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RESEARCH PAPER

Pharmacological characterization of cannabinoid receptor activity in the rat-isolated ileum myenteric plexus-longitudinal muscle preparation

R Makwana[1], A Molleman[2], and ME Parsons[2]

[1]*The Sackler Institute of Pulmonary Pharmacology, King's College London, Franklin-Wilkins Building, Waterloo Campus, London, UK, and* [2]*School of Health & Human Sciences Department of Life Sciences, University of Hertfordshire, College Lane Campus, Hatfield, Hertfordshire, UK*

Background and purpose: Cannabinoid effects on intestinal transit are commonly evaluated in rats. We characterized the cannabinoid receptors mediating the inhibitory effect of 5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-phenol (CP 55,940), (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55,212-2), arachidonylethanolamide (AEA) and Δ^9-tetrahydrocannabinol (Δ^9-THC) on contractions of the rat ileum myenteric plexus-longitudinal muscle (MPLM) preparation.
Experimental approach: The interaction of each agonist was examined with the CB_1 and CB_2 receptor antagonist rimonabant and SR 144,528 respectively, on contractions elicited by electrical field stimulation (EFS) or exogenous ACh. The interaction of AEA with capsazepine, a $TRPV_1$ receptor antagonist, was also investigated.
Key results: EFS with single and trains of pulses evoked neurogenic ACh-mediated twitch and rebound contractions respectively. The rank order of potency for inhibition was CP 55,940 = WIN 55,212-2 > AEA > Δ^9-THC and AEA > WIN 55,212-2 = Δ^9-THC = CP 55,940 respectively. The stereoisomer WIN 55,212-3 was without effect. Rimonabant antagonized the inhibition of the twitches with pK_B values of around 8.60, but only antagonized rebound contractions induced by WIN 55,212-2, AEA and Δ^9-THC, with pA_2 values of around 6.80. Rimonabant increased the twitches but inhibited the rebound contractions. Contractions to exogenous ACh were not altered. These observations extended to the guinea pig ileum MPLM.
Conclusions and implications: The rat MPLM contains CB_1 receptors and at least two non-CB_1-non-CB_2-non-$TRPV_1$ receptors attenuating EFS-evoked ACh-mediated contractions in an EFS frequency-dependent pre-synaptic and stereo-specific manner. Augmentation of the twitches by rimonabant may be through antagonism of an endocannabinoid tone or inverse agonism, whereas inhibition of the rebound contractions involved partial agonism.
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Keywords: Cannabinoid; myenteric plexus-longitudinal muscle; ileum; small intestine; contractions; cannabinoid receptor agonists; cannabinoid receptor antagonist/inverse agonist; rimonabant

Abbreviations: 2-AG, 2-arachidonylglycerol; AEA, arachidonylethanolamide; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CP 55,940, 5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-phenol; EFS, electrical field stimulation; MPLM, myenteric plexus-longitudinal muscle; rimonabant, N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxyamide; pA_2, antagonist potency; pK_B, antagonist affinity; SR 144528, N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylben-zyl)-pyrazole-3-carboxamide; Δ^9-THC, Δ^9-tetrahydrocannabinol; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate; WIN 55,212-3, S-(-)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

Introduction

A large body of evidence has emerged over the last two decades from a plethora of *in vivo* studies, which have been performed almost exclusively on the mouse and rat (Pertwee, 2001), that the predominant action of psychotropic

Correspondence: Raj Makwana, The Sackler Institute of Pulmonary Pharmacology, King's College London, 5th Floor, Franklin-Wilkins Building, Waterloo Campus, London, SE1 9NH, UK. E-mail: raj.makwana@kcl.ac.uk
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cannabinoids on the intestinal tract is a reduction in the propulsive (Colombo *et al.*, 1998; Izzo *et al.*, 1999; 2001; Landi *et al.*, 2002; Mathison *et al.*, 2004; Carai *et al.*, 2006) and secretory (Shook and Burks, 1989; Izzo *et al.*, 1999; 2003) function of the small intestine. The former action has been invariably studied by evaluating either the distance travelled or the delay in the time taken for the transit of an orally or intra-duodenally administered non-absorbable marker from the pylorus to the caecum of the animal. While *in vivo* bioassays have played an important role in characterizing the pharmacology of cannabinoids on intestinal motility under both physiological and pathophysiological states, the mechanism of the depressant action has been inferred from *in vitro* studies performed on segments or strips of the isolated ileum of the guinea pig (Pertwee *et al.*, 1996; Coutts and Pertwee, 1997; Izzo *et al.*, 1998; Mang *et al.*, 2001), and, to a lesser extent, of a human (Croci *et al.*, 1998; Manara *et al.*, 1998; Guagnini *et al.*, 2006).

Therefore, to date, the guinea pig ileum has been the standard *in vitro* bioassay for screening cannabinoids on the small intestine. The *in vitro* studies on this tissue have focussed on the ability of cannabinoids to inhibit either the peristaltic reflex in segments of whole ileum (Heinemann *et al.*, 1999; Izzo *et al.*, 2000), synaptic transmission (Lopez-Redondo *et al.*, 1997), or the low frequency electrical field simulation (EFS)-evoked release of neurotransmitters (Coutts and Pertwee, 1997; Mang *et al.*, 2001; Begg *et al.*, 2002a,b) or subsequent contraction of the longitudinal (Pertwee *et al.*, 1996; Coutts and Pertwee, 1997; Mang *et al.*, 2001) or circular (Izzo *et al.*, 1998) smooth muscle layers.

