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Characterisation of cannabinoid modulation of sensory neurotransmission in the rat isolated mesenteric arterial bed

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Running title: Cannabinoids inhibit vascular sensory neurotransmission

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Abbreviations:

ANOVA	Analysis of variance
CB	Cannabinoid
CBD	Cannabidiol
THC	Δ^9-tetrahydrocannabinol
DRG	Dorsal root ganglia
EFS	Electrical field stimulation
CGRP	Calcitonin gene-related peptide
TRPV1	Vanilloid receptor
VIP	Vasoactive intestinal polypeptide

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ABSTRACT

The present study investigated the effects of different classes of cannabinoid receptor ligands on sensory neurotransmission in the rat isolated mesenteric arterial bed. Electrical field stimulation of the mesenteric bed evoked frequency-dependent vasorelaxation due to the activation of capsaicin-sensitive sensory nerves and release of calcitonin gene-related peptide (CGRP). The CB_1/CB_2 cannabinoid agonists WIN55,212 and CP55,940 (0.01-1 μM) attenuated sensory neurogenic relaxation in a concentration-dependent manner. At 0.1 μM, WIN55,212 and CP55,940 were largely ineffective in the presence of the CB_1 antagonists SR141716A and LY320135 (1 μM) but their inhibitory actions remained in the presence of the CB_2 selective antagonist SR144528 (1 μM). The CB_1/CB_2 agonist THC (1 μM) attenuated sensory neurogenic relaxations, as did the CB_2 agonist JWH-015. The inhibitory actions of both THC and JWH–015 were still evident in the presence of SR141716A (1 μM) and SR144528 (1 μM). None of the cannabinoid agonists investigated had an effect on vasorelaxation elicited by exogenous CGRP indicating a prejunctional mechanism. These data demonstrate that different classes of cannabinoid agonists attenuate sensory neurotransmission via a prejunctional site and provide evidence for mediation by a CB_1 and/or a non CB_1/CB_2 receptor.

INTRODUCTION

Two cannabinoid receptor subtypes, both G protein-linked, have been isolated and cloned to date; the CB_1 receptor is found mainly in the central nervous system and some peripheral nerve terminals and the CB_2 receptor is associated mainly with immune tissues (Pertwee, 1999). There is evidence, however, that the cannabinoid receptor classification is incomplete. There are data which suggest the presence of multiple CB_1 subtypes in the spinal cord (Welch *et al.*, 1998) and putative CB_2-like receptors have been implicated in antinociceptive and hypotensive effects of cannabinoid agonists (Hanuš *et al.*, 1999; Calignano *et al.*, 2001). Experiments using CB receptor knockout mice have uncovered a putative novel cannabinoid receptor in the mouse brain (Breivogel *et al*, 2001; Di Marzo *et al.*, 2000; Hájos *et al.*, 2001; Monory *et al.*, 2002). In addition, studies of the rat mesentery have described a novel anandamide-sensitive, SR141716A-sensitive endothelial receptor (Járai *et al.*, 1999). Recently, Zygmunt *et al.* (2002) reported that Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) induce a CB_1/CB_2 independent release of calcitonin gene-related peptide (CGRP) from capsaicin-sensitive nerves in isolated rat mesenteric arterial rings that could be blocked by the vanilloid receptor (TRPV1) blocker ruthenium red but that was still present in TRPV1 knockout animals.

Capsaicin-sensitive sensory nerves are widely distributed in the cardiovascular system and have a dual function; an afferent function whereby they participate in reflex activation of motor nerves, and an efferent function, whereby neurotransmitter is released from the nerve terminal being stimulated (Maggi & Meli, 1988). Studies on dorsal root ganglia (DRG) and F-11 cells (DRG x neuroblastoma hybridomas) indicate that both CB_1 and CB_2 receptor proteins exist in these cells (Ross *et al.*, 2001). However, only CB_1 receptors have been demonstrated to be transported from DRG cell bodies to the periphery of the sensory nerves (Hohmann & Herkenham, 1999).

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Recently, functional evidence has been provided which suggests that non-CB_1/CB_2 cannabinoid receptors are located on capsaicin-sensitive sensory nerves in the rat mesenteric bed (Ralevic & Kendall, 2001). The potent cannabinoid CB_1/CB_2 receptor agonist HU210 was found to attenuate sensory neurogenic relaxation evoked by electrical field stimulation (EFS) in preconstricted mesenteric beds. This inhibitory action was resistant to the CB_1 selective antagonists SR141716A and LY320135 and the CB_2 selective antagonist SR144528. HU210 had no effect on the vasorelaxant response to exogenous CGRP, suggesting prejunctional modulation of CGRP release (Ralevic & Kendall, 2001).

The present study investigated the effects of different classes of cannabinoid compounds represented by the aminoalkylindoles WIN55,212, and JWH-015, the bicyclic CP55,940 and the classical cannabinoid agonist THC, on sensory neurogenic vasorelaxation to EFS in the rat isolated mesenteric arterial bed. The effects of CB_1 (SR141716A, LY320135) and CB_2 (SR144528) selective antagonists on agonist responses were determined. Electrical stimulation of sensory nerve terminals releases CGRP, the principal sensory vasorelaxant in the rat isolated mesenteric arterial bed which can be reversed by the CGRP antagonist $CGRP_{(8\text{-}37)}$ (Kawasaki *et al.,* 1988; Han *et al.,* 1990). The effects of cannabinoids on responses to exogenous CGRP were, therefore, investigated in order to determine if the actions were mediated at a pre- or postjunctional site. Since there have been reported cross-talk between cannabinoid and vanilloid receptors, the actions of capsaicin in the presence of cannabinoids were investigated. Some of this work has been published in abstract form.

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METHODS

Mesenteric arterial bed preparation.

Male Wistar rats (250-300g) were killed by decapitation after exposure to CO_2. Mesenteric beds were isolated and perfused via the superior mesenteric artery. The abdomen was opened and the superior mesenteric artery was exposed and cannulated with a blunted hypodermic needle. The superior mesenteric vein was cut, blood flushed from the preparation with 0.5 ml Krebs' solution and the gut dissected away from the mesenteric vasculature. The preparations were mounted on stainless steel grids (7 x 5 cm) in a humid chamber and perfused at a constant flow rate of 5 ml min^{-1} using a perfusion pump (model: 7554-30, Cole Parmer Ltd, 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 37ºC. In order to ascertain that the electrical circuit was complete, EFS was applied comprising a 5 s stimulus (20 Hz, 90 V, 0.1 ms) via the metal grid (negative electrode) and the hypodermic needle (positive electrode), to excite the sympathetic fibres thus eliciting a small vasoconstriction and a transient rise in perfusion pressure. Guanethidine (5 μM) was added to block sympathetic neurotransmission and after 30 min, methoxamine (5-100 μM) was added to preconstrict the preparation (30-80 mm Hg above baseline). EFS (1-12 Hz, 0.1 ms, 60 V, 30 s) was applied with a Grass S9D stimulator. The resulting vasorelaxation response has been shown to be blocked by tetrodotoxin and capsaicin (Ralevic *et al.,* 1991) consistent with it being mediated by capsaicin-sensitive sensory nerves (Kawasaki *et al.,* 1988). Moreover, the relaxation is mimicked by CGRP, whilst substance P and neurokinin A are inactive and vasoactive intestinal polypeptide (VIP) causes relaxation only at relatively high concentrations, and there are numerous CGRP-like immunoreactive fibres, but few substance P and VIP-like immunoreactive fibres present in the mesenteric arteries (Kawasaki et al., 1988). In addition, the neurogenic relaxation to electrical field stimulation is abolished by the CGRP receptor antagonist CGRP(8-37) (Han et

al., 1990). Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model: P23XL, Viggo-Spectramed, Oxnard, CA), situated on a sidearm proximal to the preparation, and recorded using A to D converter (PowerLab/400, ADI Instruments, Australia). Preparations were allowed to equilibrate for 30 min prior to experimentation.

Experimental protocol.

