmol) in the absence of guanethidine and presence of vehicle or AM404 were investigated. In another group of preparations relaxation–response curves to cumulative concentrations of anandamide, methanandamide, AM404, capsaicin and olvanil, each compound in a separate preparation, were constructed. Capsaicin pretreatment (10 μM; 1 h perfusion and superfusion, followed by 40 min washout) of mesenteric arterial beds was as described previously (Ralevic et al., 2000b).

2.6. Data analysis—recombinant vanilloid VR1 receptor expressing cells

Responses in vanilloid VR1 receptor-HEK293 cells were measured as peak fluorescence intensity minus basal fluorescence intensity, and where appropriate, were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean ± S.E.M. unless otherwise stated. Curve-fitting (4 parameter logistic fit) and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software, California, USA). pK_B values were generated from IC_{50} curves for the antagonist vs. a fixed EC_{80} concentration of agonist using the Cheng–Prusoff equation (Cheng and Prusoff, 1973). This method assumes the antagonists act competitively, which is the case for those used in this study (Jerman et al., 2000).

2.7. Data analysis—mesenteric arterial beds

Vasorelaxant responses of the mesenteric arterial beds were measured as changes in perfusion pressure (mmHg) and expressed as percentage relaxation of the methoxamine-induced increase in tone above baseline. Where the comparison was between more than two groups, data were compared by analysis of variance (ANOVA) with Tukey's post hoc test. The effects of inhibitors on AM404 modulation of sensory neurotransmission were analysed by Student's paired *t*-test. A value of $P < 0.05$ was taken to indicate a statistically significant difference. pD_2 values (negative logarithm of the dose of agonist required to elicit a half maximal response) were determined for each experiment where appropriate and are presented as the mean ± S.E.M. F_{50} is the stimulation frequency (Hz) required to elicit a response that is half of the maximal relaxation.

2.8. Drugs

AM404, anandamide, methanandamide and olvanil were purchased from Tocris (Bristol, UK). All cell culture media were obtained from Life Technologies. Capsaicin, calcitonin gene-related peptide and methoxamine (hydrochloride) were from Sigma. Guanethidine (Ismelin) was from Alliance Pharmaceuticals (Chippenham, Wiltshire). Capsazepine and all other ligands were obtained from RBI (Natick, MA, USA). Capsaicin, olvanil and LY320135 were dissolved as stock solutions of 10 mM in dimethyl sulfoxide. SR144528 and AM404 were made up as 10 mM stock solutions in ethanol.

3. Results

3.1. Effect of cannabinoids on $[Ca^{2+}]_i$ in vanilloid VR1 receptor-HEK293 cells

AM404, anandamide, olvanil and capsaicin (all at 100 pM–10 μM), caused concentration-dependent increases in $[Ca^{2+}]_i$ in rat vanilloid VR1 receptor-HEK293 cells (Fig. 1A), but were without effect in the nontransfected HEK293 cell line (data not shown). AM404 and anandamide both displayed a similar efficacy to capsaicin and olvanil (Fig. 1A) and had comparable slope factors, but were markedly less potent (Table 1). However, the AM404 and anandamide-induced Ca^{2+} responses did have similar kinetics to that of the capsaicin-induced response in the FLIPR, with an initially rapid then slowing onset (peak ~ 30 s) followed by a gradually declining secondary phase (Fig. 1B).