Results from these studies have suggested that psychotropic cannabinoids decrease intestinal motility by reducing the release of contractile neurotransmitters, such as acetylcholine (ACh) from the myenteric plexus (Coutts and Pertwee, 1997; Mang *et al.*, 2001), through an activation of the cannabinoid CB_1 receptors (nomenclature follows Alexander *et al.*, 2008) located on the somatodendritic and terminal regions of the neurones (Coutts *et al.*, 2002). Although the guinea pig ileum has long served as a useful bioassay for describing the inhibitory action of cannabinoids on intestinal transit observed in the mouse or rat *in vivo*, it is important that isolated ileal tissues from the latter species are used for studying cannabinoid effects. First, because a large number of models of disturbed intestinal motility of man have been created using the rat and mouse (Izzo *et al.*, 1999; 2000; 2001; 2003; Mascolo *et al.*, 2002; Mathison *et al.*, 2004), and second, to date, the guinea pig has not been employed for investigating cannabinoid effects on ileal transit *in vivo*.

The availability of an *in vitro* ileal bioassay from the rat and mouse would not only enable a better comparison to be made between the data obtained from either types of studies, but may offer the opportunity to understand more about the mode of action of existing and future cannabinoid drugs on ileal motility under both physiological and pathophysiological conditions. Additionally, the existence of possible novel cannabinoid receptor subtypes or targets may be revealed.

Previous studies have reported a slightly higher conservation between the mRNAs of the rat and human cannabinoid receptors compared with those of the mouse and human (Chakrabarti *et al.*, 1995; Shire *et al.*, 1996). In the rat ileum, the mRNAs of both cannabinoid receptors and their expression have been both identified and mapped in the myenteric plexus (Coutts *et al.*, 2002; Valenti *et al.*, 2005; Duncan *et al.*, 2008). Furthermore, a number of endogenous cannabinoid ligands, exemplified by arachidonylethanolamide (AEA) and 2-arachidonylglycerol (2-AG), (Gomez *et al.*, 2002; Mascolo *et al.*, 2002; Izzo *et al.*, 2003; Fegley *et al.*, 2005; Valenti *et al.*, 2005), along with the mechanisms for their enzymatic inactivation (Katayama *et al.*, 1997), have been shown to be present in the rat ileum. Therefore, the purpose of this study was to investigate whether the rat ileum myenteric plexus-longitudinal muscle (MPLM) preparation served as a suitable and robust *in vitro* ileal cannabinoid receptor bioassay by assessing the interaction between representatives of the four main classes of cannabinoid receptor agonists, that is AEA, 5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-phenol (CP 55,940), Δ^9-tetrahydrocannabinol (Δ^9-THC) and (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55,212-2), with the CB_1 and CB_2 receptor selective antagonist /inverse agonists rimonabant and SR 144 528 respectively. To aid in the interpretation of data obtained on the rat ileum MPLM, some experiments were performed on the guinea pig ileum MPLM preparation.

Preliminary accounts of some of the data have been communicated to both the British Pharmacological Society (Makwana *et al.*, 2004) and the International Cannabinoid Research Society (Makwana *et al.*, 2006).

Methods

Tissue preparation

All animal care and experimental procedures were in accordance with requirements of the Animals (Scientific Procedures) Act 1986 and the University of Hertfordshire ethics committee. Male Wistar rats (400–550 g) and Dunkin-Hartley or Heston-2 guinea pigs of either sex (500–800 g) were used. All animals were bred at the Biological Services Unit of the University of Hertfordshire, Hatfield, UK, from stock originating at Charles River Laboratories (Margate, Kent, UK) and Harlan UK (Bicester, Oxford, UK) respectively. The animals were housed in rooms with a controlled temperature (22 ± 1°C), humidity (55 ± 10%) and 12-h light-dark cycle. Food and water were available *ad libitum*. Rats were killed by carbon dioxide (CO_2) asphyxiation, while guinea pigs were killed by cervical dislocation followed by exsanguination.

A 15 cm ileum segment was excised from the small intestine of each animal and immersed in Krebs-Henseleit solution [composition (in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5], gassed with 95% O_2 and 5% CO_2 at room temperature (21 ± 4°C). A maximum of four MPLM strips were dissected from the ileal segment by the method of Paton and Zar (1968) after discarding the first 5 cm length closest to the ileo–caecal junction. Briefly, a 2 cm length of the ileum was flushed of its contents with Krebs-Henseleit solution and slipped over a glass rod of 5 mm diameter. After trimming the mesentery, the longitudinal muscle with the adherent myenteric plexus was separated

from the underlying circular muscle layer by gentle stroking with a cotton bud soaked with Krebs-Henseleit solution, starting at the mesenteric border and working along and around the circumference of whole ileum. Cotton sutures were tied at both ends of the MPLM strip, and the tissue was suspended in a 30 mL organ bath containing Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2 at 33 or 37°C. At the beginning of the study, five experiments examining the action of CP 55,940 and WIN 55,212-2 on the rebound contractions of the rat MPLM were performed at 37°C. However, because of the irregular spontaneous activity of the tissues, and therefore difficulties experienced in quantifying the evoked responses, subsequent experiments were carried out at 33°C in order to reduce the spontaneous activity. Four experiments using CP 55,940 and WIN 55,212-2 to inhibit the rebound contractions of the rat MPLM at 33°C showed that running the experiments at this temperature had no significant effect on the ability of the tissues to respond to EFS or either drug. Therefore, data from experiments carried out at the different bath temperatures have been pooled.