Three consecutive relaxant response curves to EFS at 1-12 Hz, 'EFS control', 'EFS I', and 'EFS II', were generated in each preconstricted mesenteric arterial bed. The tone of the preparation was then allowed to return to preconstricted tone before the next stimulus was applied. The first response curve acted as a control, the compound under investigation was then added to the perfusate and after 15 min, response curves EFS I and EFS II were generated. Antagonists were added at the start of the equilibration period. Only a single concentration of agonist was used per preparation. In a separate series of experiments, a dose response curve was constructed to CGRP (0.05 pmol – 0.5 nmol) and capsaicin (0.05 pmol – 5 nmol), by applying 50 μl bolus injections via norprene tubing proximal to the preparation.

Drugs.

SR141716A (N-piperidino-5- (4-chlorophenyl)-1-(2,4-dichloro phenyl)-4-methyl-3-pyrazole-carboxamide) and SR144528 (N-[1*S*)-endo-1,3,3,-trimetyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) were gifts from Sanofi (Montpellier, France). LY320135 [6-methoxy-2- (4-methoxyphenyl)benzo[*b*]-thien-3-yl][4-cyanophenyl] methanone) was a gift from Eli Lilly (U.K). CP55,940 (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol and JWH-015 (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone) were from Tocris (Bristol, U.K). (+)WIN55,212-2 R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo [1,2,3-de]-1,4-

benzoazinyl]-(1-naphthalenyl)methanone mesylate and (-)WIN55,212-3 S(-)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoazinyl]-(1-naphthalenyl)methanone mesylate were also purchased from Tocris (Bristol, U.K). Calcitonin gene-related peptide was from Sigma (Dorset, UK). Guanethidine (Ismelin) was from Alliance Pharmaceuticals (Wiltshire, UK). All cannabinoids were dissolved in ethanol at a concentration of 10^{-2} M. CGRP was dissolved in distilled water.

Data Analysis.

Vasorelaxant responses (mmHg) of the mesenteric beds were expressed as a percentage relaxation of the methoxamine-induced increase in tone above baseline. Data were compared by Student's t test and one way/two way analysis of variance (ANOVA) with Tukey's post hoc test. A value of $P<0.05$ was taken to indicate a statistically significant difference. The software package Prism GraphPad (3.0) was used to perform the analyses. R_{MAX} = maximal relaxation.

RESULTS

Effect of vehicle (0.01%) on vasorelaxant responses to EFS.

Electrical field stimulation (EFS 1-12 Hz, 0.1 ms, 60 V, 30 s) produced frequency-dependent relaxation of the rat isolated mesenteric arterial bed. Three frequency-response curves were constructed, EFS control, EFS I and EFS II. The first curve acted as a control, the compound under investigation was added for 15 min, then a further two curves were constructed. The equivalent concentration of the drug vehicle (0.01% ethanol) had no effect on the vasorelaxant response (Fig. 1a), demonstrating that the frequency response curves are reproducible under control conditions (EFS control 51.7 ± 6.3 and EFS II 43.3 ± 5.8; P>0.05).

Effect of SR141716A (1 μM) on vasorelaxant responses to EFS.

SR141716A has previously been reported to augment sensory neurogenic relaxations to EFS in the rat isolated arterial mesenteric bed (Ralevic & Kendall, 2001). In this study, SR141716A (1 μM, n=9) was found to have no significant effect on the vasorelaxant response (Fig. 1b), at a submaximal frequency of 8 Hz (EFS control 54.7 ± 5.4%, EFS I 48.6 ± 5.8 and EFS II 45.3 ± 6.1; P>0.05). The R_{MAX} was unaffected; EFS control from 56.0 ± 4.8% to EFS II 48.5 ± 5.9%. SR141716A had no significant effect on the tone of the preparations.

Effect of LY320135 (1 μM) on vasorelaxant responses to EFS.

The experiments were carried out in the presence of LY320135 (1 μM, n=3) to determine if the antagonist had any effect on the vasorelaxant response to EFS. At a submaximal frequency of 8 Hz LY320135 had no effect on the vasorelaxant response (Fig. 1c) (EFS control 50.3 ± 2.1%, EFS I 51.7 ± 6.0 and EFS II 41.0 ± 8.0; P>0.05). The R_{MAX} was

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unaffected; EFS control from 66.4 ± 5.8% to EFS II 56.7 ± 4.2%. LY320135 had no significant effect on the tone of the preparations.

Effect of SR144528 (1 μM) on vasorelaxant responses to EFS.

In order to rule out any involvement of CB_2 receptors, the experiments were repeated in the presence of the CB_2 antagonist SR144528 (1μM, n=3, Fig. 1d). At a submaximal frequency of 8 Hz SR144528 had no effect on the vasorelaxant response (EFS control 68.8 ± 7.2%, EFS I 64.2 ± 4.2 and EFS II 57.5 ± 2.5; P>0.05). The R_{MAX} was reduced slightly but significantly; EFS control from 80.3 ± 2.4%, EFS I 71.5 ± 0.8% and EFS II 66.3 ± 2.4% (P<0.05). SR144528 had no significant effect on the tone of the preparations.

Effect of WIN55,212 on vasorelaxant responses to EFS.

WIN55,212, a high potency cannabinoid agonist at both CB_1 and CB_2 receptors, attenuated EFS I and EFS II in a concentration and time-dependent manner, illustrated in the representative trace in fig. 2 and in fig. 3. At the lowest concentration, (0.01 μM, n=9), there was a small difference at 4 Hz but no significant difference in the maximal relaxation (value obtained at 12 Hz, R_{MAX}) (Fig. 3). At concentrations of 0.1 μM and 1 μM WIN55,212, the relaxation response was significantly reduced; at a submaximal frequency of 8 Hz, 0.1 μM reduced EFS control 50.5 ± 4.4% to EFS II 26.2 ± 2.8% (P<0.05, n=6) and 1 μM reduced EFS control 53.4 ± 5.5% to EFS II 24.3 ± 2.3% (P<0.05, n=6). The R_{MAX} was significantly reduced at both concentrations; at 1 μM WIN55,212 reduced EFS control from 58.8 ± 4.9% to EFS II 33.0 ± 5.1%. WIN55,212 had no significant effect on the tone of the preparations.

Effect of SR141716A and SR144528 on the inhibitory actions of WIN55,212 on the vasorelaxant response to EFS.

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In order to determine if the inhibitory actions exerted by WIN55,212 were mediated by either CB_1 or CB_2 receptors the experiments were repeated in the presence of 1 μM of the CB_1 selective antagonist, SR141716A, or the CB_2 selective antagonist SR144528 (Fig. 4). A submaximal concentration, 0.1 μM WIN55,212 was used, and 1 μM of SR141716A was used to minimise non selective effects, which have been reported at higher concentrations. In the presence of SR141716A, the inhibitory actions of WIN55,212 (0.1 μM) on sensory neurogenic vasorelaxation were largely absent. At a submaximal frequency of 8 Hz, there was no significant difference between the responses (EFS control 46.7 ± 3.6%, EFS I 39.3 ± 3.9% and EFS II 36.1 ± 3.8%; n=8, P>0.05). WIN55,212 was still able to cause inhibition of sensory neurogenic vasorelaxation in the presence of SR144528 (1 μM)**.** At 8 Hz, there was a significant inhibition of neurogenic relaxations by 0.1 μM WIN55,212 (EFS control 44.2 ± 3.9% to EFS II 17.5 ± 3.0%; n=5, P<0.05)**.**

Effect of the WIN55,212 stereoisomer (-)WIN55,212 on vasorelaxant responses to EFS.

The stereoisomer, (-)WIN55,212, was used to investigate if a stereospecificity for this compound exists; this is a useful tool to determine if the actions are receptor-mediated (Fig. 5). 1 μM (-)WIN55,212 had no significant effect on the sensory neurogenic vasorelaxation evoked by EFS, at 8 Hz EFS control 63.0 ± 7.8% and EFS II 51.2 ± 9.0%; n=6, P>0.05).