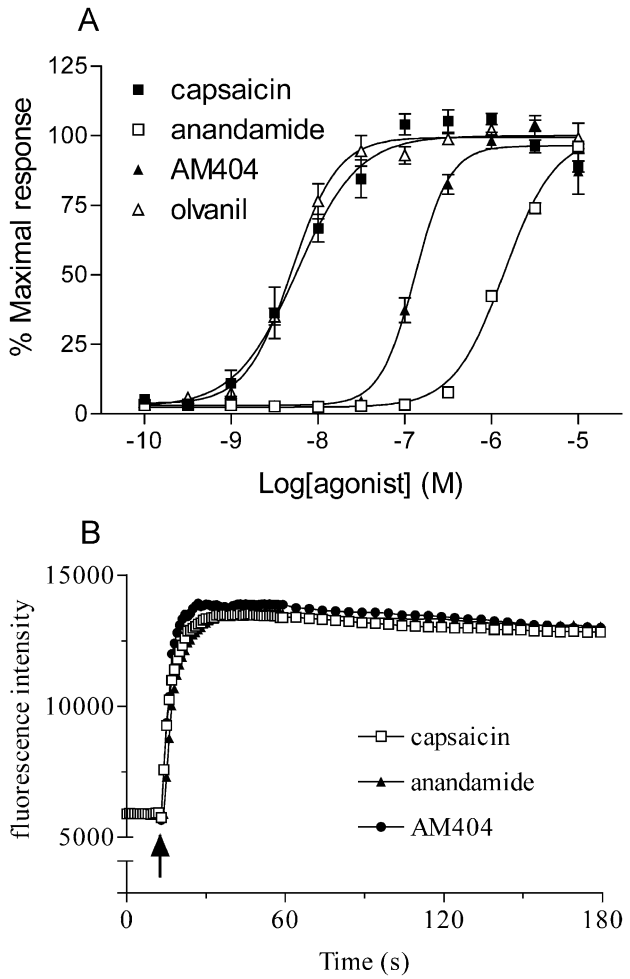

Fig. 1. (A) The AM404- and anandamide-induced Ca^{2+} responses are concentration-dependent in rat vanilloid VR1 receptor-HEK cells. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in rat vanilloid VR1 receptor-HEK293 cells before and after the addition of capsaicin (100 pM–10 μM), anandamide (100 pM–10 μM), AM404 (100 pM–10 μM) or olvanil (100 pM–10 μM). Responses were measured as peak increase in fluorescence minus basal, expressed relative to the maximum capsaicin response and are given as mean ± S.E.M., where $n = 8$. (B) AM404- and anandamide-induced Ca^{2+} responses have the same kinetics in rat vanilloid VR1 receptor-HEK293 cells. Intracellular Ca^{2+} concentrations (as fluorescence intensity) were measured in rVR1-HEK293 cells before and after the addition (at arrow) of capsaicin (100 nM), AM404 (300 nM) or anandamide (1 μM). Data shown are representative traces, typical of at least $n = 20$.

AM404 and anandamide caused similar concentration-dependent responses in human vanilloid VR1 receptor-HEK293 cells (Table 1), although the slope factors for olvanil and AM404 were lower than those of capsaicin and anandamide (Table 1). Moreover, the anandamide analogue, methanandamide, also increased $[Ca^{2+}]_i$ in these cells in a concentration-related manner, but was less potent than anandamide, only evoking a 44.6 ± 3.8% response at 10 μM ($n = 3$). Like AM404 and anandamide, this ligand was without effect in the nontransfected HEK293 cells.

Table 1
Agonist potencies at the rat and human vanilloid VR1 receptor

	Rat		Human	
	pEC_{50}	Slope	pEC_{50}	Slope
Capsaicin	7.97 ± 0.13	1.87 ± 0.26	7.10 ± 0.06	2.51 ± 0.28
Anandamide	5.73 ± 0.04	2.07 ± 0.41	5.60 ± 0.10	2.36 ± 0.28
AM404	6.53 ± 0.12	2.09 ± 0.23	6.60 ± 0.12	1.40 ± 0.12
Olvanil	8.09 ± 0.09	1.60 ± 0.23	7.73 ± 0.05	1.48 ± 0.18

Data are mean ± S.E.M., where $n = 8$-10.

Interestingly, lowering the pH of the experimental buffer from 7.4 to 6.4 enhanced the potency of capsaicin and olvanil at rat vanilloid VR1 receptor (pEC_{50} values of 7.86 ± 0.09 and 8.24 ± 0.08 at pH 6.4, $n = 3$, compared to those in Table 1 at pH 7.4), but had no effect on the potency of AM404 or anandamide (pEC_{50} values of 6.60 ± 0.12 and 5.76 ± 0.04 at pH 6.4, $n = 3$, compared to those in Table 1 at pH 7.4).