Changes in tension (as mN) of the MPLM strips was recorded isometrically using Dynamometer UF1 force transducers (Pioden Controls, Newport, Isle of White, UK) connected to either a MacLab Chart Version 3.5 data-acquisition system (AD Instruments, Chalgrove, Oxford, UK) on an Apple Macintosh computer (Apple Macintosh, UK, www.apple.com/uk) or MX216 or MultiTrace-4 chart recorders (Lectromed, Letchworth, Hertfordshire, UK)

EFS Experiments

Each MPLM strip was placed between either a pair of platinum ring (circumference 5 mm and 5 mm apart) or prong (10 mm length and 5 mm apart) electrodes connected to Grass S11 or S88 stimulators (Grass Instruments, Slough, Berkshire, UK) or a Multistim D330 stimulator (Digitimer, Welwyn Garden City, Hertfordshire, UK) via a variable cycle timer (Harvard Apparatus, Edenbridge, Kent, UK). After placing the tissue in the organ bath for 10 min, the Krebs-Henseleit solution was renewed once, and each tissue was stretched by 0.5 mN and allowed to equilibrate for a further 50 min until EFS was commenced. The rat and guinea pig MPLM strips were subjected to EFS over the entire duration of the experiment either with single pulses at a frequency of 0.05 or 0.1 Hz, respectively, or with trains of pulses for 2 s every minute at 30 Hz frequency. Each pulse at any given frequency was of 0.5 ms duration and at a voltage that was 10% greater than that required to elicit maximal contractions. Supramaximal voltage was employed to ensure that the neurones sensitive to the respective frequency within the field of stimulus were stimulated, and that the EFS conditions were comparable with previous studies (Pertwee *et al*., 1996; Coutts and Pertwee, 1997; Mang *et al*., 2001; Borelli *et al*., 2004).

No drugs were added until the amplitude of EFS-evoked contractions had become consistent for a minimum of a 30 min period. The time taken for the amplitude of the contractions to become uniform was about 3 to 4 h. It was also noted that the amplitude of the EFS-evoked responses could remain stable for a further 4 h without the need to renew the bathing Krebs-Henseleit solution. On each day, the drug treatments were randomized between the organ baths. Cumulative concentration-response curves were constructed to the cannabinoid ligands with a 20 or 30 min dosing interval on tissues stimulated with 0.05 or 0.1 and 30 Hz respectively. Only one concentration-response curve was constructed per tissue as previous studies have shown that cannabinoids cannot be washed from the tissue by replenishing the bath with drug-free Krebs-Henseleit solution (Pertwee *et al*., 1992). When competition studies were performed, the cannabinoid receptor antagonists/inverse agonists were administered 30 min prior to the addition of the cannabinoid receptor agonists. All experiments were performed in parallel with relevant vehicle treated and time controls.

ACh stimulation experiments

If a cannabinoid was found to alter the amplitude of EFS-evoked contractions, its ability to produce a similar effect on contractions elicited by exogenously applied ACh was investigated. Two experimental designs were used for carrying out these studies. The first involved administering the highest concentration of the cannabinoid, which maximally increased or decreased the amplitude of EFS-evoked contractions between two ACh cumulative concentration-response curves (10^{-10} to 10^{-5} M) constructed 30 min apart. The second experimental design involved administering a single concentration of a cannabinoid in half-logarithmic unit increments between single administrations of ACh (10^{-6} M) over 30 min intervals on a single piece of tissue. For both experimental designs, each tissue acted as its own control. All experiments were performed in parallel with relevant vehicle treated and time controls.

Data analysis

The inhibition of the EFS-evoked contractions by a cannabinoid receptor agonist was quantified in percentage terms by calculating the reduction in the amplitude of the contractions by each addition of the agonist from the amplitude immediately before the first addition of the competing ligand or its vehicle.

During some competition experiments, depending on the tissue and/or frequency of EFS employed, the presence of certain competing ligands alone either enhanced or reduced the amplitude of the EFS-evoked contractions. Therefore, the enhancement and reduction of the contractions was quantified in percentage terms by calculating the change in amplitude after each addition of the ligand from the amplitude immediately before the first addition of the same ligand.

Individual agonist concentration-response curves in the absence and presence of a competing ligand were fitted by non-linear regression to the four-parameter Hill equation (equation 1) using GraphPad PRISM 4.0 for Windows (GraphPad Software, La Jolla, CA, USA), where E denotes response, log [A] the logarithm of the concentration of an agonist A, n_H the midpoint slope of the curve, log EC_{50} the logarithm of the midpoint location parameter along the concentration axis, and E_{max} and *Basal* the upper and lower asymptotes respectively. The concentration-response data were plotted as the mean ± standard error of the mean (mean ± SEM).

$$E = Basal + \frac{E_{max} - Basal}{1 + 10^{(\log EC_{50} - \log[A])^{nH}}} \quad (1)$$

Because a significant change in basal EFS-evoked contractions was observed in the presence of some competing ligands (see Results), the concentration ratio for the rightward shift of the agonist concentration-response curve in the presence of these ligands was determined as the ratio of concentrations corresponding to the 50% equieffective agonist response level of the curves. When a series of concentration ratio values were available from experiments in which multiple concentrations of the antagonist were used, a Schild plot (Arunlakshana & Schild, 1959) was constructed to obtain an estimate of the antagonist's affinity from the negative logarithm of the equilibrium dissociation constant (pK_B) of the antagonist-receptor complex. The pK_B represented the negative logarithm of the antagonist concentration that occupied 50% of the receptors at equilibrium.