Effect of CP55,940 on vasorelaxant responses to EFS.

CP55,940, an agonist which has equivalent high potency at both CB_1 and CB_2 receptor subtypes, attenuated sensory neurogenic relaxations in a concentration-dependent manner (Fig. 6). At a concentration of 0.01 μM, CP55,940 blocked relaxation only at 8 Hz but had no effect on the maximal relaxation (R_{MAX}). At concentrations of 0.1 μM and 1 μM CP55,940, the relaxation response was significantly reduced. At a submaximal frequency of 8 Hz 0.1 μM CP55,940 reduced EFS control 38.0 ± 3.9% to EFS II 17.7 ± 3.3% (n=6, P<0.05); 1 μM

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CP55,940 reduced EFS control 38.6 ± 4.6% to EFS II 18.0 ± 2.9% (n=8, P<0.05). There was a significant reduction in R_{MAX} at both concentrations, at 1 μM CP55,940 the EFS control 46.4 ± 3.9% was reduced to 21.9 ± 3.0% (P<0.05). CP55,940 had no significant effect on the tone of the preparations at all concentrations.

Effect of LY320135 and SR144528 on inhibition by CP55,940 of the vasorelaxant response to EFS.

Since CP55,940 is equally effective at both cannabinoid receptor subtypes, to characterise the receptor mediating the inhibitory effects of CP55,940, the experiment was repeated in the presence of LY320135, a CB_1 selective antagonist, and SR144528, a CB_2 selective antagonist (Fig. 7). CP55,940 (0.1 μM) had no inhibitory actions on sensory neurogenic vasorelaxation in the presence of LY320135 (1 μM, n=6). At a submaximal frequency of 8Hz, there was no significant difference between the curves. CP55,940 (0.1 μM) was still able to cause inhibition in the presence of SR144528 (1 μM). At a submaximal frequency of 8 Hz, EFS control 54.0 ± 7.8% was reduced to EFS II 23.8 ± 5.8%; (n=6, P<0.05).

Effect of JWH-015 on vasorelaxant responses to EFS.

To further characterise the receptor involved in the inhibition of the neurogenic response the CB_2 selective agonist JWH-015 was investigated (Fig. 8a, b). At a concentration of 0.1 μM JWH-015, there was no significant difference between the frequency-response curves or the R_{MAX} values. In the presence of 1 μM JWH-015, the relaxation response was significantly reduced; at a submaximal frequency of 8 Hz, EFS control 51.7 ± 2.7% to EFS II 33.5 ± 2.8% (n=6, P<0.05). The R_{MAX} was significantly reduced, at 1 μM EFS control 56.7 ± 1.93% was reduced to EFS II 36.0 ± 3.3% (P<0.05). JWH-015 had no significant effect on the tone of the preparations

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Effect of SR141716A and SR144528 on inhibition by JWH-015 of the vasorelaxant response to EFS.

To determine if the inhibitory actions of JWH-015 were mediated by the CB_1 or CB_2 receptor we challenged with selective antagonists for CB_1 and CB_2 (Fig. 8c, d). JWH-015 (1 μM) was still able to cause inhibition of sensory neurogenic vasorelaxation in the presence of the CB_2 selective antagonist SR144528 (1 μM). At a submaximal frequency of 8 Hz, EFS control 59.4 ± 6.6% was reduced to EFS II 33.4 ± 5.1%; n=9, $P<0.05$). In the presence of the CB_1 selective antagonist SR141716A (1 μM), JWH-015 (1 μM) was still able to inhibit sensory neurogenic vasorelaxation, although this effect appeared to be attenuated compared to control responses in the absence of SR141716A. At a submaximal frequency of 8 Hz, there was a significant difference between the curves (EFS control 74.8 ± 5.1%, EFS I 57.4 ± 7.3% and EFS II 40.7 ± 8.9%; n=5, $P<0.05$).

Effect of THC on vasorelaxant responses to EFS.

The classical plant-derived cannabinoid THC, which acts as a partial agonist at both CB_1 and CB_2 receptors, attenuated sensory neurogenic relaxation (Fig. 9a, b). In the presence of 0.1 μM THC, at a submaximal frequency of 8 Hz, the response was reduced from EFS control 60.6 ± 7.6% to EFS II 31.1 ± 3.9% (n=6, $P<0.05$). 1 μM THC reduced 8 Hz EFS control 53.1 ± 5.0% to EFS II 11.1 ± 1.6%. The maximal response was also significantly reduced, from 59.3 ± 7.5% to 17.3 ± 2.0% (n=4, $P<0.05$). THC initially produced a transient pressor effect (30.14 ± 2.6 mmHg) followed by a reduction in the tone of the preparation. The secondary vasorelaxation required methoxamine to maintain tone.

Effect of SR141716A and SR144528 on inhibition by THC of the vasorelaxant response to EFS.

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THC (1 μM) was challenged with antagonists for both the CB_1 and CB_2 receptors to determine its site of action (Fig. 9c, d). THC was still able to cause inhibition of sensory neurogenic vasorelaxation in the presence of the CB_1 selective antagonist SR141716A (1 μM) At a submaximal frequency of 8 Hz, there was a significant difference between the curves (EFS control 71.78 ± 6.9%, EFS I 33.2 ± 3.2% and EFS II 27.9 ± 5.7%; n=5, $P<0.05$). THC was still able to cause inhibition of sensory neurogenic vasorelaxation in the presence of the CB_2 selective antagonist SR144528 (1 μM). At a submaximal frequency of 8 Hz, EFS control 52.1 ± 4.7% was reduced to EFS II 24.0 ± 7.2%; (n=8, $P<0.05$).

Effects of WIN55,212, CP55,940, JWH-015 and THC on the vasorelaxant response to exogenous CGRP

In order to determine if the inhibitory actions on sensory neurotransmission by the cannabinoid agonists are via a pre or postjunctional site; dose-response curves to CGRP, the principal motor neuropeptide involved in sensory neurogenic relaxation were constructed, in the presence of 1 μM cannabinoid agonists. The pD_2 value was unaltered in the presence of all cannabinoid agonists investigated (Table 1).

Effects of WIN55,212, CP55,940, JWH-015 and THC on the vasorelaxant response to capsaicin

In order to investigate the possibility of cross-talk between actions of the cannabinoid agonists and TRPV1 receptors, dose-response curves to capsaicin, which elicits CGRP release via TRPV1 activation, were constructed in the presence of 1 μM cannabinoid agonists. The pD_2 value for capsaicin was unaltered in the presence of CP55,940, THC and JWH-015, however, the capsaicin response was enhanced in the presence of WIN55,212 (Table 2).

DISCUSSION

The present study has shown that cannabinoid agonists representative of three different chemical classes attenuate sensory neurogenic relaxation elicited by EFS in the rat isolated mesenteric arterial bed. The presence of a cannabinoid-sensitive site on sensory nerves is in agreement with previous studies by Richardson *et al.,* and Ellington *et al.,* in which CP55,940 and anandamide were demonstrated to inhibit capsaicin-evoked CGRP release in rat paw skin, which is richly innervated with sensory nerves, in a SR141716A-sensitive manner (Richardson *et al.,* 1998b, Ellington *et al*., 2002). Furthermore, studies using ^{3}H-CP55,940 in radioligand binding assays have uncovered a saturatable binding site using the trigeminal ganglia as a model for sensory nerves (Richardson *et al.,* 1998a). This would indicate that a CP55,940-sensitive receptor is present on sensory nerves, in agreement with the functional pharmacological studies. CB_1 mRNA has been located in DRG cells, and CB_1 receptors are trafficked to peripheral sensory nerve terminals (Hohmann & Herkenham, 1999) and so cannabinoids would be expected to exert their inhibitory effects by prejunctional modulation of neurotransmitter release from the peripheral nerve terminal. This is in agreement with our findings that none of the cannabinoids investigated exerted an inhibitory effect on vasorelaxation in response to exogenous CGRP.

