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The effects of Δ^9-tetrahydrocannabinol in rat mesenteric vasculature, and its interactions with the endocannabinoid anandamide

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1 Δ^9-tetrahydrocannabinol (THC) produces varying effects in mesenteric arteries: vasorelaxation (third-order branches, G3), modest vasorelaxation (G2), no effect (G1) and vasoconstriction (the superior mesenteric artery, G0).

2 In G3, vasorelaxation to THC was inhibited by pertussis toxin, but was unaffected by the CB_1 receptor antagonist, AM251 (1 μM), incubation with the TRPV1 receptor agonist capsaicin (10 μM, 1 h), the TRPV1 receptor antagonist capsazepine (10 μM) or de-endothelialisation.

3 In G3, vasorelaxation to THC was inhibited by high K^+ buffer, and by the following K^+ channel inhibitors: charybdotoxin (100 nM), apamin (500 nM) and barium chloride (30 μM), but not by 4-aminopyridine, glibenclamide or tertiapin.

4 In G3, THC (10 and 100 μM) inhibited the contractile response to Ca^{2+} in a Ca^{2+}-free, high potassium buffer, indicating that THC blocks Ca^{2+} influx.

5 In G0, the vasoconstrictor responses to THC were inhibited by de-endothelialisation and SR141716A (100 nM), but not by the endothelin (ET_A) receptor antagonist FR139317 (1 μM).

6 THC (1 and 10 μM) antagonised vasorelaxation to anandamide in G3 but not G0. THC did not antagonise the noncannabinoid verapamil, capsaicin or the CB_1 receptor agonist CP55,940. THC (10 and 100 μM) inhibited endothelium-derived relaxing factor (EDHF)-mediated responses to carbachol in a manner similar to the gap junction inhibitor 18α-glycyrrhetinic acid.

7 These data show that THC causes vasorelaxation through activation of K^+ channels and inhibition of Ca^{2+} channels, and this involves non-CB_1, non-TRPV1 but G-protein-coupled receptors. In G0, THC does not cause relaxation and at high concentrations causes contractions. Importantly, THC antagonises the effects of anandamide, possibly through inhibition of EDHF activity.
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Abbreviations: AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; CB, cannabinoid; ChTX, charybdotoxin; EDHF, endothelium-derived hyperpolarising factor; G0, the superior mesenteric artery; G3, third-order branch of the superior mesenteric artery; 18α-GA, 18α-glycyrrhetinic acid; L-NAME, N^G-nitro-L-arginine methyl ester; NADA, *N*-arachidonoyl-dopamine; PTX, pertussis toxin; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; THC, Δ^9-tetrahydrocannabinol

Introduction

In the early 1990s, a receptor for the active constituent of marijuana, Δ^9-tetrahydrocannabinol (THC) was identified, and this was shortly followed by the discovery of an endogenous ligand for this receptor, anandamide (Devane *et al*., 1992). The first *in vitro* cardiovascular study showed that both anandamide and THC were capable of relaxing rabbit cerebral arterioles (Ellis *et al*., 1995), initiating a wealth of research into the cardiovascular effects of anandamide. It has now been widely shown that anandamide causes vasorelaxation through a number of mechanisms involving the endothelium, sensory nerves and modulation of ion channels (for a review, see Randall *et al*., 2004). Interestingly, the contribution of the first identified cannabinoid (CB) receptor, CB_1, to the vasorelaxant effects of anandamide remains controversial.

By contrast, the effects of THC on blood vessels have been largely neglected. While early research showed that THC produced prostaglandin-mediated vasorelaxation of rabbit cerebral vessels (Ellis *et al*., 1995), subsequent studies showed both a vasoconstrictor effect of THC in the whole perfused rat mesenteric bed (Wagner *et al*., 1999) and endothelium-independent relaxation in the rabbit superior mesenteric artery (Fleming *et al*., 1999). More recent work has indicated that THC relaxes rat hepatic and mesenteric arteries entirely through its actions on sensory nerves, but not through the archetypal TRPV1 receptor (Zygmunt *et al*., 2002). In this work, THC was inhibited by depletion of sensory neurotransmitters by incubation with the TRPV1 receptor agonist capsaicin, and was inhibited by antagonism of the vasodilator

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neurotransmitter, calcitonin gene-related peptide (CGRP). However, vasorelaxation to THC persisted in TRPV1 receptor knockout mice, and was not inhibited by the TRPV1 receptor antagonist capsazepine. The authors therefore speculated that THC may act at another member of the TRP ion channel family. Indeed, a recent paper shows that THC and cannabinol activate TRPA1 (Jordt *et al*., 2004), a member of the TRP family that is present in a subpopulation of capsaicin-sensitive sensory nerves (Story *et al*., 2003; Jordt *et al*., 2004).

The potentially important consequences of interactions between endogenous and exogenous CB compounds are still largely unknown, although some evidence in the literature suggests that CBs may antagonise each other. For example, Petitet *et al*. (1998) showed that cannabidiol (10 μM) or THC (10 μM) antagonised the [^{35}S] GTP-γ-S binding of the CB_1 receptor agonist CP55,940. Similarly, Bayewitch *et al*. (1996) showed that THC (1 μM) is capable of binding to transfected CB_2 receptors in COS 7 and CHO cells without an intracellular effect, and that THC antagonised the effects of CB_2 receptor agonists. More recently, Kelley & Thayer (2004) reported that THC (100 nM) antagonised the inhibitory effects of the endogenous CB 2-arachidonoyl glycerol (2-AG) on synaptic firing in rat hippocampal neurons. It has not yet been established whether such interactions between CB compounds also occur in the vasculature.

Given the lack of consensus on the vascular effects of THC, this study aimed first to further investigate the effects of THC in isolated resistance and conduit mesenteric vessels, concentrating on mechanisms already known to be involved in vasorelaxation to the endogenous CB anandamide. Secondly, we sought to determine the effects of an exogenous CB (THC) on vasorelaxation to an endogenous CB (anandamide), and investigate the mechanisms involved.

Methods

Blood vessel preparation

Male Wistar rats (250–350 g, Charles River, U.K.) were stunned by a blow to the back of the head and killed by cervical dislocation. The superior mesenteric artery and mesenteric arterial bed were removed rapidly and placed into cold Krebs-Henseleit buffer (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10). From the mesenteric arterial bed, 2 mm segments of branches of the superior mesenteric artery (G3 or G2) were dissected free of adherent connective and adipose tissue. G3/G2 vessels were mounted on fine tungsten wires (40 μM diameter) on a Mulvany–Halpern myograph (Myo-Interface Model 410A, Danish Myo Technology, Denmark) (Mulvany & Halpern, 1977). The superior mesenteric artery (G0; 3–4 mm in length) was also cleaned of adherent tissue and was mounted on fixed segment support pins using the Multi Myograph system, as were G1 vessels (Model 610 M, Danish Myo Technology, Denmark). Tension was measured and was recorded on a MacLab 4e recording system (ADInstruments, U.K.).

Once mounted, all vessels were kept at 37°C in Krebs–Henseleit buffer and gassed with 5% CO_2 in O_2. The mesenteric vessels were stretched to an optimal passive tension of 5 mN (O'Sullivan *et al*., 2004a, b). All vessels were allowed to equilibrate and the contractile integrity of each was initially tested by its ability to contract to 60 mM KCl by at least 5 mN.

In some preparations, the endothelium was removed by abrasion with a human hair. Preparations were considered denuded when relaxation to 10 μM carbachol after pre-contraction with U46619 was less than 20% relaxation (see White & Hiley, 1997). All other preparations were endothelium-intact.

Experimental protocol

Viable vessels were contracted with a combination of U46619 (10–100 nM, a thromboxane prostanoid (TP) receptor agonist) and methoxamine (1–3 μM, an α-adrenoceptor agonist) to increase tension by at least 5 mN. Once a stable contraction was achieved, the vasorelaxant effects of THC (or other compounds) were assessed as cumulative concentration–response curves. The steady-state response was taken at each concentration and expressed as a percentage relaxation of the imposed contraction. For each manipulation, data were compared with control concentration–response curves to THC obtained at the same time. Vasorelaxation to THC was examined in G0, G1, G2 and G3. Since vasorelaxation to THC was greatest in G3, all subsequent experiments exploring the mechanisms of vasorelaxation were examined in G3.

To characterise the vasorelaxant effects of THC in G3 vessels, the involvement of a number of potential receptors and mechanisms were investigated. To assess if THC acts at a $G_{(i/o)}$ protein-coupled receptor, vessels were incubated for 2 h with 400 ng ml^{-1} pertussis toxin (PTX; White & Hiley, 1997). The involvement of the CB_1 receptor was assessed using the CB_1 receptor antagonist AM251 (1 μM; Gatley *et al*., 1996) added to the preparations 10 min before preconstriction. The involvement of TRPV1 receptors was assessed by incubating vessels for 1 h with the TRPV1 agonist capsaicin (10 μM) to deplete the sensory nerves of vasoactive neurotransmitters, followed by a 20-min washout (Zygmunt *et al*., 1999). This protocol was also performed in the presence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) and the cyclooxygenase inhibitor indomethacin (10 μM). Also, the TRPV1 receptor antagonist capsazepine and the channel blocker ruthenium red (both 10 μM) were added to the preparations 10 min before contraction. To investigate the role of the endothelium, the endothelial layer was removed using gentle abrasion with a human hair. The role of vasorelaxant prostanoids was investigated by performing experiments in the presence of the cyclooxygenase inhibitor indomethacin (10 μM).