3.2. Effect of capsazepine on cannabinoid-induced Ca^{2+} responses in vanilloid VR1 receptor HEK293 cells

The competitive vanilloid VR1 receptor antagonist (Szallasi and Blumberg, 1999), capsazepine (100 pM–10 μM), inhibited the AM404 (300 nM)-, anandamide (3 μM)- and capsaicin (100 nM)-induced Ca^{2+} responses in both rat and human vanilloid VR1 receptor expressing HEK293 cells (Table 2).

3.3. Effect of cannabinoids on tone of the rat mesenteric arterial preparations

The cannabinoids and vanilloids caused a concentration-dependent relaxation of methoxamine-preconstricted preparations with a potency order of: olvanil > capsaicin > AM404 > anandamide > methanandamide (Fig. 2). The pEC_{50} values were: 8.75 ± 0.05 ($n = 6$), 8.16 ± 0.07 ($n = 8$), 7.14 ± 0.12 ($n = 5$), 6.32 ± 0.04 ($n = 7$) and 5.98 ± 0.05 ($n = 5$), respectively. AM404 (0.1–3 μM) additionally caused a transient (maximum duration about 10 min) concentration-dependent increase in perfusion pressure (16.3 ± 3.2, 35.5 ± 8.5 and 48 ± 17.5 mmHg at 0.1, 1 and 3 μM AM404, respectively; $n = 4$).

Table 2
Affinity of capsazepine vs. various agonists at the vanilloid VR1 receptor

	pK_B at rat	pK_B at human
Vs. capsaicin	7.21 ± 0.06	7.31 ± 0.03
Vs. anandamide	7.40 ± 0.12	7.40 ± 0.02
Vs. AM404	7.18 ± 0.03	7.27 ± 0.04
Vs. olvanil	7.35 ± 0.06	–

Data are mean ± S.E.M., where $n = 5$.

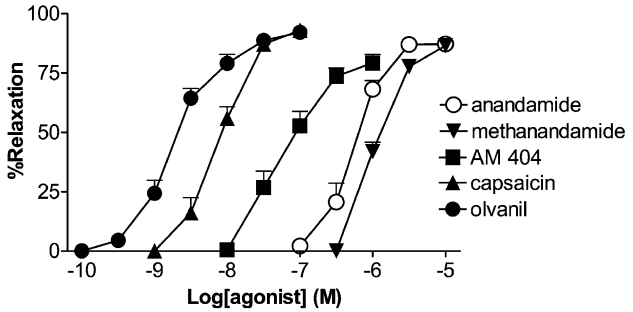

Fig. 2. Concentration vasorelaxation response curves to olvanil ($n = 6$), capsaicin ($n = 8$), AM404 ($n = 5$), anandamide ($n = 7$) and methanandamide ($n = 6$) in the rat isolated mesenteric arterial bed. Data are given as mean ± S.E.M.

3.4. Effect of capsaicin pretreatment on relaxations mediated by AM404

Capsaicin pretreatment (10 μM, 1 h) of the mesenteric arterial beds virtually abolished vasorelaxation to AM404 (0.01–1 μM) ($n = 4$). AM404 still induced vasoconstriction at the highest concentrations.

3.5. Effect of AM404 on sensory neurogenic vasorelaxation in the rat mesenteric arterial preparations

Electrical field stimulation (2–12 Hz) elicited frequency-dependent relaxations of the mesenteric arterial beds (Fig. 3). AM404 (0.1–3 μM) elicited concentration-dependent inhibition of the maximal response, but had no significant effect on the F_{50} values (Fig. 3). The R_{max} and F_{50} values in the presence of 0.1 μM AM404 were $R_{max} = 52 \pm 5.4\%$, $F_{50} = 5.3 \pm 0.34$ Hz, and the control values were $R_{max} = 61 \pm 4.5\%$, $F_{50} = 5.2 \pm 0.44$ Hz ($n = 4$). In the presence of 1 μM AM404, $R_{max} = 36 \pm 7.3\%$ ($P < 0.01$), $F_{50} = 6.0 \pm 0.44$ Hz, and the control values were $R_{max} = 66 \pm 4.1\%$, $F_{50} = 5.6 \pm 0.2$ Hz ($n = 4$). In the presence of 3 μM AM404, $R_{max} = 15 \pm 3.3\%$ ($P < 0.01$), $F_{50} = 6.5 \pm 0.78$ Hz, and the control values were $R_{max} = 49 \pm 7.6\%$, $F_{50} = 5.4 \pm 0.33$ Hz ($n = 4$).