The concentrations of cannabinoid agonist used in this study are in the micromolar range, much higher than concentrations used in some other bioassay preparations such as mouse vas deferens and CB receptor-transfected cell lines (Pertwee, 1999; Howlett *et al.,* 2002). However, the concentrations used were in agreement with previous studies on isolated blood vessels (White & Hiley, 1998). Since cannabinoids are highly lipophilic compounds, the true concentration at the site of action could be much lower than the micromolar range. Indeed, the time-dependency of the inhibitory action (EFS II more sensitive to the inhibitory actions

than EFS I) could be, in part, due to the slow diffusion of the compounds through the blood vessel wall.

The inhibitory actions of both WIN55,212 and CP55,940 in the rat mesenteric bed, which were selectively antagonised by CB_1 but not CB_2 antagonists, indicate the likely involvement of CB_1 or CB_1-like receptors. Neither of the two compounds are agonists at TRPV1 receptors (Zygmunt *et al.*, 1999). Moreover, the inactivity of the stereoisomer (-)WIN55,212 indicates a clear structure activity relationship. Whilst we cannot rule out the possibility that, at least part of the action of SR141716A was due to functional antagonism (Ralevic & Kendall, 2001), this is unlikely to account for all its inhibitory actions as another CB_1 antagonist LY320135, was similarly effective. However, there is growing evidence indicating the existence of novel cannabinoid receptors. Indeed, WIN55,212 is reported to be active at the putative novel cannabinoid receptor identified in the brain. This receptor, described by Breivogel *et al* (2001) was SR141716A-sensitive, whereas the novel WIN55,212-sensitive receptors reported by Hájos *et al*. (2001) and Monory *et al*. (2002) were SR141716A-resistant. Our data describing the effects of the other cannabinoid agonists, suggest that CB_1/CB_1-like receptors are not the only subtype expressed on the perivascular sensory nerves.

JWH-015, a CB_2 selective agonist, inhibited sensory neurogenic vasorelaxation, which was unexpected as there is little evidence for the expression of CB_2 receptors in sensory nerves. However, this action was not blocked by a selective CB_2 antagonist SR144528. Griffin *et al.*, (1997) reported that JWH-015 inhibited electrically evoked contractions in the mouse vas deferens and the guinea-pig myenteric plexus and that this effect could be reversed by SR141716A in the myenteric plexus, suggesting that the inhibitory actions are CB_1-mediated (Griffin *et al.*, 1997). This is in agreement with our study in that the inhibitory actions of

JWH-015 appeared to be attenuated in the presence of the CB_1 antagonist SR141716A. Recently, Ross *et al*, (2001), described CB_2 receptor protein in F-11 (DRG x neuroblastoma hybridoma) cells using antibodies raised against the N-terminus. However, in nerve ligation studies using antisense cRNA probes, mRNA for CB_2 receptors could not be detected in the DRG and CB_2 receptors were not found to be transported to peripheral nerve endings (Hohmann & Herkenham, 1999). Thus, the pharmacological and molecular studies do not indicate expression of CB_2 receptors in sensory nerves. There is evidence for cardiovascular actions of CB_2 receptors as HU-308, a bicyclic CB_2 specific agonist has been shown to significantly reduce blood pressure *in vivo* and this action can be blocked by the CB_2 antagonist SR144528 (Hanuš *et al.*,1999). Therefore, a novel JWH-015-sensitive, SR144528-resistant cannabinoid receptor could be involved in the regulation of perivascular sensory nerves. However, an indirect action cannot be ruled out; JWH-015 could be activating cannabinoid receptors and releasing vasoactive mediators from cells associated with sensory nerves such as mast cells, which have been reported to express CB_2 receptors, although these inhibitory effects were SR144528-sensitive (Facci *et al.*, 1995).

THC, a plant-derived cannabinoid that acts equally well at both CB receptor subtypes, also inhibited vasorelaxation to EFS. This effect could not be blocked by either SR141716A or SR144528. A postjunctional effect was ruled out, as THC had no effect on CGRP vasorelaxation. It is, therefore, possible that THC is mediating its inhibitory actions via a novel prejunctional cannabinoid receptor. In contrast to the other cannabinoids investigated, THC produced a vasoconstriction when added to the perfusate; this is not unexpected as THC has been reported to produce a pressor response followed by a prolonged hypotension *in vivo* (Wagner *et al.*, 1998). The hypotensive effect can be abolished by surgical/pharmacological removal of sympathetic tone and this effect is also sensitive to SR141716A, leading to the possibility of sympatho-inhibition by presynaptic CB_1 receptors. The pressor response could

not be abolished by the elimination of sympathetic tone and so is thought to be due to a peripheral vasoconstriction; it is therefore possible, that the constriction we observed in the mesenteric bed contributes to the pressor effect observed *in vivo*. Vasoconstriction is unlikely, however, to have caused a functional antagonism of the neurogenic relaxation responses, as it was not sustained (the relaxation response curves were generated after reversal of the constriction) and, as relaxation responses to CGRP were unaffected.

THC and cannabidiol have recently been described as acting via a novel receptor subtype to release CGRP in capsaicin-sensitive sensory nerves (Zygmunt *et al.,* 2002). The ability of THC to release CGRP was blocked by the vanilloid blocker ruthenium red, but was present in TRPV1 knockout mice, which raises the possibility of THC activating a transient receptor potential (TRP) ion channel to release CGRP. It is possible that the constriction we observed to THC could mask the effect of the co-incident CGRP release and, indeed, a relaxation followed. Thus, vasorelaxation to EFS could be attenuated because the sensory nerves were depleted of CGRP, or the CGRP receptors were desensitised; this would explain why the inhibitory action could not be blocked by SR141716A. However, ruthenium red (1 μM) does not block the inhibitory actions of THC on sensory neurotransmission in the rat isolated mesenteric arterial bed (Duncan *et al.,* 2003), so a vasorelaxant mechanism dependent on CGRP depletion is unlikely. Interestingly, Zygmunt *et al.,* did not report a constrictor effect of THC, but their study was carried out in arterial segments, whereas the present experiments were in whole vascular beds, so more complex mechanisms could be involved such as the release of endothelial vasoconstrictors.

From the data presented, THC appears to mediate the inhibition of sensory neurotransmission via a non-CB_1CB_2 site. The apparently differential sensitivities of JWH-015 and THC to the actions of SR141716A would suggest they are not acting at a common site. We recently

reported that the inhibitory actions of the putative endocannabinoid noladin ether on sensory neurotransmission could not be blocked by CB_1 and CB_2 antagonists (Duncan *et al.*, 2004), or ruthenium red (Duncan *et al.*, 2003), but could be abolished by the pretreatment of animals with pertussis toxin (PTX). The inhibitory actions of THC are also abolished by PTX-pretreatment (unpublished observations). Thus, there does appear to be a Gi/o protein-coupled, cannabinoid-sensitive receptor on the capsaicin-sensitive perivascular sensory nerves.

In this study, the antagonists alone had no effect on mesenteric arterial contraction, indicating a potential lack of effect of endogenous cannabinoid tone as a modulator in the vascular beds. This is in contrast with a previous report from this laboratory showing that SR141716A augmented sensory neurotransmission in the same model (Ralevic & Kendall, 2001). The reason for this is not clear as the sex, strain and ages of the rats, and the experimental conditions were similar.

In the investigation of possible cross-talk between the cannabinoid and vanilloid systems, we found no modulation of the capsaicin response by CP55,940, THC and JWH-015. WIN55,212 enhanced the capsaicin response, for reasons which are not clear (but which are unrelated to its inhibitory effects on electrically-evoked sensory neurotransmitter release), indicating possible complex effects of this compound on sensory nerves.