To investigate the effects of THC on calcium influx, concentration–response curves to $CaCl_2$ (10 μM to 100 mM) were obtained in the absence and presence of THC (10 or 100 μM). The vessels were first allowed to equilibrate in a calcium-free Krebs' solution, and were then bathed in calcium-free, high potassium (100 mM) Krebs' solution. After 10 min incubation with THC, a concentration–response curve to $CaCl_2$ (causing contraction) was constructed (Tep-areenan *et al*., 2003). These experiments were also performed in the presence of SR141716A (100 nM) to determine any involvement of the CB_1 receptor in this response.

To investigate the involvement of K^+ channels, vasorelaxation to THC was performed in vessels precontracted with a high potassium (60 mM) Krebs' solution to inhibit potassium flux. Concentration–response curves to THC were also performed in the presence of 100 nM charybdotoxin (ChTX) (an inhibitor of large calcium-activated K^+ channels and voltage-sensitive K^+ channels; Randall & Kendall, 1998), 500 nM apamin (a small calcium-activated K^+ channel inhibitor; Randall & Kendall, 1998), 30 μM barium chloride (a voltage-dependent inward rectifier K^+ channel inhibitor; Harris *et al*., 2002), 10 μM glibenclamide (a selective K_{ATP} channel inhibitor; Randall & Kendall, 1997), 1 mM 4-aminopyridine (a K_V channel inhibitor; Honda *et al*., 1999) and 100 nM tertiapin (an inhibitor of G-protein-coupled inwardly rectifying potassium channels (GIRKs); Jin & Lu, 1998; Chauhan *et al*., 2003).

To characterise the vasoconstrictor effects of THC in G0, some experiments were performed in the presence of SR141716A (100 nM), or in the presence of indomethacin (10 μM), after removal of the endothelium, or in the presence of the endothelin receptor (ET_A) antagonist FR139317 (1 μM; Sogabe *et al*., 1993).

To investigate the effects of THC on vasorelaxation to anandamide in G3, vessels were incubated with THC (1 or 10 μM) for 10 min before preconstriction, and concentration–response curves to anandamide performed. This was also done in the presence of L-NAME (300 μM). To establish whether THC inhibits anandamide in G3 through sensitisation of receptors involved, concentration–response curves to anandamide were performed after exposing the same vessels to anandamide (1 μM) for 1 h. To test whether the effects of THC were through nonspecific effects on vasorelaxation, concentration–response curves were performed to a noncannabinoid vasorelaxant, verapamil, in the presence and absence of THC (10 μM). The effects of 10 μM THC on anandamide-induced vasorelaxation were also assessed in G0 vessels.

To investigate the putative receptor at which THC acts, vessels were treated with THC (10 μM, 10 min) and concentration–response curves performed to the TRPV1 receptor agonist capsaicin, and the CB_1/CB_2 receptor agonist CP55,940. The effects of THC (10 μM) on vasorelaxation to anandamide were also tested after endothelial denudation.

To investigate whether THC inhibits endothelium-derived hyperpolarising factor (EDHF) activity, the vasorelaxant response to 1 μM carbachol in the presence of indomethacin (10 μM) and L-NAME (300 μM) was examined in the presence of THC (10 and 100 μM) and in the presence of the gap junction inhibitor 18α-glycyrrhetinic acid (18α-GA, 100 μM; Harris *et al*., 2002). The EDHF response was determined using five separate successive doses of carbachol, each separated by 10 min.

Gender studies

To investigate whether arteries from female rats have a greater sensory component to vasorelaxation, concentration–response curves to capsaicin, THC, and THC after capsaicin pretreatment were performed in mesenteric vessels (G3) obtained from female Wistar rats (Charles River, U.K.) and compared with those from male rats.

Statistical analysis

The concentration of vasorelaxant giving the half-maximal response (EC_{50}) was obtained from the concentration–response curve fitted to a sigmoidal logistic equation with the minimum vasorelaxation set to zero using the GraphPad Prism package (Tep-areenan *et al*., 2003). Maximal responses and pEC_{50} (negative logarithm of the EC_{50}) values are expressed as mean $\pm$ s.e.m. The number of animals in each group is represented by n. Data were compared, as appropriate, by Student's t-test or by analysis of variance (ANOVA) with statistical significance between manipulations and controls determined by Dunnett's *post-hoc* test.

Drugs

All drugs were supplied by Sigma Chemical Co. (Poole, U.K.), except where stated. Anandamide, CP55,940, AM251, tertiapin, FR139317 and VDM11 were obtained from Tocris (U.K.). SR141716A was supplied by Research Biochemicals International as part of the Chemical Synthesis Programme of the National Institute of Mental Health contract (NOIMH3003). Carbachol and L-NAME were dissolved in Krebs–Henseleit solution. Indomethacin was dissolved first in 100 μl ethanol and then dissolved in the Krebs–Henseleit solution. Tertiapin was dissolved in distilled water. THC, CP55,940, anandamide, capsaicin, capsazepine and SR141716A were dissolved in ethanol at 10 mM, with further dilutions made in distilled water. AM251, FR139317, ruthenium red and 18α-GA were dissolved in DMSO to 10 mM, with further dilutions in distilled water.

Results

Vascular effects of THC in mesenteric arteries

THC caused vasorelaxation of G3 vessels, with potency and efficacy lower than the endocannabinoid anandamide (anandamide $R_{max} = 82.2 \pm 3.1\%$ relaxation, $pEC_{50} = 6.61 \pm 0.09$, $n = 10$; THC $R_{max} = 64.8 \pm 2.2\%$ relaxation, $P < 0.01$, $pEC_{50} = 5.37 \pm 0.07$, $n = 16$, $P < 0.01$, Figure 1a, d). Anandamide also produced vasorelaxation in G0 (the superior mesenteric artery) ($R_{max} = 31.4 \pm 5.2\%$ relaxation, $pEC_{50} = 5.39 \pm 0.29$, $n = 7$); however, at concentrations up to 10 μM, THC had no effect on preconstricted superior mesenteric arteries (Figure 1b). At higher concentrations (10–100 μM), THC produced additional contraction in precontracted preparations (100 μM, $14.3 \pm 6.4\%$ contraction, $n = 7$, Figure 1b). In G2, the potency of THC was considerably lower than in G3 ($pEC_{50} = 3.75 \pm 0.12$, $n = 6$), but with a similar maximal relaxation at the highest concentration of THC ($66.9 \pm 5.6\%$ relaxation, $n = 6$, Figure 1c). In G1, there was no significant change in tone imposed by THC such that at the highest concentration of THC tested, relaxation was $1.6 \pm 6.4\%$ ($n = 7$, Figure 1c).

Effects of THC in G3 vessels

Vasorelaxation to THC in G3 vessels was significantly inhibited by treatment of the vessels with PTX (400 ng ml^{-1}, 2 h) to block $G_{(i/o)}$-protein-coupled receptors (control