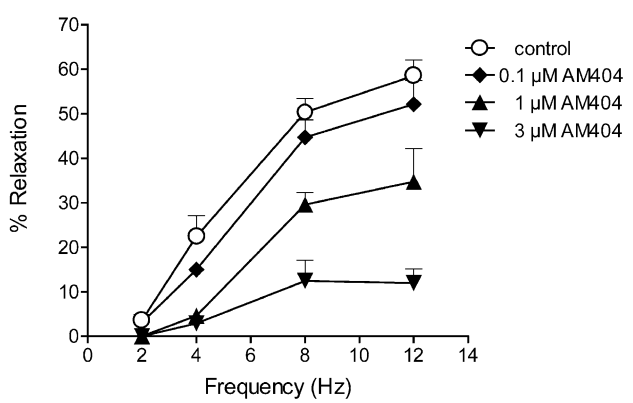

Fig. 3. Effect of AM404 (0.1–3 μM) on sensory neurogenic vasorelaxation to electrical field stimulation (2–12 Hz, 60 V, 0.1 ms, 30 s) of the rat isolated mesenteric arterial bed. Control ($n = 16$); 0.1 μM AM404 ($n = 4$); 1 μM AM404 ($n = 4$); 3 μM AM404 ($n = 4$). The maximal relaxation was significantly inhibited at both 1 and 3 μM AM404. Data are presented as means and bars indicate S.E.M.

3.6. Effect of methanandamide on sensory neurogenic vasorelaxation in the rat mesenteric arterial preparations

Methanandamide (0.1 and 1 μM) mimicked the effects of AM404 on sensory neurotransmission. Methanandamide (1 μM) attenuated the R_{max}, from 66 ± 2.4% to 22 ± 3.0% ($P < 0.001$), but there was no significant difference in the