In conclusion, it is not possible to fully characterise the receptors involved in cannabinoid-mediated attenuation of sensory nerve mediated vasorelaxation using the antagonists presently available, and the inhibitory prejunctional effects cannot be readily characterised as CB_1 or CB_2 mediated. However, we propose that a heterogeneous population of cannabinoid receptors exist on capsaicin-sensitive nerves in the rat mesenteric bed. Our findings suggest at

least two types of receptor, one sensitive to SR141716A and LY320135, possibly CB_1 or CB_1-like, the other resistant to SR141716A, but distinct from SR144528-sensitive CB_2 receptors.

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REFERENCES

Breivogel CS, Griffin G, Di Marzo V & Martin BR. (2001) Evidence for a new G protein coupled cannabinoid receptor in the mouse brain. *Mol Pharmacol* **60:**155-163.

Calignano A, La Rana G & Piomelli D (2001) Antinociceptive activity of the endogenous fatty acid amide, palmitoylethanolamide. *Eur J. Pharmacol* **419:**191-198.

Di Marzo V. Breivogel CS, Tao Q, Bridgen DT, Razdan RK, Zimmer AM, Zimmer A & Martin BR (2000) Levels, metabolism and pharmacological activity of anandamide in CB_1 cannabinoid receptor knockout mice: evidence for non CB_1, non CB_2 receptor-mediated actions of anandamide in the mouse brain. *J Neurochem* **75:**2434-2344.

Duncan M, Kendall DA & Ralevic V. (2004) Noladin ether, a putative endocannabinoid, attenuates sensory neurotransmission in the rat isolated mesenteric arterial bed via a non-CB_1/CB_2 $G_{i/o}$ linked receptor. *Br J Pharmacol* advance online publication, May 17, 2004; doi:10.1038/sj.bjp.0705789

Duncan M, Kendall DA & Ralevic V. (2003) The effects of ruthenium red on the inhibitory actions of noladin ether, THC & HU210 on sensory neurotransmission in the rat isolated mesenteric arterial bed. 1st European Workshop on Cannabinoid Research. http://www.ucm.es/info/seic-web/abstract-book.pdf

Ellington HC, Cotter MA, Cameron NE, & Ross RA. (2002) The effect of cannabinoids on capsaicin-evoked calcitonin gene-related peptide (CGRP) release from the isolated paw skin of diabetic and non-diabetic rats. *Neuropharmacology* **42:**966-975.

Facci L, Dal Toso R, Romanello S, Buriani A, Skaper SD & Leon A. (1995) Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc Nat Acad Sci U.S.A* **92:**3376-3380.

Griffin G, Fernando SR, Ross RA, McKay NG, Ashford MLJ, Shire D, Huffman JW, Yu S, Lainton JAH, & Pertwee RG. (1997) Evidence for the presence of CB_2-like cannabinoid receptors on peripheral nerve terminals. *Eur J Pharmacol* **339:**53-61.

Hájos N, Ledent C & Freund TF. (2001) Novel cannabinoid-sensitive receptor mediates inhibition of glutamateric synaptic transmission in the hippocampus. *Neuroscience* **106:**1-4.

Han SP, Naes L & Westfall TC. (1990) Inhibition of periarterial nerve stimulation-induced vasodilation of the mesenteric arterial bed by CGRP (8-37) and CGRP receptor desensitization. *Biochem Biophys Res Commun* **168:**786-91.

Hanuš L, Breuer A, Tchilibon S, Shiloah S, Goldenberg D, Horowitz M, Pertwee RG, Ross RA, Mechoulam R & Fride E. (1999) HU-308: A specific agonist for CB_2, a peripheral cannabinoid receptor. *Proc. Nat. Acad. Sci. U.S.A* **96:**14228-14233.

Hohmann AG & Herkenham M. (1999) Cannabinoid receptors undergo axonal flow in sensory nerves. *Neuroscience* **92:**1171-1175.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellass P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R. & Pertwee RG. (2002) International union of

Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54:**161-202

Járai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, Razdan RK, Zimmer A & Kunos G. (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB_1 or CB_2 receptors. *Proc. Nat Acad Sci U.S.A* **96:**14136-14141.

Kawasaki H, Takasaki K, Saito A & Goto K. (1988) Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature* **335:**164-167

Maggi CA & Meli A. (1988) The sensory-efferent function of capsaicin-sensitive neurons. *Gen Pharmacol* **19:**1-43.

Monory K, Tzavara EH, Leximе J, Ledent C, Parmentier M, Borsodi A & Hanoune J. (2002) Novel, non-adenylyl cyclase-coupled cannabinoid binding site in cerebellum of mice. *Biochem Biophys Res Commun* **292:**231-235.

Pertwee RG. (1999) Pharmacology of Cannabinoid Receptor Ligands *Curr Med Chem* **6:**635-664.

Ralevic V, Aberdeen JA & Burnstock G. (1991) Acrylamide-induced autonomic neuropathy of rat mesenteric vessels: histological and pharmacological studies. *J Auton Nerv Syst* ***34*:**77-88.

Ralevic V & Kendall DA. (2001) Cannabinoid inhibition of capsaicin-sensitive sensory neurotransmitter in the rat isolated mesenteric arterial bed. *Eur J Pharmacol* **418:**117-125.

Richardson JD, Aanonsen L & Hargreaves KM. (1998a) Antihyperalgersic effects of spinal cannabinoids. *Eur J Pharmacol* **345:**145-153.

Richardson JD, Kilo S & Hargreaves KM. (1998b) Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB_1 receptors. *Pain* **75:**111-119.

Ross RA, Coutts AA, McFarlane SM, Anavi-Goffer S, Irving AJ, Pertwee RG, MacEwan DJ, Scott RH. (2001) Actions of cannabinoid receptor ligands on rat cultured sensory neurones: implications for antinociception. *Neuropharm* **40:**221-232.

Wagner JA, Varga K & Kunos G. (1998) Cardiovascular actions of cannabinoids and their generation during shock. *J Mol Med* **76:**824-836.

Welch SP, Huffman JW & Lowe J. (1998) Differential blockade of the antinociceptive effects of centrally administered cannabinoids by SR141716A. *J Pharmacol Exp Ther* **286:**1301-1308.

White R & Hiley CR. (1998) The actions of come cannabinoid receptor ligands in the rat isolated mesenteric artery. *Br J Pharmacol* **125:**533-541.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgard M, Di Marzo V, Julius D & Högestätt ED. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400:**452-456.

Zygmunt PM, Andersson DA & Högestätt ED. (2002) Δ^9-tetrahydrocannabidiol and Cannabidiol activate capsaicin-sensitive sensory nerves via a CB_1 and CB_2 cannabinoid receptor-independent mechanism. *J Neuroscience* **22:**4720-4727.

FOOTNOTES

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Figure 1 The effects of A) 0.01% ethanol (n=6), B) 1 μM SR141716A (n=9), C) 1 μM LY320135 (n=3) and D) 1 μM SR144528 (n=3) on frequency-dependent vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS; the first acts as a control, the compound under investigation was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

Figure 2 Representative traces showing the inhibitory effect of the high affinity cannabinoid agonist WIN55,212 (1 μM) on frequency-dependent sensory neurogenic vasorelaxation in a methoxamine-preconstricted rat mesenteric bed. Electrical field stimulation (EFS 1-12 Hz, 0.1 ms, 60 V, 30 s) evoked frequency-dependent vasorelaxation. The curves are shown as; A) Control conditions, WIN55,212 was then added and two further curves were constructed, B) EFS I and C) EFS II.