If the line of best fit of the plot had a slope of unity, the antagonism was taken as being simple competitive. However, if the slope was not equal to unity, but the 95% confidence intervals (CI) of the slope included unity, the regression was re-fitted to a unit slope to determine the pK_B of the antagonist. When a single value for the concentration ratio was available from experiments in which only a single concentration of the antagonist was used, the antagonist potency (pA_2) was calculated from the Gaddum–Schild equation (Schild, 1949). The pA_2 represents the negative logarithm of the concentration of the antagonist, which produces a shift of the agonist concentration-response curve to the right by two linear units to give a concentration ratio of two.

The amplitude of the contractions to cumulative additions of ACh in the presence of a cannabinoid receptor ligand or its vehicle was expressed as a percentage of the maximal contraction to ACh (10^{-5} M) from the initial ACh concentration-response curve constructed on each tissue. Where appropriate, shifts of a concentration-response curve by the presence of the competing ligand were compared by a one-way ANOVA followed by a Dunnett's *post hoc* test for multiple comparisons or a Student's unpaired *t*-test for comparisons of individual means. The probability $P < 0.05$ was taken to be statistically significant.

Materials

ACh (acetylcholine chloride), atropine sulphate, hexamethonium bromide, L-NAME (N_ω-nitro-L-arginine methyl ester hydrochloride), Δ^9-THC, and WIN 55,212-3 were purchased from Sigma-Aldrich, Poole, Dorset, UK. AEA (anandamide, arachidonylethanolamide), capsazepine, TTX (tetrodotoxin) and WIN 55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) were purchased from Tocris Biosciences, Avon, Bristol, UK. CP 55,940 was a kind gift from Pfizer, Sandwich, Kent, UK, whereas rimonabant (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxyamide) and SR 144528 (N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylben-zyl)-pyrazole-3-carboxamide) were generous gifts from Sanofi-Recherché (Montpellier, France). All other chemicals were purchased from Fisher Scientific UK (Loughborough, Leicestershire, UK). Atropine, hexamethonium, nicotine and TTX were dissolved in distilled water. WIN 55,212-3 was dissolved in 100% dimethylsulphoxide. All other drugs were prepared in 100% ethanol. The total volume of the solvents added to the organ baths did not exceed 1% of the bath volume. The presence of the solvents did not have a significant effect on the evoked responses of the tissues.

Results

Responses of the rat and guinea pig MPLM to EFS

On tissues from both species, EFS with single pulses at 0.05 Hz for the rat MPLM and 0.1 Hz for the guinea pig MPLM elicited a transient twitch contraction during each pulse (Figure 1A).

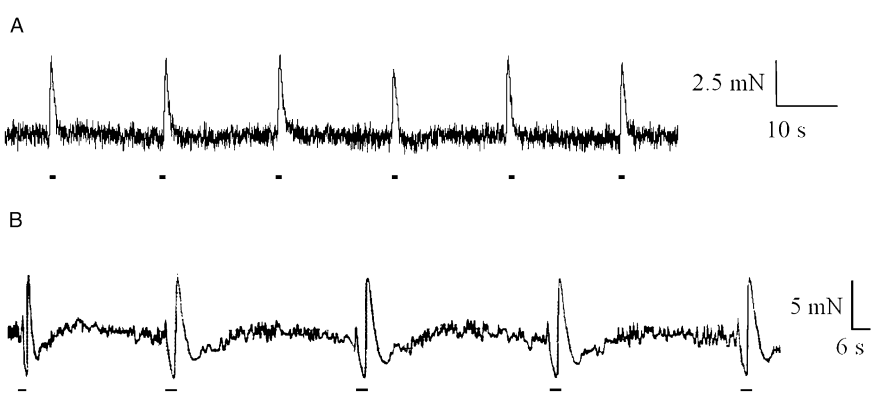

Figure 1 Representative traces of the electrical field stimulation (EFS)-evoked responses of the rat ileum myenteric plexus-longitudinal muscle. (A) Twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110% supramaximal voltage. Each upward deflection represents a contraction. Upward deflections represent the contractions. (B) Biphasic responses to EFS at 30 Hz for 2 s every min, 0.5 ms duration and 110% supramaximal voltage. Note the transient relaxations during the 2 s stimulation periods and the subsequent rebound contractions. The downward deflections denote relaxations, while upward deflections represent contractions. In both traces, the short bars below the records represent the time at which EFS was applied.