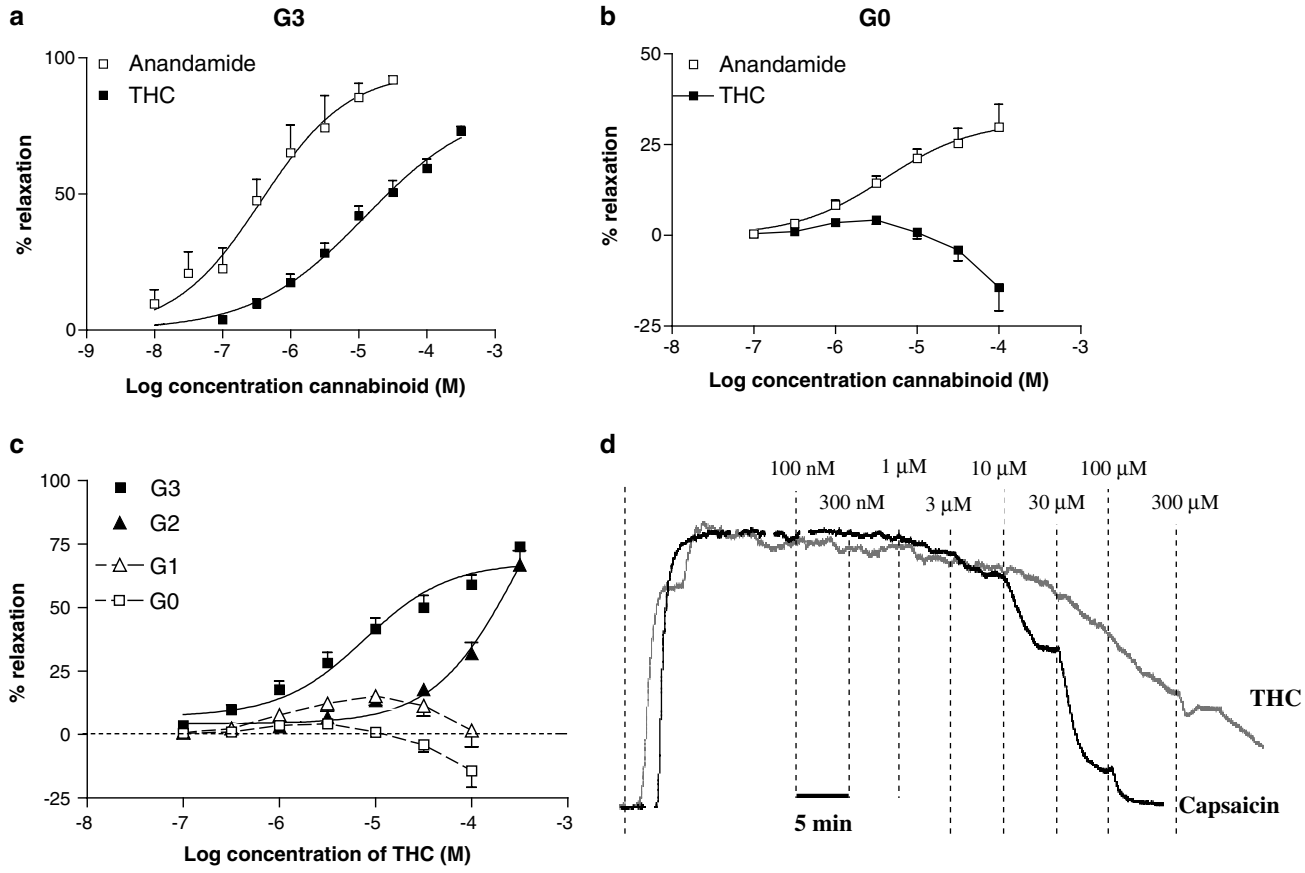

Figure 1 The vascular effects of THC ($n=16$) compared with the endocannabinoid anandamide ($n=10$) in third-order branches of the mesenteric artery (G3, a) and in the superior mesenteric artery (anandamide $n=7$; THC $n=7$, b). A comparison of the effects of THC in GO ($n=7$), G1 ($n=7$), G2 ($n=6$) and G3 ($n=16$, c), and raw data showing the vasorelaxant effects of THC in G3 compared to the more potent vasorelaxant, capsaicin (d). Data are given as means, with error bars representing s.e.m.

$R_{max}=61.8\pm4.3\%$ relaxation, $n=7$; PTX $R_{max}=40.2\pm5.1\%$ relaxation, $n=7$, $P<0.01$, Figure 2a). Vasorelaxation to THC was not affected by the CB_1 receptor antagonist AM251 at 1 μM (control $R_{max}=62.3\pm4.9\%$ relaxation, $pEC_{50}=6.11\pm0.32$, $n=7$; AM251 $R_{max}=59.9\pm7.7\%$ relaxation, $pEC_{50}=5.93\pm0.39$, $n=7$, Figure 2b). Pretreatment with the TRPV1 receptor agonist capsaicin (10 μM) for 1 h did not affect vasorelaxation to THC (control $R_{max}=71.6\pm4.1\%$ relaxation, $n=9$; capsaicin pretreatment $R_{max}=62.6\pm4.5\%$ relaxation, $n=11$, Figure 2c). In the presence of L-NAME and indomethacin, however, there was a significant reduction in the maximal relaxation caused by THC after capsaicin treatment compared with either control or capsaicin treatment alone ($R_{max}=40.4\pm4.4\%$ relaxation, $n=8$, $P<0.001$, ANOVA, Figure 2c). However, the TRPV1 receptor antagonists capsazepine (10 μM) (control $R_{max}=59.8\pm3.1\%$ relaxation, $pEC_{50}=5.22\pm0.14$, $n=6$; capsazepine $R_{max}=48.7\pm4.5\%$ relaxation, $pEC_{50}=5.34\pm0.28$, $n=7$) and ruthenium red (10 μM) (ruthenium red $R_{max}=62.3\pm6.8\%$ relaxation, $pEC_{50}=4.81\pm0.25$, $n=7$, Figure 2d) did not affect vasorelaxation to THC. The vasorelaxant response to THC in G3 vessels was endothelium-independent (control $R_{max}=65.4\pm4.1\%$ relaxation, $pEC_{50}=5.26\pm0.17$, $n=7$; endothelium-denuded $R_{max}=59.6\pm4.0\%$ relaxation, $pEC_{50}=5.0\pm0.16$, $n=7$, Figure 2e). In the presence of indomethacin (10 μM), there was a small significant inhibitory effect on the maximal vasorelaxant response to THC (control $R_{max}=64.4\pm4.34\%$ relaxation, $n=6$; indomethacin $R_{max}=52.6\pm3.8\%$ relaxation, $n=7$, $P<0.05$, Figure 2f).

Effects of THC on calcium re-introduction

The potency of the contractile response to the re-introduction of calcium in a calcium-free, high-potassium (100 mM) Krebs–Hensleit solution was significantly reduced in the presence of 10 μM THC (control $pEC_{50}=3.22\pm0.05$, $n=8$; THC 10 μM $pEC_{50}=2.75\pm0.06$, $n=7$, $P<0.01$, Figure 3). In the presence of 100 μM THC, this response was further inhibited, with an additional significant effect on the maximum contraction achieved (control $R_{max}=13.6\pm0.5$ mN tension; THC 100 μM $R_{max}=6.6\pm0.9$ mN tension, $n=8$, $P<0.001$, $pEC_{50}=2.58\pm0.06$, $P<0.001$, Figure 3a). This inhibition was not affected by the presence of the CB_1 receptor antagonist SR141716A (100 nM) (THC 10 μM and SR141716A $pEC_{50}=2.88\pm0.07$, $R_{max}=13.2\pm0.7$ mN tension $n=7$) (THC 100 μM and SR141716A $pEC_{50}=2.54\pm0.18$, $R_{max}=5.1\pm0.9$ mN tension $n=7$, Figure 3b).

Effects of THC on potassium channels

When arteries were contracted with a high-potassium Krebs' solution instead of with U46619 and methoxamine, the vasorelaxant potency of THC was greatly reduced (control

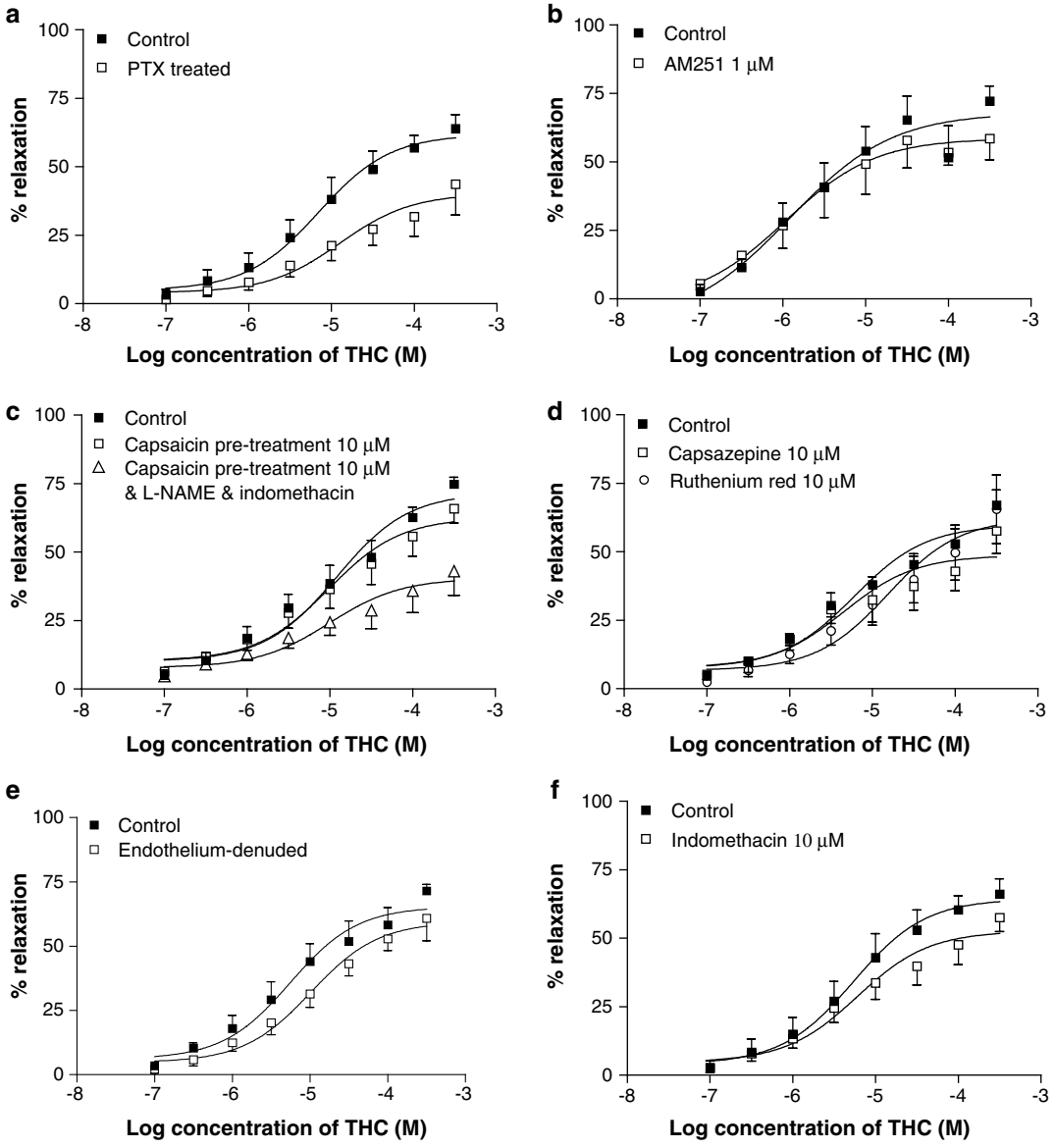

Figure 2 The effects of PTX treatment (400 ng ml^{-1}, 2 h, $n=7$, a), the CB_1 receptor antagonist AM251 (1 μM, $n=7$, b), capsaicin pretreatment for 1 h (10 μM, $n=11$, c) or capsaicin treatment in the presence of L-NAME and indomethacin ($n=8$), TRPV1 receptor antagonism with capsazepine or ruthenium red (both 10 μM, $n=6$, d), removal of the endothelium ($n=7$, e), and the presence of indomethacin (10 μM, $n=7$, f) on the vasorelaxant response to THC in G3 mesenteric vessels. Data are given as means, with error bars representing s.e.m.