Figure 3 The effects of A) 0.01 μM WIN55,212 (n=9), B) 0.1 μM WIN55,212 (n=6) and C) 1 μM WIN55,212 (n=6) on frequency-dependent vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS, the first acts as a control, WIN55,212 was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

Figure 4 The effect of A) CB_1 selective antagonist SR141716A (1 μM, n=8) and B) CB_2 selective antagonist SR144528 (1 μM, n=5), on the inhibitory actions of 0.1 μM WIN 55,212 on frequency-dependent vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS, the first acts as a control, WIN55,212 was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

Figure 5 The effects of the stereoisomer (-)WIN55,212 (1 μM, n=6) on frequency-dependent vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS, the first acts as a control, EFS control, (-) WIN55,212 was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

Figure 6 The effects of cannabinoid agonist A) 0.01 μM CP55,940 (n=6), B) 0.1 μM CP55,940 (n=6) and C) 1 μM CP55,940 (n=8) on frequency-dependent vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS, the first acts as a control, CP55,940 was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

Figure 7 The effect of A) CB_1 selective antagonist LY320135 (1 μM, n=6) and B) CB_2 selective antagonist SR144528 (1 μM, n=6), on the inhibitory actions of CP55,940 (0.1 μM) on frequency-dependent vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS, the first acts as a control, CP55,940 was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

Figure 8 The effects of A) 0.1 μM JWH-015 (n=7) and B) 1 μM JWH-015 (n=6) on frequency-dependent vasorelaxation to electrical field stimulation; and the effect of C) CB_1 selective antagonist SR141716A 1 μM (n=5) and D) CB_2 selective antagonist SR144528 1 μM (n=9) on the inhibitory actions of JWH-015 (1 μM) on frequency-dependant vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS, the first acts as a control, JWH-015 was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

Figure 9 The effects of A) 0.1 μM THC (n=6) and B) 1 μM THC (n=4) on frequency-dependent vasorelaxation to electrical field stimulation; and the effect of C) CB_1 selective antagonist SR141716A 1 μM (n=5) and D) CB_2 selective antagonist SR144528 1 μM (n=8), on the inhibitory actions of 1 μM THC on frequency-dependent vasorelaxation to electrical field stimulation in the rat isolated mesenteric arterial bed. Three consecutive curves were constructed to EFS, the first acts as a control, EFS control, THC was added to the perfusate, then EFS I and EFS II constructed. Data are presented as means±s.e.m.

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Table 1 pD_2 values of WIN55,212, CP55,940, THC and JWH-015 on the vasorelaxation to exogenous CGRP in the rat isolated mesenteric bed

Agonist	**pD_2**	**95% Confidence limits**
Control	10.5 ± 0.1	10.67-10.31
WIN55,212	10.7 ± 0.1	10.85-10.47
CP55,940	10.7 ± 0.1	10.86-10.48
THC	10.1 ± 0.1	10.35-9.88
JWH-015	10.1 ± 0.2	10.58-9.68

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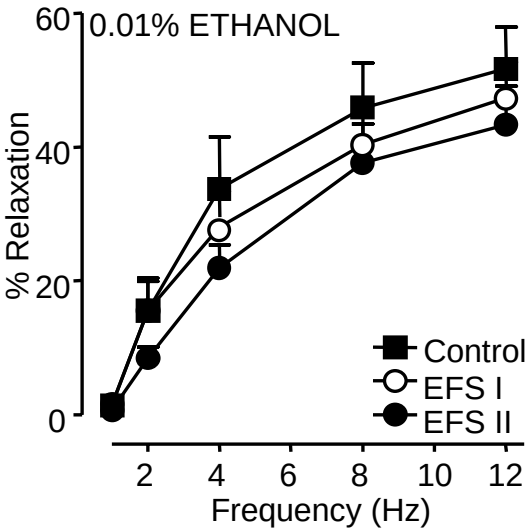
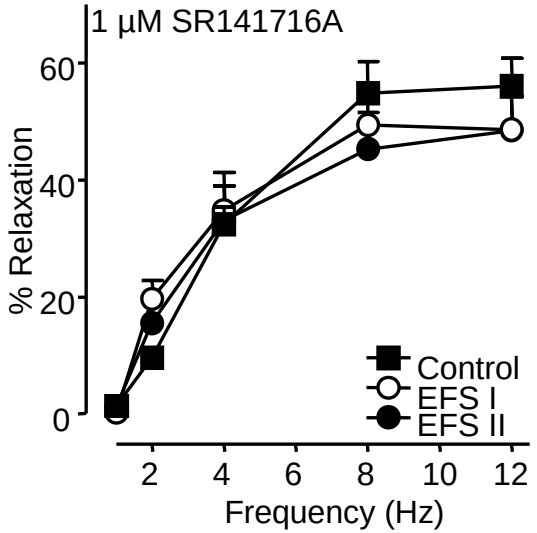
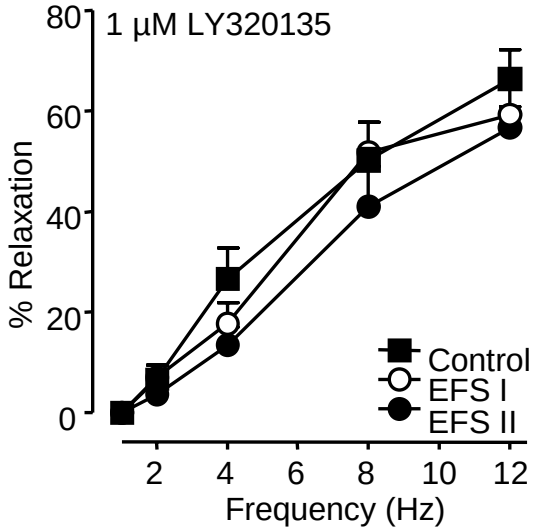
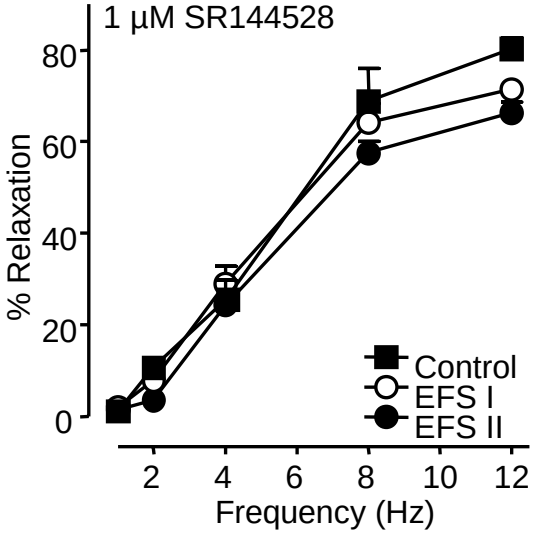
Table 2 pD_2 values of WIN55,212, CP55,940, THC and JWH-015 on the vasorelaxation to capsaicin in the rat isolated mesenteric bed