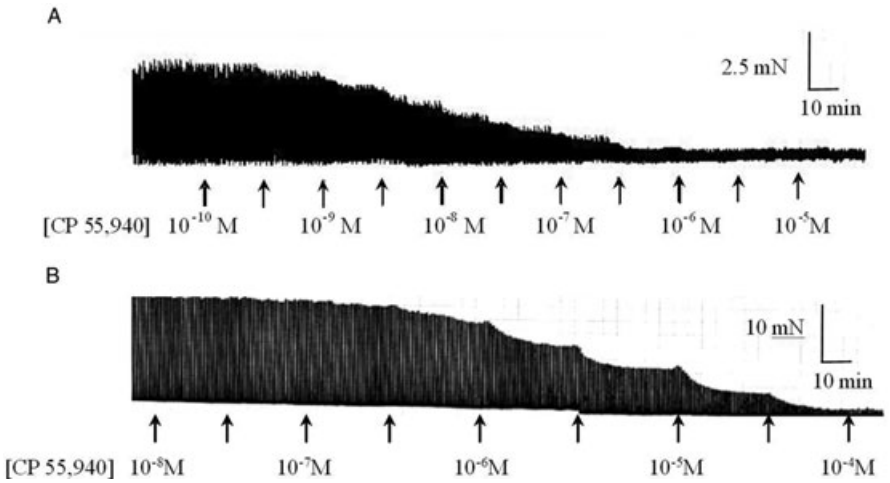

Figure 2 Representative traces of the electrical field stimulation (EFS)-evoked contractions of the rat myenteric plexus-longitudinal muscle in the absence and presence of CP 55,940 added in half-logarithmic unit increments. Unlabelled arrows represent intermediate concentrations. (A) Trace illustrating the twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110% supramaximal voltage in the absence and presence CP 55,940 (B) Trace illustrating the rebound contractions to EFS at 30 Hz for 2 s every min, 0.5 ms duration and 110% supramaximal voltage in the absence and presence of CP 55,940.

In contrast, EFS with 2 s trains of pulses at 30 Hz every minute evoked a transient rebound contraction immediately on termination of the train of pulses (Figure 1B). In contrast to the guinea pig MPLM, each rebound contraction of the rat MPLM was preceded by a transient relaxation during the train of pulses. However, the amplitude of the relaxations of the rat MPLM diminished over the first 20 min of stimulation, as the basal tension of the tissues decreased from 5 mN to about 2 mN. By contrast, the amplitude of the rebound contractions gradually increased during this period and continued to increase for up to 3 to 4 h. The amplitude of the twitch and rebound contractions before the addition of any cannabinoid drugs was 4.41 ± 1.27 mN (n = 100) and 13.93 ± 3.33 g (n = 100) on the rat MPLM, respectively, and 15.89 ± 7.35 mN (n = 60) and 32.46 ± 4.71 mN (n = 60) on the guinea pig MPLM respectively.

The responses of both tissues to all EFS parameters were abolished immediately by treatment with the voltage gated Na^+ channel blocker TTX (10^{-6} M, data not shown, both n = 6). The EFS-evoked contractions of both tissues were abolished by treatment with the muscarinic ACh receptor antagonist atropine (10^{-6} M, data not shown, both n = 6), whereas the relaxant response of the rat MPLM during the trains of pulses 30 Hz EFS was abolished by treatment with the nitric oxide synthase inhibitor L-NAME (10^{-4} M, data not shown, n = 6). In the presence of L-NAME, the rebound contractions of the rat MPLM continued to occur at the end of the trains of pulses. The presence of L-NAME had no effect on the amplitude of the subsequent rebound contractions. The nicotinic ACh receptor antagonist hexamethonium (10^{-4} M) had no effect on the responses of the MPLM tissues under either EFS condition (data not shown, both n = 6).

Effect of the cannabinoid receptor agonists on the EFS-evoked contractions of the rat MPLM

Cumulative additions of AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 resulted in a concentration-related inhibition of both the EFS-evoked twitch and rebound contractions of the rat MPLM (Figures 3 and 5). Figure 2 shows representative traces of both types of the contractions in the absence and presence of CP 55,940. Although all four agonists caused a similar maximal inhibition of both types of the contractions (Figures 3 and 5, Table 1), a comparison of the pEC_{50} values of each agonist under the two EFS conditions indicated that each agonist was a significantly ($P < 0.05$, unpaired t-test) more potent inhibitor of the twitch contractions than of the rebound contractions (Table 1). A comparison between the rank orders of the pEC_{50} values of the four agonists on tissues subjected to the two EFS conditions showed no correlation. The rank order of the pEC_{50} values of the agonists for the inhibition of the twitch and rebound contractions was CP 55,940 = WIN 55,212-2 > AEA > Δ^9-THC and AEA > CP 55,940 = Δ^9-THC = WIN 55,212-2 respectively. Table 1 shows that the pEC_{50} value of CP 55,940 and WIN 55,212-2 was reduced by around one logarithmic unit greater than the pEC_{50} value of AEA and Δ^9-THC as a result of the increase in the frequency of EFS.

The rate of onset of the inhibition of both types of contractions by each agonist was both slow and dependent on the frequency of EFS employed. The maximal inhibition of the twitch and rebound contractions at each concentration of an agonist was achieved within 20 and 30 min of administration respectively. Table 1 also lists the time taken ($t_{1/2}$) for all four agonists to inhibit the twitch and rebound contractions by 50% of the maximum at 10^{-6} M and 10^{-4} M respectively. Despite the differences in the rank orders of the pEC_{50} values of the four agonists under the two EFS conditions, the rank orders of rate of onset of the inhibition of the contractions were similar, that is WIN 55,212-2 > CP 55,940 > Δ^9-THC > AEA.