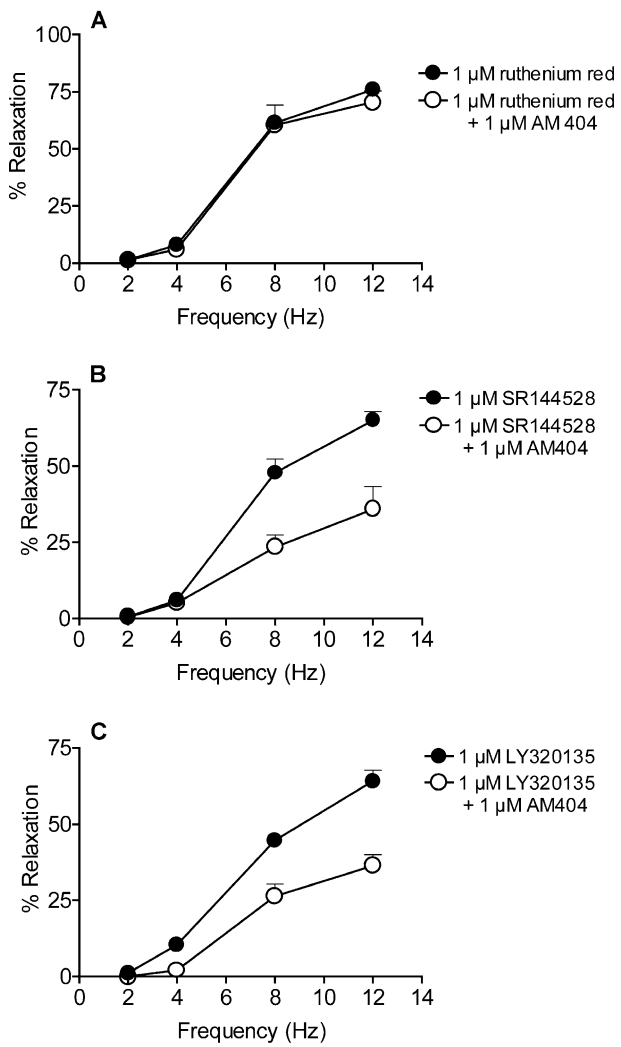

Fig. 4. Effect of (A) ruthenium red ($n = 4$), (B) SR 144528 ($n = 4$) and (C) LY 320135 ($n = 4$) on inhibition by AM404 (1 μM) of sensory neurotransmission in the rat isolated mesenteric arterial bed. Filled circles indicate responses in the presence of antagonist/inhibitor alone and open circles indicate responses in the additional presence of AM404. Inhibition by AM404 of sensory neurotransmission was blocked by ruthenium red, but was unaffected by SR 144528 and LY 320135. Data are presented as means and bars indicate S.E.M.

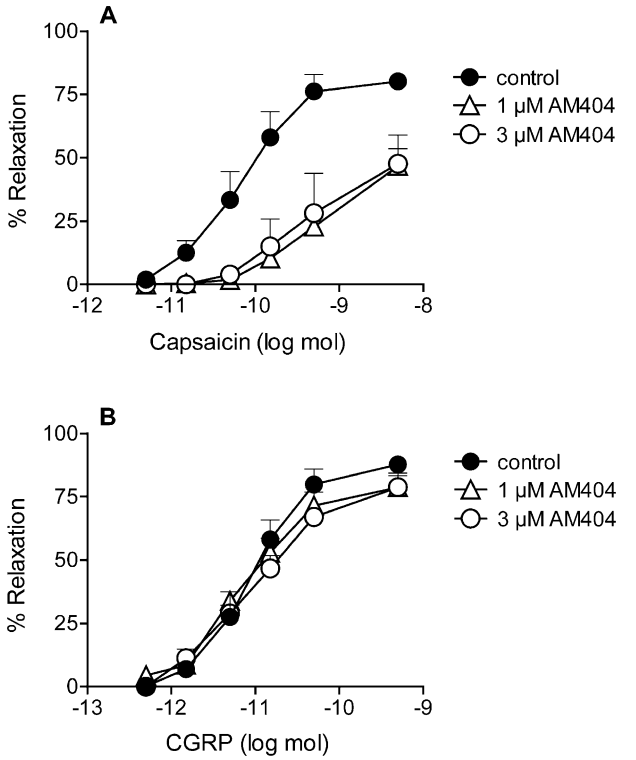

Fig. 5. Relaxant responses to doses of (A) capsaicin (5 pmol–5 nmol), (B) calcitonin gene-related peptide (CGRP; 0.5–500 pmol), of the rat isolated mesenteric arterial bed in the absence of agents ($n = 6$) and in the presence of 1 and 3 μM AM404 ($n = 4$–5). Relaxation to capsaicin was attenuated, but there was no significant effect of AM404 on relaxation to calcitonin gene-related peptide. Data are presented as means and bars indicate S.E.M.

F_{50} values at 5.9 ± 0.08 and 5.6 ± 0.09 Hz in the absence and presence of methanandamide, respectively ($n = 4$). Methanandamide (0.1 μM) also reduced the maximal response to electrical field stimulation, 49 ± 1.5% ($P < 0.05$; $n = 5$). Methanandamide at 0.1 μM, a concentration subthreshold for vasorelaxation, had no significant effect on relaxant responses to capsaicin ($n = 6$).

3.7. Effect of vanilloid and cannabinoid receptor antagonists on inhibition by AM404 of sensory neurotransmission in the rat mesenteric arterial bed

Ruthenium red (1 μM), a channel blocker and inhibitor of vanilloid responses, blocked inhibition by AM404 of sensory neurogenic vasorelaxation (ruthenium red alone, R_{max} 76 ± 3.5%, F_{50} 6.3 ± 0.05 Hz; ruthenium red plus AM404, R_{max} 70 ± 4.9%, F_{50} 6.3 ± 0.39 Hz; $n = 4$) (Fig. 4A). Neither the cannabinoid CB_1 receptor-selective antagonist, LY 320135 (1 μM; $n = 4$; Fig. 4C), nor the cannabinoid CB_2 receptor-selective antagonist, SR 144528 (1 μM; $n = 4$; Fig. 4B), blocked inhibition by AM404 of sensory neurotransmission.