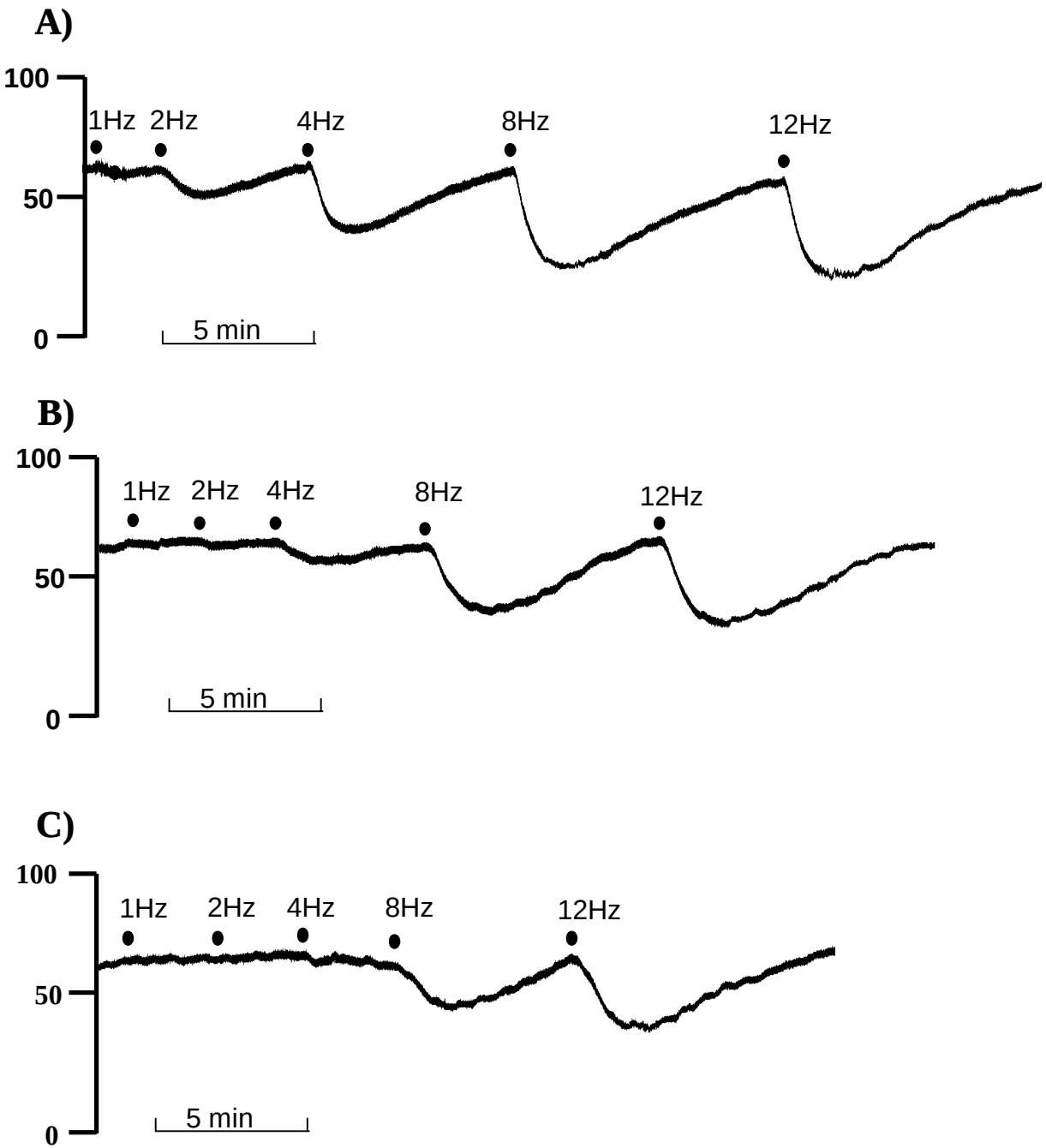
Agonist	**pD_2**	**95% Confidence limits**
Control	10.1 ± 0.2	10.51-9.7
WIN55,212	11.7 ± 0.4	12.41-10.84
CP55,940	10.4 ± 0.1	10.62-10.26
THC	10.1 ± 0.1	10.31-9.85
JWH-015	10.5 ± 0.3	11.0-9.98











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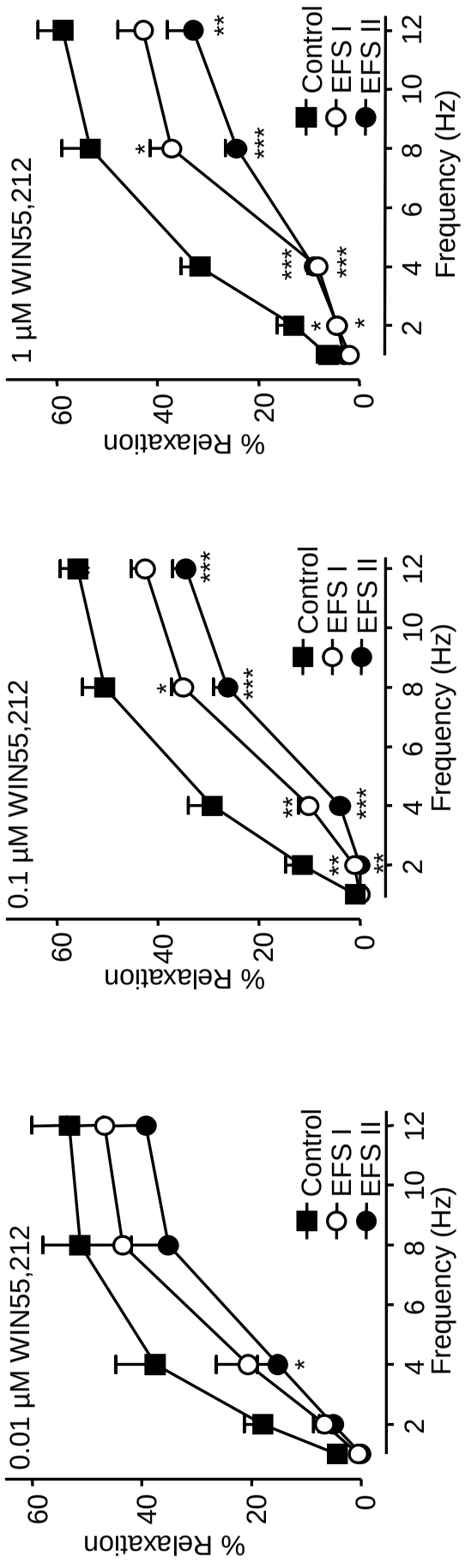

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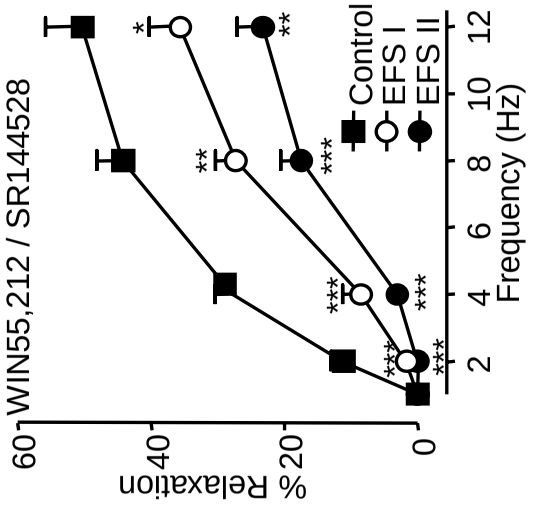

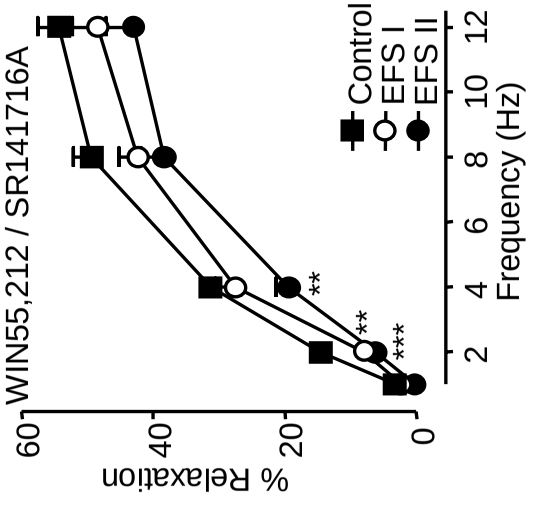

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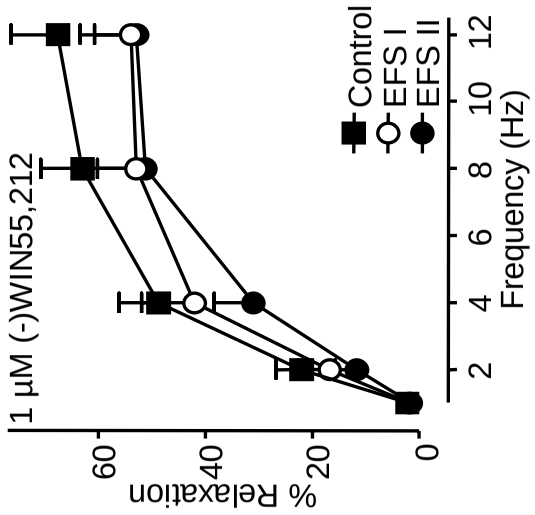