Unlike WIN 55,212-2, its stereoisomer WIN 55,212-3 was a significantly ($P < 0.05$ unpaired t-test) less efficacious inhibitor of both types of the EFS-evoked contractions over the same concentration range (Table 1). The relaxations of the rat MPLM preceding the rebound contractions were not altered

Table 1 A comparison of the potency (pEC_{50}), tissue maximal response (E_{max}) and potency ratios of the various cannabinoid receptor agonists for the inhibition of the EFS-evoked contractions of the rat ileum MPLM

Cannabinoid agonist	$pEC_{50\ (0.05\ Hz)}$	$E_{max\ (0.05\ Hz)}$ (%)	$pEC_{50\ (30\ Hz)}$	$E_{max\ (30\ Hz)}$ (%)	$log\left[\frac{EC_{50\ (30\ Hz)}}{EC_{50\ (0.05\ Hz)}}\right]$	$t_{1/2\ (0.05\ Hz)}$ (min)	$t_{1/2\ (30\ Hz)}$ (min)
AEA	7.91 ± 0.02	87.28 ± 3.45	5.90 ± 0.03*	91.59 ± 3.48	2.0	9 ± 2	12 ± 1
CP 55,940	8.42 ± 0.02	96.64 ± 3.32	5.37 ± 0.04*	90.77 ± 1.63	3.0	5 ± 1	9 ± 2
WIN 55,212-2	8.31 ± 0.04	97.25 ± 1.12	5.49 ± 0.01*	92.10 ± 1.36	2.8	4 ± 1	7 ± 1
Δ^9-THC	7.56 ± 0.02	95.78 ± 7.88	5.47 ± 0.01*	88.24 ± 7.84	2.1	7 ± 1	11 ± 1
WIN 55,212-3	7.45 ± 0.04	4.02 ± 2.12#	6.03 ± 0.12	8.65 ± 3.53#	1.4	ND	ND

Agonist pEC_{50}, E_{max} values were derived by non-linear regression analysis for tissues stimulated electrically with either single pulses of 0.5 ms duration, 110% supramaximal voltage at 0.05 Hz frequency or 2 s trains of pulses of 0.5 ms duration and 110% supramaximal voltage at a frequency of 30 Hz every minu The $t_{1/2\ (0.05\ Hz)}$ and $t_{1/2\ (30\ Hz)}$ values represent the time taken in min for 50% of maximal inhibition of the contractions evoked by 0.05 and 30 Hz using 10^{-6} and 10^{-4} M of the agonists respectively. ND represents not determined. Values are represented as mean ± SEM, $n \geq 6$.
*Represents a significantly ($P < 0.05$, unpaired *t*-test) lower pEC_{50} compared with pEC_{50} of the same agonist in tissues stimulated at 0.05 Hz.
#Represents a significantly ($P < 0.05$, unpaired *t*-test) lower E_{max} compared to the E_{max} of WIN 55,212-2 on tissues under similar EFS conditions.
EFS, electrical field stimulation; MPLM. myenteric plexus-longitudinal muscle.

by the presence of the cannabinoid agonists at 10^{-6} M (data not shown, each $n = 4$).

Effect of the cannabinoid agonists on the EFS-evoked contractions of the rat MPLM in the presence of rimonabant, SR 144528 or capsazepine

The presence of increasing concentrations of rimonabant (10^{-8} to 10^{-6} M) caused significant ($P < 0.05$, ANOVA and Dunnett's test), progressive degrees of rightward shift of the AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 concentration-response curves with no significant ($P < 0.05$, paired *t*-test) effect on the E_{max} of each agonist (Figures 3 and 5). A sum-of-squares *F*-test on the Hill slope parameters of each family of agonist concentration-response curves in the absence and presence of rimonabant indicated that the slope of each curve was not significantly ($P > 0.05$) different from unity. Hence, the rightward shifts of the agonist concentration-response curves caused by rimonabant were considered parallel. A sum-of-squares *F*-test on the E_{max} parameter of the agonist concentration-response curves in the absence and presence of rimonabant showed no significant ($P > 0.05$) difference; therefore, each family of curves was re-fitted to equation (1) with the E_{max} shared between each data set to a common value. Figure 3 represents the agonist concentration-response curves in the absence and presence of rimonabant after constraining the E_{max} to be shared between each family of curves for all four agonists.

Schild analysis (Figure 4) of the rightward displacements of the concentration-response curves of each agonist by rimonabant (Figure 3) yielded the estimates of the pK_B value and slope shown in Table 2. In all instances, the slope of the Schild plots were significantly ($P < 0.05$, unpaired *t*-test) less than unity. But the estimates of the pK_B value obtained from these slopes were similar irrespective of the agonist that was used. Because the experimentally determined concentration ratios represented a sample from the complete population of the infinite concentration ratios that could be determined using infinite concentrations of rimonabant, the 95% CI of the slope of each Schild analysis was calculated to investigate whether random sample variation may have produced the non-unit slopes, and if the sample data came from the population describing simple competitive antagonism, that is unit slope. As shown in Table 2, the 95% a CI value of the slopes of each Schild analysis contained unity. Therefore, each Schild plot was re-plotted by constraining the slope to unity to estimate the pK_B value of rimonabant with the assumption that the interaction between rimonabant and each of the agonists was simple competitive, and that random variation caused the deviation from unit slope. The estimates of the pK_B value of rimonabant obtained before and after constraining the slopes of the Schild plots to unity are shown in Table 2. The estimates of the pK_B value of rimonabant yielded from the Schild plots with the unit slopes were slightly higher than those obtained from the non-unit slopes. Nonetheless, the pK_B values of rimonabant obtained were similar irrespective of the agonist that was used.