$pEC_{50}=5.32\pm0.12$, $n=6$; high K^+ $pEC_{50}=3.67\pm0.11$, $n=6$, $P<0.001$, Figure 4a). The vasorelaxant potency to THC was also significantly inhibited by 100 nM ChTX (control $pEC_{50}=5.32\pm0.13$, $n=6$; ChTX $pEC_{50}=4.66\pm0.12$, $n=6$, $P<0.01$, Figure 4b), by 500 nM apamin (control $pEC_{50}=5.50\pm0.16$, $n=6$; apamin $pEC_{50}=4.75\pm0.17$, $n=6$, $P<0.01$, Figure 4c) and by 30 μM barium chloride (control $pEC_{50}=5.41\pm0.14$, $n=6$; barium chloride $pEC_{50}=4.47\pm0.20$, $n=6$, $P<0.01$, Figure 4d). Vasorelaxation to THC in G3 vessels was not inhibited by 10 μM glibenclamide (control $pEC_{50}=4.59\pm0.12$, $n=6$; glibenclamide $pEC_{50}=5.06\pm0.31$, $n=6$, Figure 4e) or by 1 mM 4-aminopyridine (control $pEC_{50}=5.50\pm0.23$, $n=6$; 4-aminopyridine $pEC_{50}=5.36\pm0.17$, $n=6$, Figure 4f). Tertiapin (100 nM) did also not inhibit vasorelaxation to THC (control $pEC_{50}=5.6\pm0.16$, $n=7$; tertiapin $pEC_{50}=5.1\pm0.18$, $n=7$).

Effects of THC in the superior mesenteric artery (G0)

In the presence of indomethacin (10 μM), G0 vessels showed enhanced vasorelaxant responses to THC in the lower concentration range (see Figure 5b), with a maximal vasorelaxant effect of $16.1\pm3.1\%$ relaxation ($n=7$) at 3 μM THC compared with the control value of $4.2\pm1.1\%$ relaxation ($n=9$, $P<0.01$). Neither the CB_1 receptor antagonist SR141716A (100 nM) nor de-endothelialisation affected responses to THC in the lower concentration range.

The vasoconstrictor response to THC in G0 vessels seen at high concentrations (from 10 to 100 μM) was converted to vasorelaxation by de-endothelialisation (at 100 μM, control $14.3\pm6.4\%$ contraction, $n=9$; de-endothelialisation $11.5\pm2.2\%$ relaxation, $n=6$, $P<0.05$, Figure 5b). The presence of indomethacin (10 μM) did not affect the vasoconstrictor

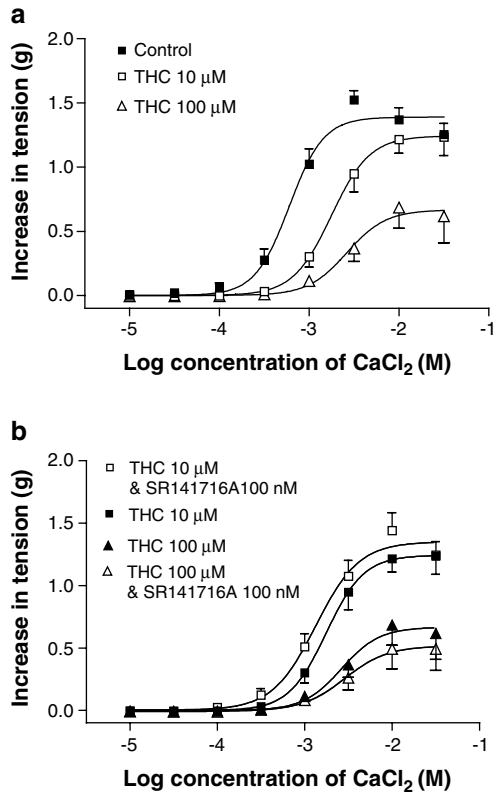

Figure 3 The effects of Ca^{2+} re-introduction in G3 isolated arteries bathed in a Ca^{2+}-free, depolarised solution in the presence or absence of 10 μM THC ($n=7$) or 100 μM THC ($n=8$, a), and in the presence of SR141716A (100 nM, $n=7$, b). Data are given as means, with error bars representing s.e.m.

responses to THC (17.3±5.2% increase in tone at 100 μM THC). In the presence of the CB_1 receptor antagonist SR141716A (100 nM), THC did not cause vasoconstriction at 100 μM (1.3±5.3% relaxation). The vasoconstrictor effects of THC in G0 were not inhibited by the endothelin receptor antagonist FR139317 (1 μM, $n=6$, see Figure 5c).

Interactions of THC and anandamide

The presence of THC caused significant rightward shifts in the concentration–response curve to anandamide in G3 vessels at 1 μM (control $pEC_{50}=6.43\pm0.22$, $n=8$, cf $pEC_{50}=5.09\pm0.57$, $n=8$, $P<0.05$, ANOVA) and at 10 μM ($pEC_{50}=4.49\pm0.20$, $n=6$, $P<0.01$, ANOVA, Figure 6a), with a K_B value calculated by Schild analysis of 10 nM ($pK_B=-8$). There was no inhibitory effect on the vasorelaxant responses to anandamide after exposing the vessels to anandamide (1 μM) for 1 h (control $R_{max}=95.0\pm10.8\%$ relaxation, $pEC_{50}=6.43\pm0.22$, $n=8$; anandamide 1 μM $R_{max}=94.5\pm9.5\%$ relaxation, $pEC_{50}=6.64\pm0.20$, $n=6$, Figure 6b). THC (10 μM) did not affect the noncannabinoid vasorelaxant, verapamil (control $pEC_{50}=7.01\pm0.12$, $n=5$; verapamil and THC 10 μM $pEC_{50}=7.22\pm0.09$, $n=6$, Figure 6c). THC (10 μM) did not affect the actions of anandamide in the superior mesenteric artery (G0; control $R_{max}=31.4\pm5.2\%$ relaxation, $pEC_{50}=5.39\pm0.29$, $n=7$; and THC 10 μM $R_{max}=28.5\pm5.5\%$ relaxation, $pEC_{50}=5.44\pm0.34$, $n=7$, Figure 6d).

Although the concentration–response curve to the TRPV1 agonist capsaicin was significantly inhibited by the TRPV1 receptor antagonist capsazepine (10 μM), ruthenium red (10 μM) and capsaicin pretreatment (capsaicin $pEC_{50}=5.23\pm0.07$, $n=8$; capsazepine $pEC_{50}=4.61\pm0.08$, $n=6$, $P<0.01$; ruthenium red $pEC_{50}=4.63\pm0.16$, $n=6$, $P<0.01$; capsaicin pretreatment $pEC_{50}=4.42\pm0.09$, $n=6$, $P<0.01$, ANOVA, Figure 7a), it was not affected by THC (10 μM) $pEC_{50}=5.30\pm0.08$, $n=6$, Figure 7a). THC did also not antagonise the vasorelaxant effects of the CB_1 receptor agonist CP 55,940 (CP 55,940 $pEC_{50}=5.86\pm0.09$, $n=5$; THC 10 μM $pEC_{50}=5.77\pm0.18$, $n=5$, Figure 7b). When THC (10 μM) was applied in combination with endothelial denudation in G3 vessels, both manipulations significantly inhibited anandamide ($P<0.01$, ANOVA); however, THC did not inhibit the vasorelaxant effects of anandamide further than was seen with denudation alone (denuded vessels $pEC_{50}=5.32\pm0.16$, $n=7$; denuded vessels and THC 10 μM $pEC_{50}=4.85\pm0.11$, $n=6$, Figure 7c).

THC and EDHF activity

In general, both the magnitude and duration of the carbachol response in the presence of indomethacin and L-NAME, that is, the EDHF response, was significantly reduced in the presence of THC, becoming more pronounced with each dose of carbachol. This was similar in profile to the effects of the gap junction inhibitor, 18α-GA (see Figure 8).

The EDHF response to the first dose of carbachol was significantly inhibited by 18α-GA (control 82.2±5.2% relaxation cf 42.8±11.3% relaxation, $P<0.01$, Figure 8b). The second dose of carbachol was significantly inhibited by 18α-GA and 100 μM THC (control 81.9±4.2% relaxation; 18α-GA 37.3±11.1% relaxation; 100 μM THC 40.0±9.1% relaxation, $P<0.01$, Figure 8b). The EDHF response to the third, fourth and fifth doses of carbachol was significantly inhibited by 18α-GA, 10 and 100 μM THC (see Figure 8b).