3.8. Effect of AM404 on responses to capsaicin and calcitonin gene-related peptide in the rat mesenteric arterial bed

Capsaicin (5 pmol–5 nmol) elicited dose-dependent relaxation in the mesenteric arterial beds; the R_{max} was 86 ± 7% and the pD_2 was 10.2 ± 0.15 ($n = 5$). AM404 (1 and 3 μM) inhibited vasorelaxation to capsaicin (Fig. 5A). Dose-dependent vasorelaxation to calcitonin gene-related peptide (0.5–500 pmol) was not significantly affected by AM404 (1 and 3 μM) (Fig. 5B).

3.9. Effect of AM404 on responses to acetylcholine and sodium nitroprusside in the rat mesenteric arterial bed

There was a significant difference between the relaxation dose–response curves to acetylcholine in the absence and presence of AM404 (1 and 3 μM; $P < 0.01$, ANOVA), although the pD_2 and R_{max} values were not significantly different between the groups (Fig. 6A). AM404 (1 and 3 μM) had no significant effect on vasorelaxation elicited by sodium nitroprusside (Fig. 6B).

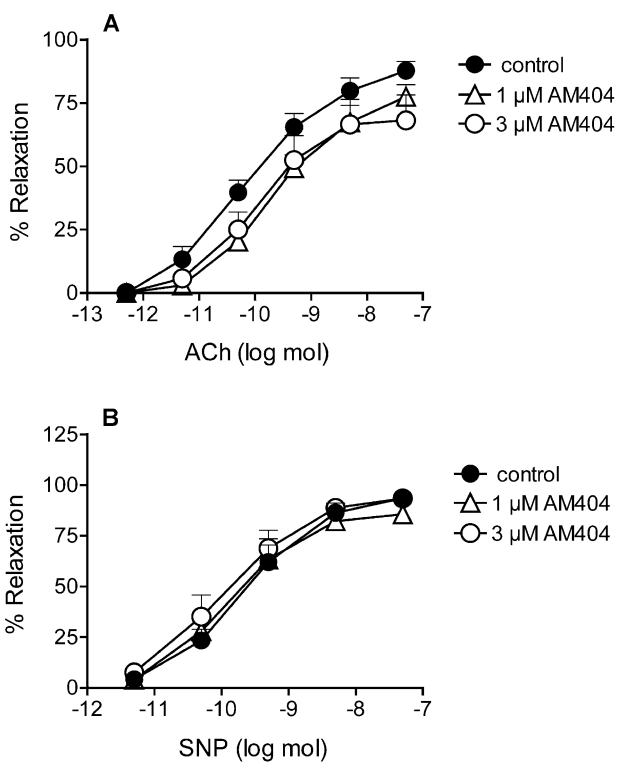

Fig. 6. Relaxant responses to doses of (A) acetylcholine (ACh, 0.5–50 pmol), (B) sodium nitroprusside (SNP; 0.5–50 pmol), of the rat isolated mesenteric arterial bed in the absence of agents ($n = 7$) and in the presence of 1 and 3 μM AM404 ($n = 5$–7). AM404 at 3 μM attenuated vasorelaxation to acetylcholine, but had no significant effect on vasorelaxation to sodium nitroprusside. Data are presented as means and bars indicate S.E.M.

4. Discussion

This study has demonstrated that the structurally related cannabinoids, AM404, anandamide and methanandamide, are agonists at recombinant vanilloid VR1 receptors and endogenous vanilloid receptors in the rat isolated mesenteric arterial bed. The potency order relative to vanilloids in both assays was: olvanil ≥ capsaicin > AM404 > anandamide > methanandamide.