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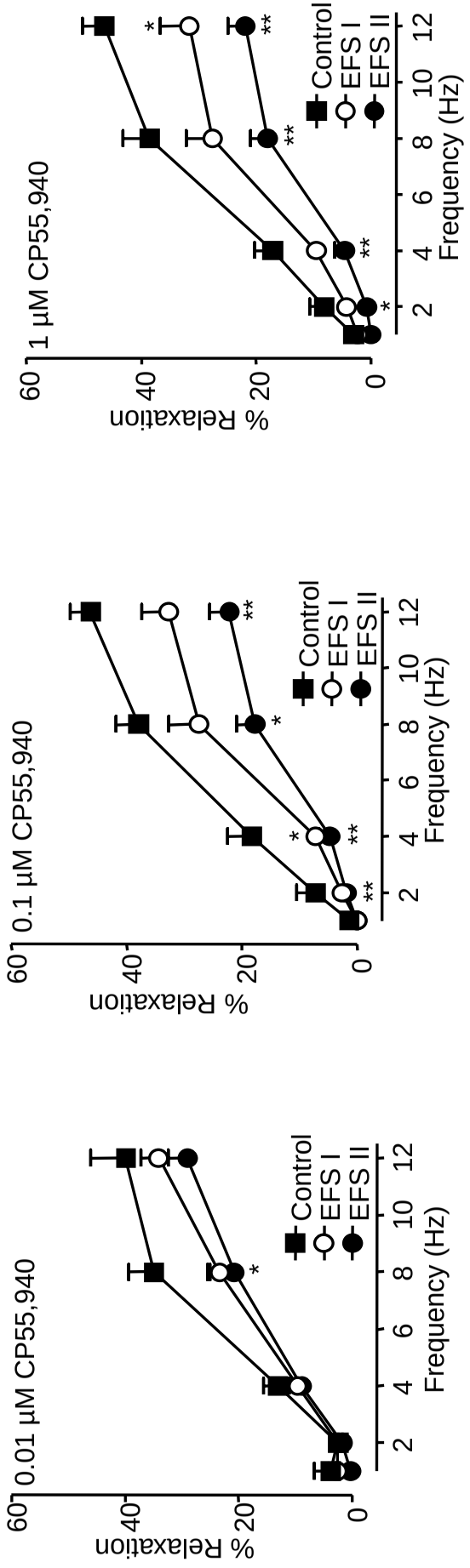

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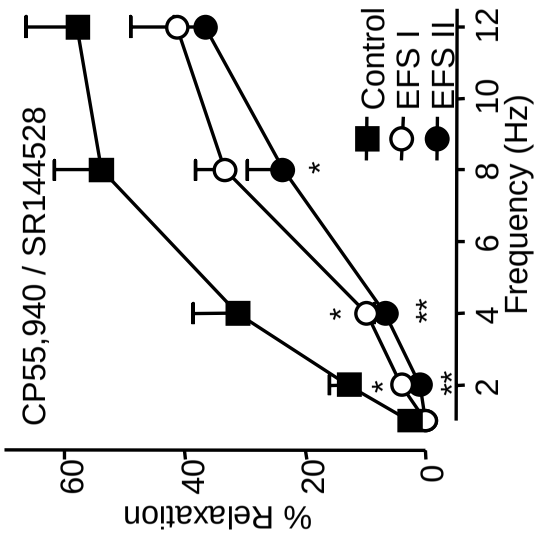

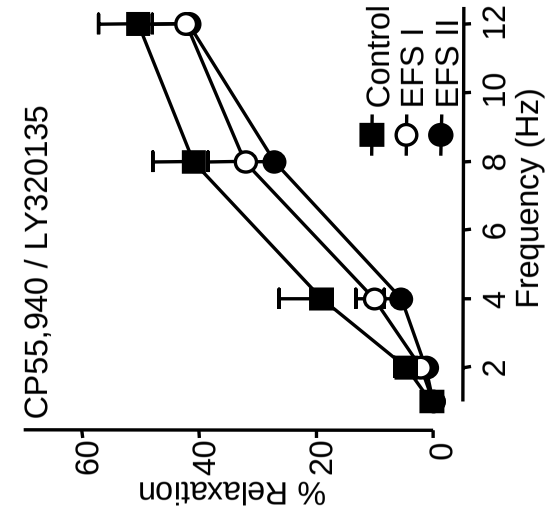

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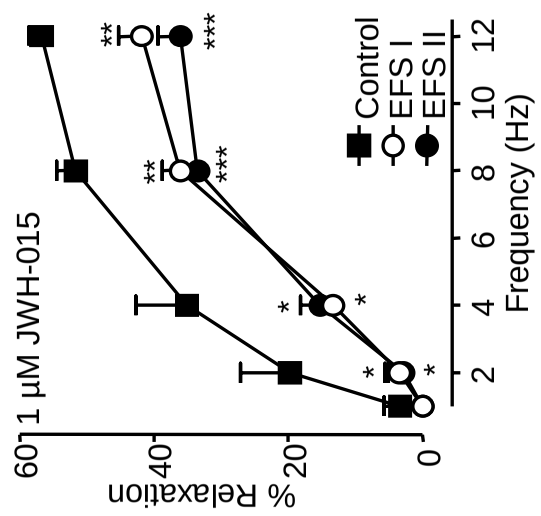

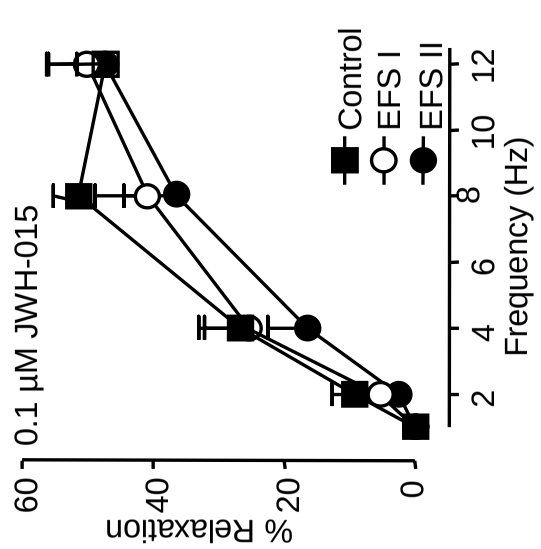

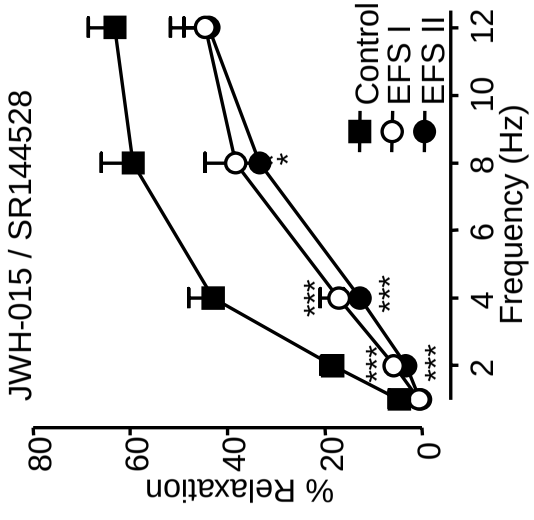

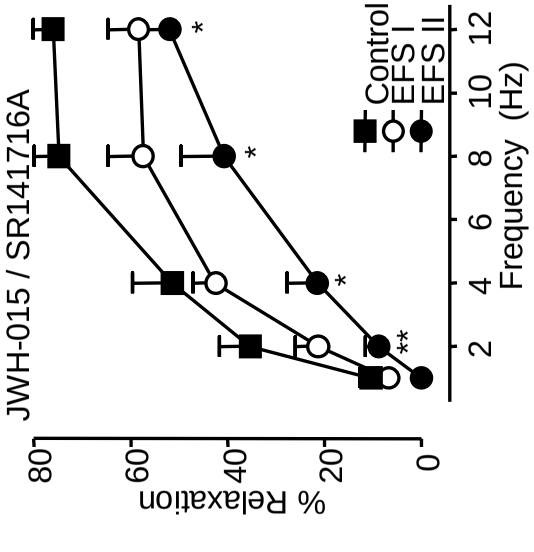

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