18α-GA (100 μM) significantly inhibited vasorelaxation to anandamide (control $pEC_{50}=6.55\pm0.13$, $n=8$; 18α-GA $pEC_{50}=5.14\pm0.17$, $n=7$, $P<0.01$, Figure 9a). The inhibitory effect of 1 μM THC on vasorelaxation to anandamide was significantly enhanced in the presence of 300 μM L-NAME (control $pEC_{50}=6.43\pm0.22$, $n=8$; THC 1 μM $pEC_{50}=5.59\pm0.13$, $n=8$; THC 1 μM and L-NAME $pEC_{50}=3.22\pm0.93$, $n=5$, Figure 9b). By contrast, the effect of THC 10 μM on vasorelaxation to anandamide was not different in the presence of L-NAME (10 μM of THC $pEC_{50}=4.49\pm0.20$, $n=6$; THC 10 μM and L-NAME $pEC_{50}=4.16\pm0.46$, $n=4$; Figure 9c).

Gender studies There was no significant difference in the vasorelaxant response to THC between males and females (female $R_{max}=86.6\pm11.9\%$ relaxation, $n=5$; male $R_{max}=77.1\pm6.01\%$ relaxation, $n=5$, Figure 10a). The vasorelaxant response to THC in females was not significantly inhibited by pretreating vessels with capsaicin to delete sensory neurotransmitters ($R_{max}=82.7\pm15.1\%$ relaxation, $n=5$, Figure 10a). There was also no gender difference in the vasorelaxant response to capsaicin (female $R_{max}=102\pm9.6\%$ relaxation, $n=5$; male $R_{max}=113\pm6.6\%$ relaxation, $n=6$, Figure 10b).

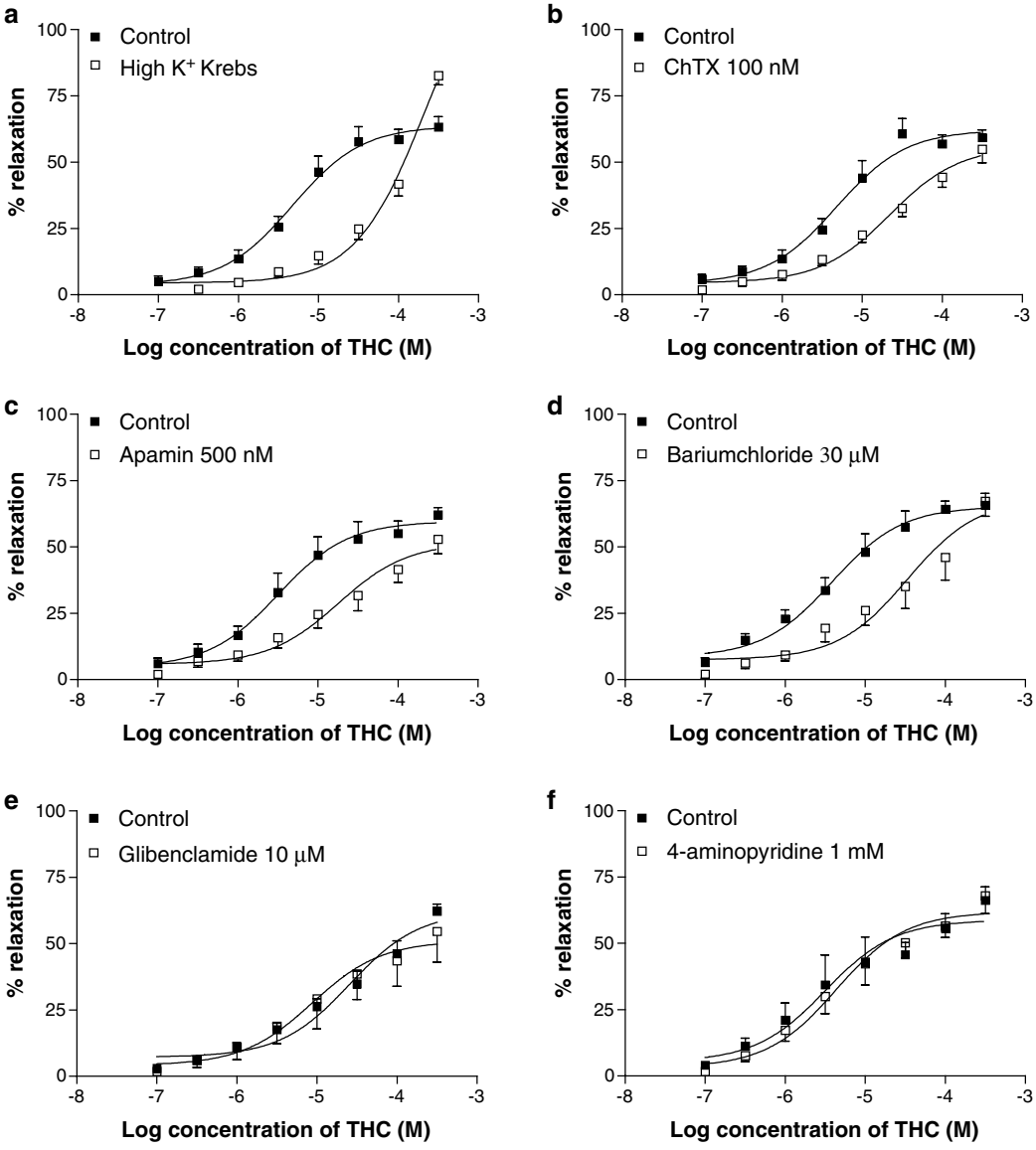

Figure 4 The effects of a high-potassium Krebs' solution ($n=6$, a), the large calcium-activated K^+ channel inhibitor ChTX (100 nM, $n=6$, b), the small calcium-activated K^+ channel inhibitor apamin (500 nM, $n=6$, c), the voltage-dependent inward rectifier K^+ channel inhibitor, barium chloride (30 μM, $n=6$, d), the K_{ATP} channel inhibitor, glibenclamide (10 μM, $n=6$, e), and the K_V channel inhibitor, 4-aminopyridine (1 mM, $n=6$, f) on vasorelaxation to THC in G3 vessels. Data are given as means, with error bars representing s.e.m.

Discussion

In this study, we have investigated the vascular effects of the plant CB THC. THC causes vasorelaxation of small resistance mesenteric vessels through an unidentified $G_{(i/o)}$-protein-coupled receptor, activation of K^+ channels and inhibition of Ca^{2+} channels. By contrast, THC causes vasoconstriction of the superior mesenteric artery through at least two separate mechanisms. We have also shown for the first time that THC antagonises the vascular effects of the endogenous CB anandamide, not through the CB_1 or TRPV1 receptor, but possibly through inhibition of EDHF and/or intercellular communication.

Vasorelaxation to THC in mesenteric resistance arteries

THC was found to cause vasorelaxation of G3 arteries, but with lower potency and efficacy than the endogenous CB, anandamide. Furthermore, anandamide caused vasorelaxation in the superior mesenteric artery (G0), while THC caused vasoconstriction. In G2 and G1, THC was found to have small (G2) and no (G1) vasorelaxant effects. The target sites underlying vasorelaxation to THC in G3 vessels were further investigated. Vasorelaxation to THC was not inhibited by CB_1 receptor antagonism, TRPV1 receptor antagonism, capsaicin pretreatment, or de-endothelialisation, all of which have been implicated in vasorelaxation to anandamide in the same vessel preparation (Zygmunt *et al*., 1999; O'Sullivan *et al*., 2004b).

It is important to highlight that the lack of sensory nerve involvement in vasorelaxation to THC in G3 is in contrast with recent work by Zygmunt and colleagues (2002), who found that although capsazepine did not affect vasorelaxation to THC, there was a significant reduction by capsaicin treatment, CGRP antagonism and ruthenium red. Thus, these authors demonstrated that THC at submicromolar concentra-

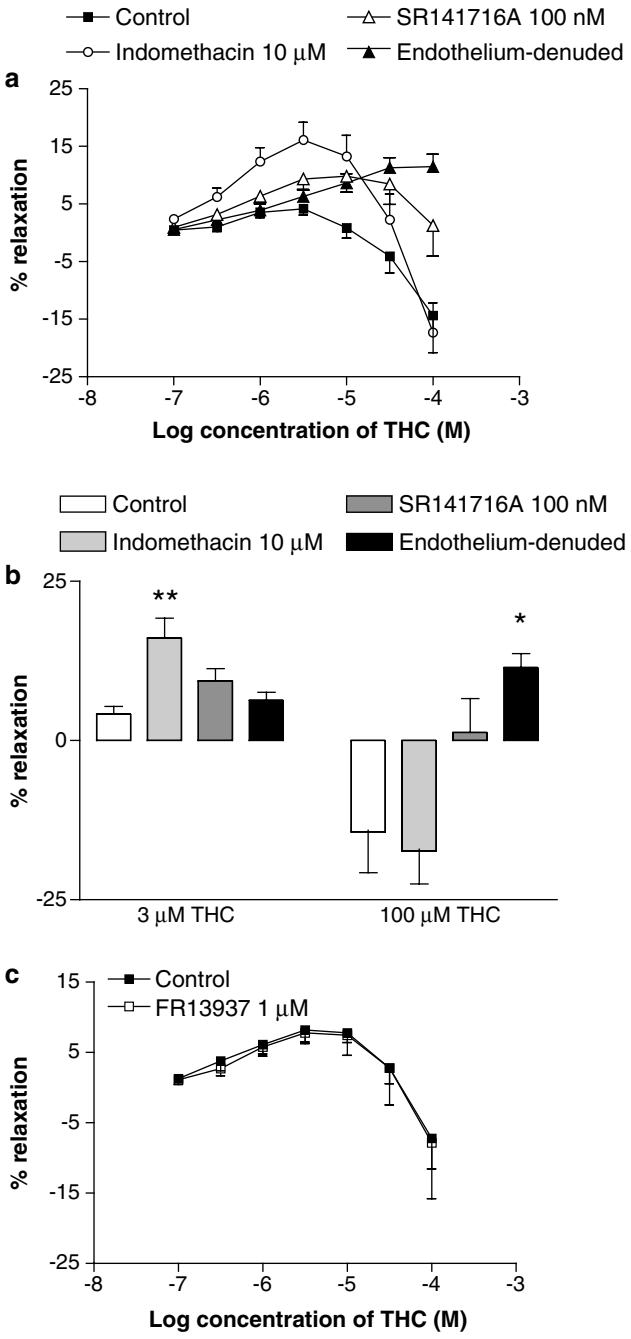

Figure 5 The effects of indomethacin (10 μM, $n=7$), the CB_1 receptor antagonist SR141716A (100 nM, $n=7$) and removal of the endothelium ($n=9$), and the endothelin antagonist FR139317 (1 μM, $n=6$) on the vasoconstrictor effects of THC in the superior mesenteric artery (G0). Data are given as means, with error bars representing s.e.m.