The concentrations of each agonist required to inhibit the rebound contractions were significantly higher than those necessary for inhibiting the twitch contractions. Additionally, it was impossible to prepare very highly concentrated stock solutions of each cannabinoid agonist. Therefore, the pA_2 values of rimonabant were calculated using the Gaddum–Schild equation following incubation of a single concentration of 10^{-6} M. Rimonabant (10^{-6} M) caused small but significant ($P < 0.05$, ANOVA and Dunnett's test) rightward displacements of the concentration-response curves of AEA, Δ^9-THC and WIN 55,212-2, but not CP 55,940 (Figure 5). As shown in Table 2, the estimates of the pA_2 values of rimonabant for the antagonism of WIN 55,212-2, AEA and Δ^9-THC were similar against these three agonists, but lower than the pK_B value obtained in the twitch contraction experiments.

In addition to its antagonist activity, the presence of rimonabant alone caused a significant ($P < 0.05$, paired *t*-test) concentration-dependent augmentation of the amplitude of the EFS-evoked twitch contractions. The magnitude of the augmentation of the twitch contractions can be seen in Figure 3 as the progressive concentration-dependent downward shift of the bottom asymptote of each agonist concentration-response curve in the presence rimonabant. Figure 6 shows a concentration-response curve for the augmentation of the twitch contractions by rimonabant (10^{-8} to 10^{-5} M). The amplitude of the contractions in the presence of rimonabant at 10^{-6} and 10^{-5} M were significantly ($P < 0.05$,

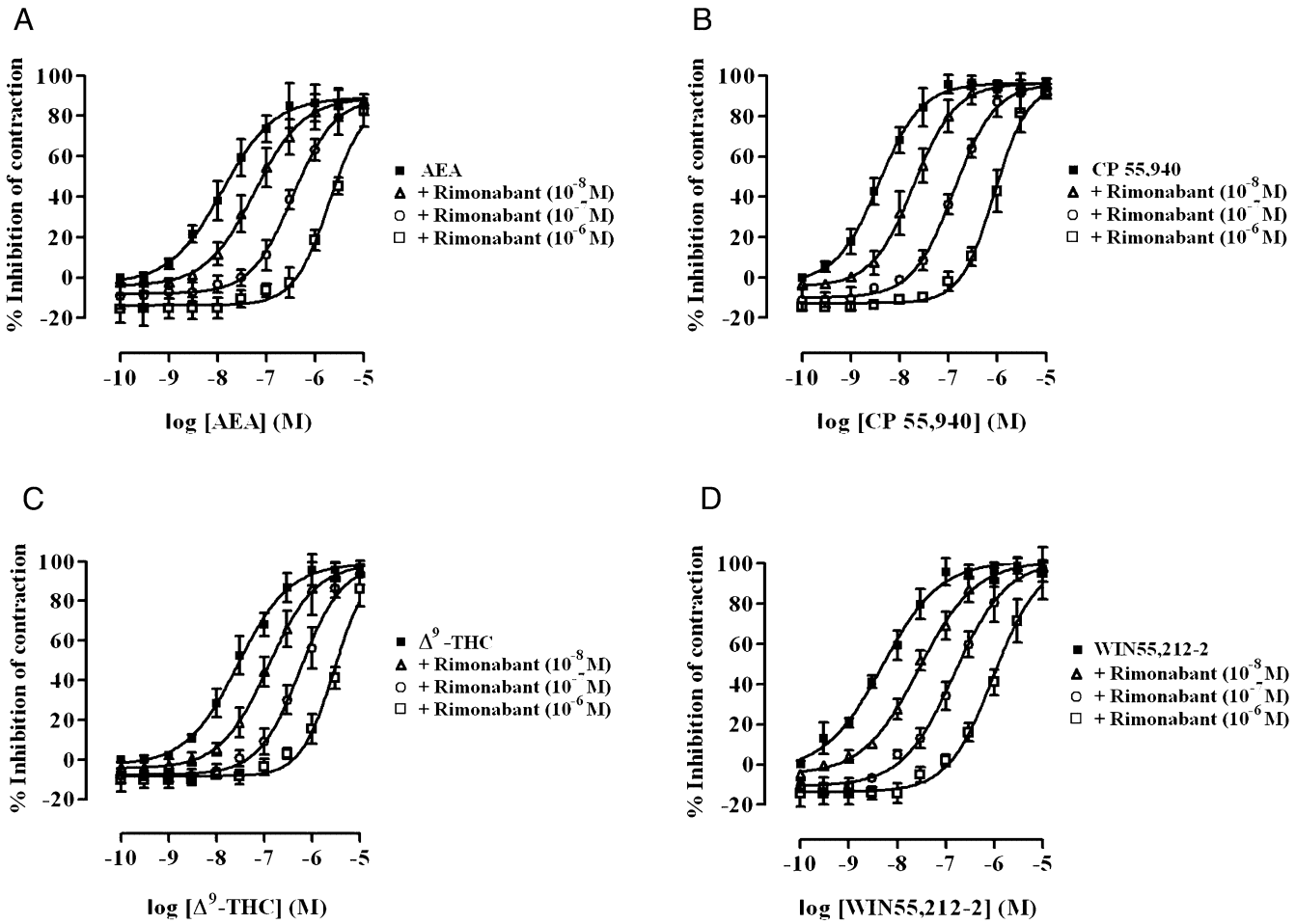

Figure 3 Concentration-response curves for the inhibition of the electrical field stimulation (EFS) (0.05 Hz frequency, 0.5 ms duration, 110% supramaximal voltage) evoked twitch contractions of the rat ileum myenteric plexus-longitudinal muscle by (A) AEA, (B) CP 55,940, (C) Δ^9-THC and (D) WIN 55,212-2 constructed in the presence of vehicle or rimonabant (10^{-8} M, 10^{-7} M or 10^{-6} M). Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the twitch response measured immediately before the addition of any drug to the organ bath. Vertical lines indicate SEM, $n = 6$ for each curve. Rimonabant or vehicle (ethanol, <1% final concentration) was added 20 min before the first addition of an agonist.