AM404 and anandamide increased $[Ca^{2+}]_i$ in vanilloid rat and human vanilloid VR1 receptor-expressing, but not nontransfected, HEK293 cells. This is the first demonstration of AM404 activation of the human vanilloid VR1 receptor, confirming and extending the recent report of AM404 activation of the rat vanilloid VR1 receptor (Zygmunt et al., 2000). Moreover, this is the first demonstration that anandamide is a full agonist at the rat vanilloid VR1 receptor, although partial agonism at the rat vanilloid VR1 receptor (Zygmunt et al., 1999) and full agonism at the human vanilloid VR1 receptor (Smart et al., 1999) has previously been reported. The apparent partial agonism in the electrophysiological study (Zygmunt et al., 1999) probably reflects the technical difficulties in applying sufficiently high concentrations of such a lipophilic ligand in that system, as demonstrated for the human vanilloid VR1 receptor (Smart et al., 1999). The effect of the cannabinoids on $[Ca^{2+}]_i$ was specific to the vanilloid VR1 receptor as there was no effect in wild-type cells lacking the vanilloid VR1 receptor. Furthermore, the effects of AM404 and anandamide were fully inhibited by the vanilloid receptor antagonist capsazepine. In addition, the kinetics of AM404 and anandamide were identical to the kinetics of the vanilloid VR1 receptor response to capsaicin.

At the vanilloid VR1 receptor, all of the cannabinoids were considerably less potent than the archetypal vanilloid agonist capsaicin, and olvanil, with a potency order relative to the vanilloids of: olvanil ≥ capsaicin > AM404 > anandamide > methanandamide. A similar order of potency of these agonists was observed for mediation of relaxation in the rat isolated mesenteric arterial bed. The EC_{50} values were similar between the assays and to those reported for anandamide and methanandamide at endogenous vanilloid receptors on sensory nerves in rat isolated mesenteric arteries and mesenteric arterial bed (EC_{50} values approximately 1 μM) (Zygmunt et al., 1999; Ralevic et al., 2000b). The potencies of the cannabinoids are unlikely to be strongly influenced by their stabilities as methanandamide is metabolically more stable than anandamide. Different potencies of anandamide versus methanandamide at endogenous vanilloid receptors has been reported previously in a range of different bioassays (Ralevic et al., 2000a).

Evidence for AM404 activation of sensory nerves in the rat mesenteric arterial bed came from the demonstration that the relaxation response was abolished by capsaicin pretreatment to desensitize and/or deplete sensory nerves of neurotransmitter. This is as described for methanandamide in the same preparation (Ralevic et al., 2000b). Moreover, AM404 elicited pronounced inhibition of the relaxant response mediated by electrical stimulation of capsaicin-sensitive sensory nerves, and this action was abolished by ruthenium red, a channel blocker and inhibitor of vanilloid responses, indicating a likely involvement of vanilloid receptors. AM404 also inhibited vasorelaxation due to capsaicin, but had no effect on vasorelaxation to exogenous calcitonin gene-related peptide, the principal endogenous sensory neurotransmitter in the rat mesenteric arterial bed (Kawasaki et al., 1988), indicating that its effect was indeed prejunctional. The effect of AM404 was mimicked by the endogenous cannabinoid methanandamide. AM404-mediated inhibition of sensory neurotransmission likely involves depolarization and desensitization of the sensory nerves following activation of endogenous vanilloid receptors on the sensory nerve axons and nerve terminals. In this respect, AM404 mimics the action of capsaicin on sensory neurotransmission as a blocker of impulse transmission and axonal flow of neuropeptides (Maggi and Meli, 1988).

HU210, a synthetic cannabinoid receptor agonist that does not act at vanilloid receptors (Smart et al., 1999; Zygmunt et al., 1999), has been shown to inhibit sensory neurotransmission in the rat mesenteric arterial bed (Ralevic and Kendall, 2001). This suggests that there may be cannabinoid receptors, as well as vanilloid receptors, on perivascular sensory nerves in the mesentery and raises the question of whether cannabinoid receptors are involved in mediating the inhibitory effect of AM404 on sensory neurotransmission. The potent and selective cannabinoid receptor antagonists, LY 320135 and SR 144528 (both at 1 μM), which have nanomolar affinity for cannabinoid CB_1 and CB_2 receptors (Felder et al., 1998; Rinaldi-Carmona et al., 1998), had no effect on inhibition by AM404 of sensory neurotransmission. Thus, our data indicate that cannabinoid CB_1 and CB_2 receptors are unlikely to be involved in inhibition of sensory neurotransmission by AM404. The vanilloid VR1 receptor-mediated action of AM404 contrasts with the vanilloid receptor-independent inhibition that we have observed previously with HU210 (Ralevic and Kendall, 2001) and identifies pronounced differences in effects on sensory nerves of these two cannabinoids.