tions causes sensory-nerve-mediated vasodilation of rat and mouse mesenteric arteries, which is in line with the ability of THC to activate rat and human TRPA1 (Jordt *et al*., 2004) and the presence of TRPA1 in mouse TRPV1 containing dorsal root ganglion neurons (Story *et al*., 2003). Since all experiments in this work were carried out in the presence of L-NAME and indomethacin, we examined whether the effects of capsaicin were enhanced under these conditions and found that there was indeed a significant reduction in vasorelaxation to THC. This could suggest that when other pathways of vasorelaxation are suppressed, a sensory component of vasorelaxation to THC is revealed. However, it should also be remembered that we found a small decrease in the maximum vasorelaxant effects of THC in the presence of indomethacin alone, indicating a partial involvement of vasorelaxant prostanoids, as previously shown by Ellis *et al*. (1995). Another possible cause of discrepancy may be that the animals by Zygmunt *et al*. (2002) were females, and there is recent evidence to suggest that female rats have a greater sensory component to vasorelaxation to either a TRPV1 agonist or to the endocannabinoid anandamide in the whole mesenteric bed (Peroni *et al*., 2004). To test this, we examined vasorelaxation to both THC and capsaicin obtained from female rats. However, we did not find that there was any evidence of a greater sensory component to vasorelaxation in females in isolated resistance mesenteric arteries. Finally, it should be noted that the potency of capsaicin in the present study is considerably lower than previously reported (Zygmunt *et al*., 1999; 2002; Jerman *et al*., 2000; Smart *et al*., 2001), which suggests that there may be an alteration in sensory nerve function in our preparations, we therefore cannot exclude the possibility that THC does activate sensory nerves. However, we have previously revealed a sensory-nerve-mediated component to vasorelaxation to anandamide and NADA by similar mechanisms (O'Sullivan *et al*., 2004a, b).

Vasorelaxation to THC was sensitive to pretreatment with PTX, implicating the involvement of a $G_{(i/o)}$-protein-coupled receptor. In addition to the CB_1 and CB_2 receptors, there is recent evidence for a novel endothelial CB receptor (Jarai *et al*., 1999; Offertaler *et al*., 2003), and it is suggested that still other unidentified vascular CB receptors exist (see Pertwee, 2002). Since vasorelaxation to THC was not endothelium-dependent, the $G_{(i/o)}$-protein-coupled receptor activated by THC is likely to be located on the smooth muscle. It has been previously shown that cannabidiol (CBD) antagonises THC and other CB compounds (Pertwee *et al*., 2002) at a prejunctional site that is not the CB_1 or CB_2 receptor. However, in the present study, CBD (1 μM) did not affect vasorelaxation elicited by either anandamide or THC (data not shown), ruling out this particular target.

Although the receptor sites through which THC causes vasorelaxation acts were not identified, we explored whether THC causes vasorelaxation through modulation of ion channels. We found that THC inhibits the contractile response to the re-introduction of calcium in a calcium-free depolarised solution, suggesting that THC inhibits calcium entry. A similar finding was shown by Gebremedhin *et al*. (1999), who found that another CB_1 receptor agonist, WIN55,212, inhibited L-type calcium channel currents in cerebral smooth muscle cells in an SR141716A-sensitive manner. To test the possibility that the inhibition of calcium channels by THC is mediated through the CB_1 receptor in our preparation, we repeated the experiment in the presence of 100 nM SR141716A, but did not find that SR141716A restored the calcium contractile response. It should also be noted that vasorelaxation to THC was also not inhibited by CB_1 receptor antagonism; therefore it is unlikely that the inhibition of calcium channels by THC is coupled to CB_1 receptors in this arterial preparation.

When vessels were contracted with a high-potassium solution, it was found that the vasorelaxant effects of THC were greatly reduced, implicating the activation of K^+

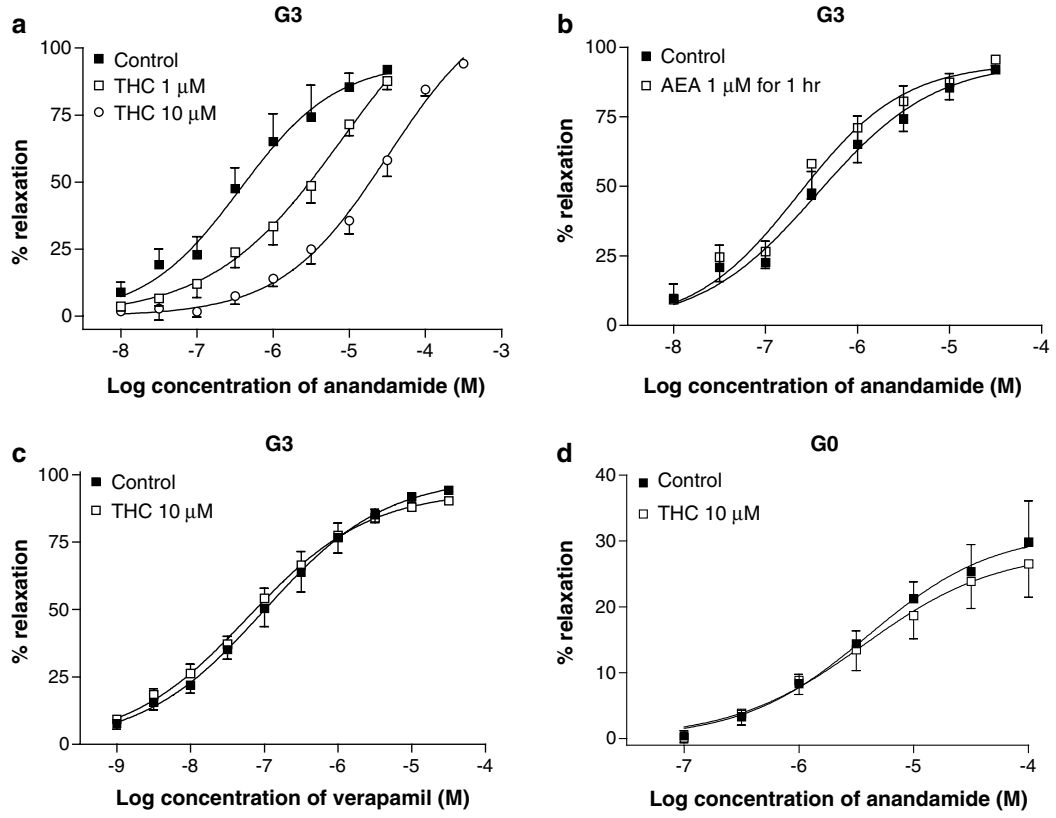

Figure 6 The effects of 1 μM THC ($n=8$) and 10 μM THC ($n=6$) on vasorelaxation to anandamide in G3 (a), and the effects of 10 μM THC on vasorelaxation to anandamide in G0 (d, $n=7$), and on vasorelaxation to verapamil (c, $n=6$), and the effects of prior exposure to anandamide on vasorelaxation to anandamide in G3 vessels (b, $n=6$). Data are given as means, with error bars representing s.e.m.

channels during vasorelaxation. Similar findings have been made for the endocannabinoids anandamide and NADA (Randall *et al*., 1996; White & Hiley, 1997; O'Sullivan *et al*., 2004a). Interestingly, contracting vessels with a high-potassium solution had little or no effect on vasorelaxation caused by the synthetic CBs CP55,940, HU210 and WIN55,212–2 (White & Hiley, 1998). Similarly, HU210 and palmitoylethanolamide (PEA) have not been found to cause hyperpolarisation of arteries (Chataigneau *et al*., 1998), highlighting that the mechanisms of actions between various CB compounds are not homogeneous.

To further investigate the activation of potassium channels by THC, a number of selective K^+ channel inhibitors were used. It appears that THC activates both large and small calcium-activated K^+ channels, which has also been observed for anandamide (Plane *et al*., 1997; Randall & Kendall, 1998). It was also found that THC activates voltage-dependent inward rectifier K^+ channels, as does anandamide (White & Hiley, 1997), and like anandamide (White & Hiley, 1997) inhibition of either the K_{ATP} channel or K_V channel had no effect on vasorelaxation to THC. These data suggest that THC is similar to anandamide with regard to potassium channel activation, at least in mesenteric arteries. In this regard, the K_{ATP} channel has been implicated in vasorelaxation to anandamide in the pig carotid artery, but not in rat mesenteric arteries in the same study (Chataigneau *et al*., 1998), emphasising regional variations in the roles of various potassium channels.

Since vasorelaxation to THC was inhibited by PTX treatment and by oubain, it was examined whether THC stimulates G-protein-coupled inward rectifier K^+ channels (GIRK channels) using tertiapin (Jin & Lu, 1998; Chauhan *et al*., 2003), a GIRK channel inhibitor. However, tertiapin had no effect on vasorelaxation to THC in G3 vessels.

Vasoconstriction to THC in the superior mesenteric artery

In the superior mesenteric artery, THC caused vasoconstriction in the high micromolar range. This is in contrast with the effects of THC in G3 vessels, and the effects of anandamide in the superior mesenteric artery. However, vasoconstrictor effects of THC have previously been reported in the central artery of the rabbit ear (Barbosa *et al*., 1981), in the rat mesenteric bed (Wagner *et al*., 1999), and in the rat aorta (O'Sullivan *et al*., 2003). In order to establish the mechanism of vasoconstriction in G0, some experiments were performed in the presence of the cyclooxygenase inhibitor indomethacin, and it was found that the vasoconstrictor response to high concentrations of THC was unaffected, but vasorelaxation to THC in the lower concentration was enhanced. This would indicate that, at lower concentrations, THC stimulates release of vasoconstrictor prostanoids that oppose vasorelaxation. After removal of the endothelium, the vasoconstrictor response to THC in the higher concentration ranges was

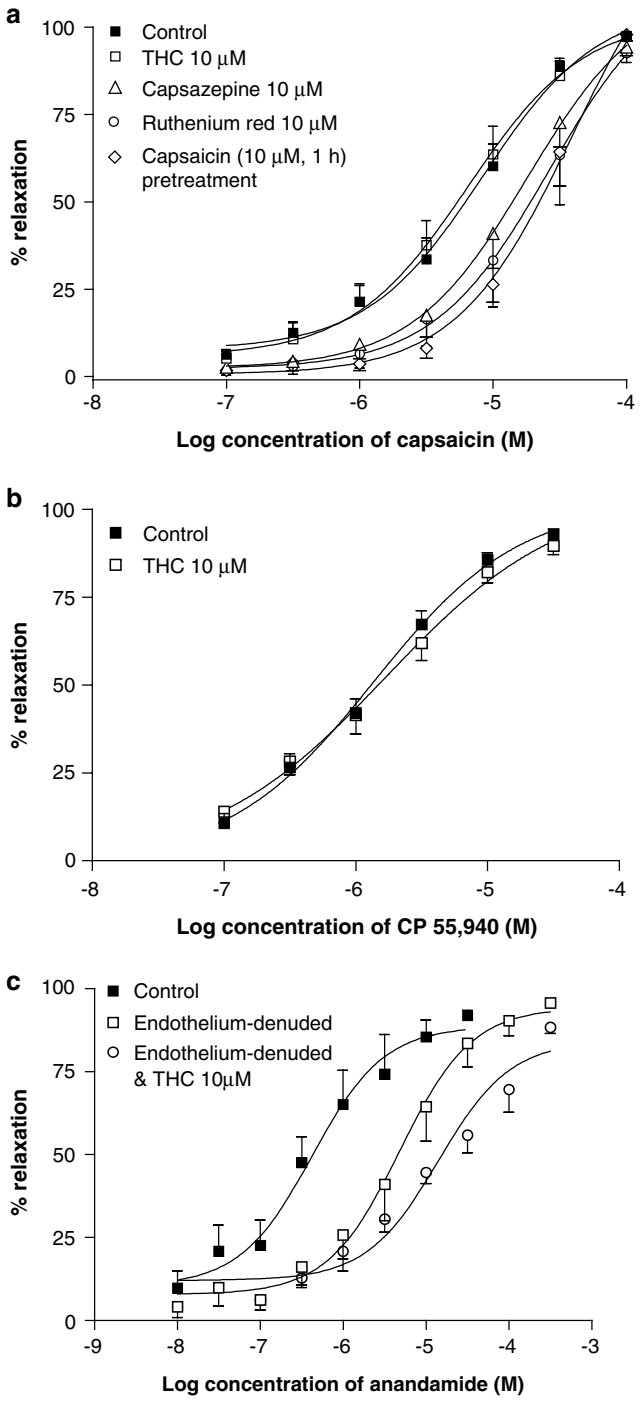

Figure 7 The effects of THC (10 μM) on vasorelaxation to the TRPV1 agonist capsaicin ($n=6$, a) and to the CB_1 receptor agonist CP55,940 ($n=5$, b) in G3 isolated arteries. (c) the antagonist effects of THC on anandamide after removal of the endothelium ($n=6$). Data are given as means, with error bars representing s.e.m.

converted to vasorelaxation, indicating that an endothelium-derived vasoconstrictor is responsible for constricting the artery at high concentrations of THC. It was thus investigated whether this could be through THC-stimulated release of endothelin; however, an endothelin antagonist did not affect the vasoconstrictor response to THC. It was also found that vasoconstriction to THC was reduced in the presence of the CB_1 receptor antagonist SR141716A, consistent with the notion that CB_1 receptors are coupled to vasoconstriction (Holland *et al*., 1999). However, it should also be considered that intrinsic vasorelaxant effects of SR141716A may counteract the contractile effects of THC (White & Hiley, 1998).

The absence of a vasorelaxant response to THC in the superior mesenteric artery might indicate that the $G_{(i/o)}$-protein-linked receptor responsible for vasorelaxation to THC in G3 is not expressed and/or functional in the larger conduit vessels. Indeed, we have previously shown that vasorelaxation to endocannabinoids (anandamide and NADA) occurs partly at an endothelial CB receptor that is only expressed and/or functional in the smaller mesenteric arteries (O'Sullivan *et al*., 2004a, b).

Antagonism of vasorelaxation to anandamide by THC

There is evidence in the literature that CBs may antagonise each other (Bayewitch *et al*., 1996; Petitet *et al*., 1998; Kelley & Thayer, 2004), and we sought to establish the effects of an exogenous CB, THC, on the vascular effects of an endocannabinoid, anandamide. We have shown for the first time that THC inhibits vasorelaxation to anandamide, which was not due to nonspecific inhibition of vasorelaxation, as it had no effect on the noncannabinoid vasorelaxant verapamil. The effects of THC were also not due to desensitisation of CB receptors, as pre-exposure of vessels to anandamide had no effect on subsequent concentration–response curves. Since some of the vasorelaxant effects of anandamide are mediated through the TRPV1 receptors and CB_1 receptors (Zygmunt *et al*., 1999; O'Sullivan *et al*., 2004b), we examined whether THC affects vasorelaxation to the TRPV1 receptor agonist capsaicin, or the CB_1 receptor agonist CP55,940. We have previously shown that vasorelaxation to CP55,940 is antagonised by 100 nM SR141716A (O'Sullivan *et al*., 2004b), and is thus partly mediated by the CB_1 receptor. We found that THC did not affect the vasorelaxant responses to either capsaicin or CP55,940, suggesting that its actions on anandamide are not through competition at the TRPV1 or CB_1 receptor.

We have previously shown that vasorelaxation to anandamide in G3 is partly mediated by a pathway that is not present in G0 that involves stimulation of an endothelial receptor, EDHF release and gap junctions (O'Sullivan *et al*., 2004b). Since THC antagonised the actions of anandamide in G3 and not G0, this could implicate one of these as the site of action for THC. First, we tested the effects of THC after de-endothelialisation, and found that THC did not have an inhibitory effect on anandamide after removal of the endothelium. We also found that the antagonist effects of THC on anandamide were enhanced in the presence of L-NAME. Since the involvement of EDHF activity is enhanced when nitric oxide activity is inhibited, this could suggest that THC inhibits anandamide through inhibition of EDHF activity, which is proposed to be involved in vasorelaxation to anandamide (Jarai *et al*., 1999; O'Sullivan *et al*., 2004b). Additionally, THC has been previously shown to inhibit EDHF in rabbit mesenteric arteries (Fleming *et al*., 1999). We found that, in G3, THC inhibited both the magnitude and duration of the vasorelaxant response to carbachol in the