AM404 inhibited endothelium-dependent relaxation to acetylcholine, but not to the smooth muscle vasodilator sodium nitroprusside. This is consistent with a previous study showing that AM404 attenuates vasorelaxation to carbachol mediated by endothelium-derived hyperpolarizing factor (Harris et al., 1998). This indicates that AM404 can have complex vascular effects. Moreover, it indicates further functional differences between structurally related cannabinoids, as anandamide has been reported to evoke endothelium-dependent vasorelaxation (Wagner et al.,

1999) and to induce Ca^{2+} mobilization in cultured endothelial cells (Mombouli et al., 1999). The mechanism by which AM404 attenuates endothelium-dependent vasorelaxation is unclear, but this action is not specific to acetylcholine as endothelium-dependent relaxation to ATP was also attenuated (unpublished observations). Interestingly, AM404 caused a pronounced, albeit transient vasoconstriction at high concentrations, an effect which was small or nonexistent with methanandamide and anandamide (White and Hiley, 1998; present study). Clearly, these actions of AM404, as well as its actions at vanilloid receptors, means that it must be used with caution as an inhibitor of cannabinoid uptake.

Olvanil, a capsaicin analogue, was a full agonist at the rat vanilloid VR1 receptor, and was equipotent with capsaicin. At the human vanilloid VR1 receptor olvanil was more potent than capsaicin. Olvanil was also more potent than capsaicin at eliciting vasorelaxation in the rat mesenteric arterial bed. Previous reports of the potency of olvanil relative to capsaicin in biological tissue are conflicting. Whilst olvanil was 10-fold more potent than capsaicin at increasing blood flow when injected intradermally (Hughes et al., 1992), it produced only a small release of calcitonin gene-related peptide-like immunoreactivity compared to capsaicin (Dickenson et al., 1990) and was a partial agonist at the vanilloid receptor in the rat dorsal spinal cord (Wardle et al., 1997). Olvanil is known to be nonpungent and it was suggested that this is because it activates different subtypes of receptors compared with capsaicin and/or that its activation kinetics compared with capsaicin are slower than the rate at which it inhibits action potentials from polymodal nociceptors (Liu et al., 1997). Our data with the recombinant vanilloid VR1 receptor, showing that olvanil is at least as efficacious as capsaicin, suggest that the latter of these possibilities may be more likely. Whether the greater potency of olvanil compared to capsaicin in the mesenteric arterial bed is due to additional actions of olvanil at nonvanilloid receptor sites remains to be determined.

Interestingly, differences were observed between cannabinoids and vanilloids with respect to pH. Lowering the pH of the buffer from 7.4 to 6.4 enhanced the potency of capsaicin and olvanil, as reported for capsaicin at rat and human recombinant vanilloid VR1 receptor (Caterina et al., 1997; Smart et al., 1999). In contrast, the potency of AM404 or anandamide was unaffected by pH. The reason for this is not entirely clear. It is possible that cannabinoids bind to different sites on the vanilloid VR1 receptor or activate the channel by different mechanisms. On the other hand, similar antagonistic effects were observed with capsazepine for both cannabinoid and capsaicin responses. An alternative possibility is that the change in pH to 6.4 may affect preferentially the vanilloid receptor ligand rather than the vanilloid receptor.

In conclusion, the present study has demonstrated that the structurally related cannabinoids, AM404, anandamide and methanandamide, are agonists at rat and human vanilloid VR1 receptors. Moreover, AM404 activates endogenous vanilloid receptors to cause vasorelaxation and inhibition of capsaicin-sensitive sensory neurotransmission in the rat isolated mesenteric arterial bed.

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