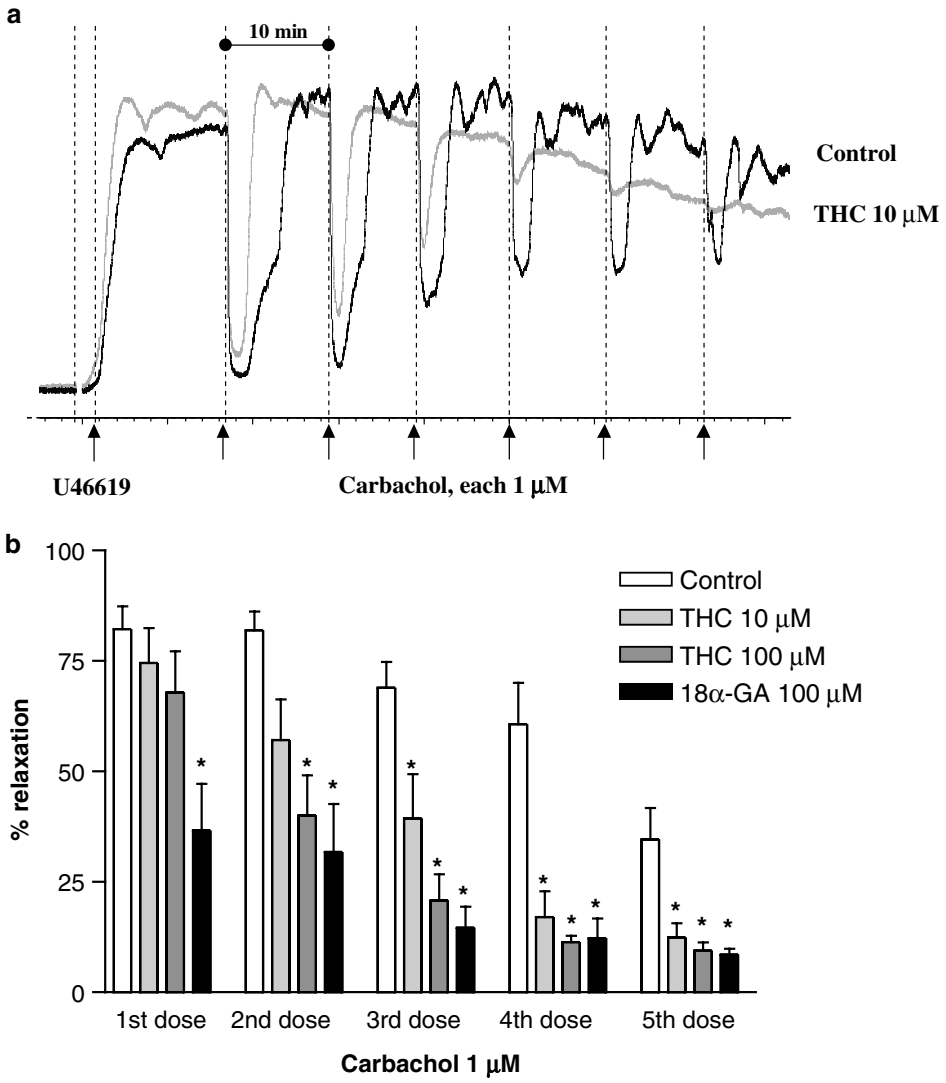

Figure 8 Superimposed tracings from adjacent segments of the same G3 artery showing the typical response to carbachol (1 μM) in the presence of L-NAME (30 μM) and indomethacin (10 μM). Both the magnitude and duration of the EDHF response were inhibited in the presence of THC (10 μM) compared with the control response (a). The EDHF response to repeated doses of carbachol in G3 vessels in the presence of 18α-GA (100 μM, $n=8$) and THC at 10 μM ($n=8$) and 100 μM ($n=9$, b). *denotes a significant difference between manipulation and control as determined by ANOVA. Data are given as means, with error bars representing s.e.m.

presence of nitric oxide synthase and cyclooxygenase inhibition. The inhibition of the EDHF-mediated response to carbachol by THC is compatible with the notion that THC inhibits anandamide through inhibition of the EDHF component of vasorelaxation. Although we have not excluded the possibility that THC antagonises the endothelial site of action of anandamide (Jarai *et al*., 1999; O'Sullivan *et al*., 2004b), since THC also inhibits carbachol, the inhibitory effects of THC on EDHF activity are at least independent of any possible additional actions at this target.

In view of the central role of gap junctions in EDHF-type response (Chaytor *et al*., 1999), we compared the actions of THC with that of the gap junction inhibitor 18α-GA. It was found that the effects of THC were similar in profile to 18α-GA, which might suggest that THC inhibits intracellular activity. Previous work has shown that THC (30 μM) inhibits gap junctional communication in endothelial cell cultures through activation of ERK and subsequent phosphorylation of connexin proteins involved in EDHF-type responses (Brandes *et al*., 2002). Similarly, Upham *et al*. (2003) showed recently in rat epithelial cells that THC (15 μM) inhibits gap junctional intracellular communication using Lucifer yellow dye transfer, again through stimulation of ERK. Interestingly, in the work by Upham and colleagues, this effect was independent of either the CB_1 or CB_2 receptor. Inhibition of gap junctional activity in mesenteric vessels is thus a possible mechanism by which THC may inhibit vasorelaxation to anandamide.

In conclusion, we have shown that THC, in addition to activating sensory nerves (Zygmunt *et al*., 2002), produces vasorelaxation in resistance mesenteric arteries through an unidentified PTX-sensitive receptor and involves activation of potassium channels and inhibition of calcium channels. By contrast, THC contracts the larger superior mesenteric artery through at least two vasoconstrictor pathways. Furthermore,

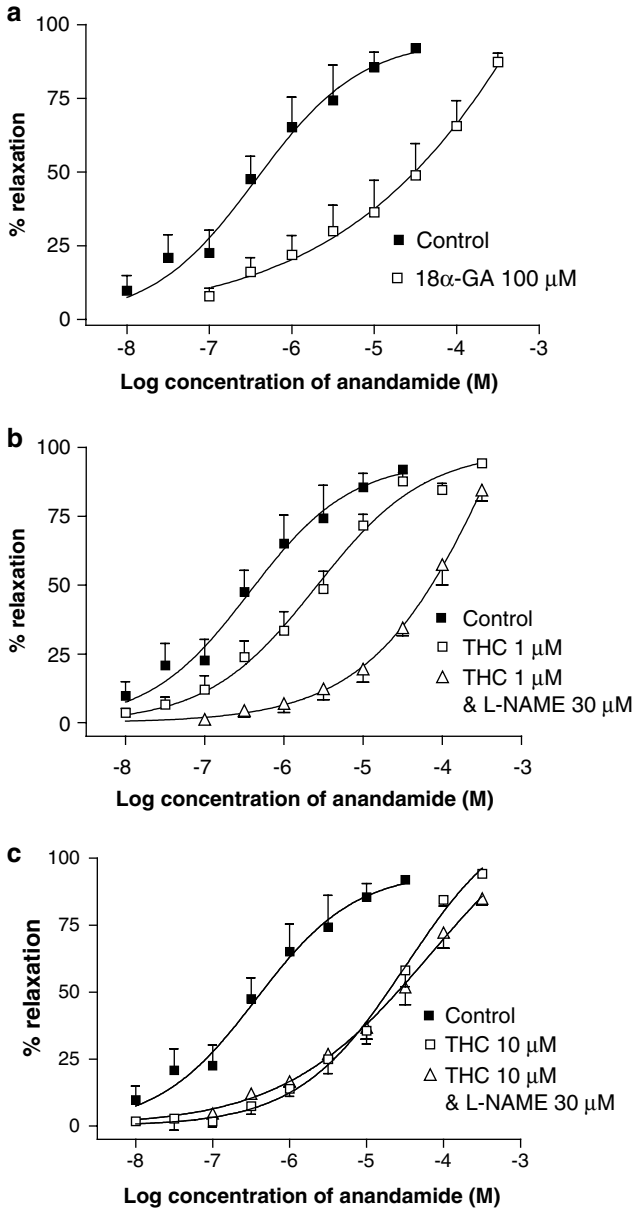

Figure 9 The effects of 18α-GA (100 μM, $n=7$, a), and the effects of L-NAME (30 μM, $n=5$) on the inhibitory effects of THC (1 μM, $n=5$, b) and 10 μM ($n=4$, c) on vasorelaxation to anandamide in G3 vessels. Data are given as means, with error bars representing s.e.m.

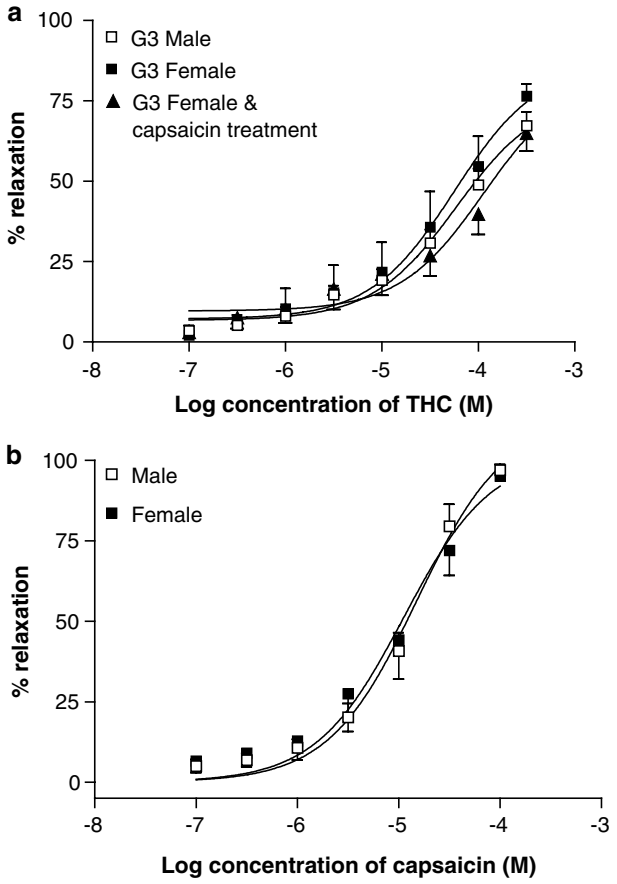

Figure 10 Vasorelaxation to capsaicin ($n=5$, a), THC ($n=5$) and THC after capsaicin pretreatment for 1 h (10 μM, $n=5$, b) in isolated G3 mesenteric arteries obtained from female rats compared with male rats. Data are given as means, with error bars representing s.e.m.

THC inhibits the vasorelaxant activities of the endogenous CB anandamide, which may be through inhibition of EDHF activity, possibly through actions on intercellular communication.

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