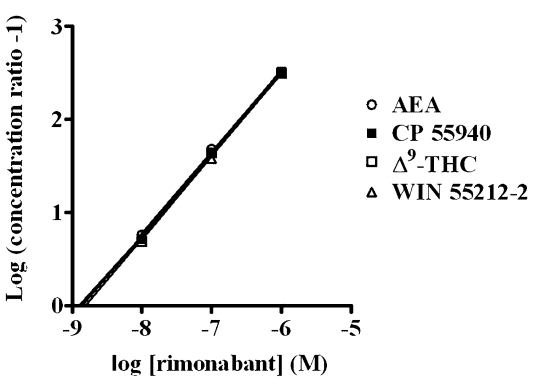

Figure 4 Schild plot for rimonabant for the antagonism of the inhibition of the electrical field stimulation (EFS) (0.05 Hz frequency, 0.5 ms duration, 110% supramaximal voltage)-evoked twitch contractions of the rat ileum myenteric plexus-longitudinal muscle by AEA, CP 55,940, Δ^9-THC and WIN 55,212-2. The concentration ratios were calculated using the agonist concentrations corresponding to the 50% equieffective inhibition level in the absence and presence of rimonabant (10^{-8} to 10^{-6} M). The intersection of the slope with the abscissa where log (concentration ratio – 1) = 0 represents the pK_B value of rimonabant against each of the four agonists.

paired *t*-test) larger than those in the presence of its vehicle. The maximum potentiation of the contractions at 10^{-5} M corresponded to an increase of 12.45 ± 4.1% (n = 6). The pEC_{50} value of rimonabant from this data was 7.52 ± 0.02. In contrast to the augmentation of the twitch contractions, rimonabant alone significantly ($P < 0.05$, paired *t*-test) inhibited the EFS-evoked rebound contractions. The inhibition of the rebound contractions can be seen in Figure 5 as the elevation of the bottom asymptote of the agonist concentration-response curves in the presence of rimonabant (10^{-6} M). Higher concentrations of rimonabant were not used for antagonism studies because this cannabinoid produced a further concentration-related inhibition of the rebound contractions (Figure 6). The inhibition of the contractions caused by rimonabant at 10^{-6} M was 28.3 ± 4.4% (n = 9), whereas that at the maximal concentration used, that is 10^{-5} M, was 76.1 ± 3.7% (n = 9). The pEC_{50} value of rimonabant from this data was 5.70 ± 0.01. The maximal effect at each concentration of rimonabant was achieved within 20 and 30 min of administration on tissues stimulated at 0.05 and 30 Hz respectively (data not shown, each n = 6).

Table 2 A comparison of the estimates of the pK_B and pA_2 values of rimonabant against AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 for the inhibition of the EFS-evoked contractions of the rat ileum MPLM

Cannabinoid receptor agonist	*Experimentally fitted slope*	*Constrained slope*	*pK_B from the experimentally fitted slope*	*pK_B from the constrained slope*	*pA_2*
AEA	0.88 ± 0.03* (0.55–1.21)	1.00	7.78 ± 0.18 (5.45–10.1)	8.65 ± 0.07 (8.33–8.97)	6.91
CP 55,940	0.91 ± 0.02* (0.65–1.16)	1.00	7.96 ± 0.14 (6.15–9.77)	8.63 ± 0.06 (8.39–8.87)	–
Δ^9-THC	0.90 ± 0.03* (0.53–1.27)	1.00	7.91 ± 0.20 (5.32–10.5)	8.60 ± 0.06 (8.35–8.87)	6.83
WIN 55,212-2	0.88 ± 0.03* (0.60–1.18)	1.00	7.78 ± 0.16 (5.77–9.90)	8.61 ± 0.06 (8.33–8.89)	6.62

The experimentally fitted slope column represents the slopes of the Schild plots of rimonabant obtained from a line of best fit for the antagonism of the four cannabinoid receptor agonists in tissues subjected to EFS at 0.05 Hz frequency, 0.5 ms duration, 110% supramaximal voltage. The constrained slope column represents the unit slopes of the Schild plots of rimonabant for the same experiments. Where appropriate, the 95% CI values are shown in parenthesis. The pK_B values of rimonabant represent the negative logarithmic concentrations of rimonabant on the Schild plot where the experimentally fitted and constrained slopes of the Schild plot intersected with the abscissa. The pA_2 values represent the negative logarithmic concentrations of rimonabant calculated using the Gaddum–Schild equation for the antagonism of the cannabinoid receptor agonists by rimonabant (10^{-6} M) on tissues stimulated with 30 Hz frequency, 2 s every minute, 0.5 ms duration, 110% supramaximal voltage–represents no antagonism. Where appropriate values represent mean ± SEM.
*Represents significantly ($P < 0.05$, unpaired *t*-test) lower than unity.

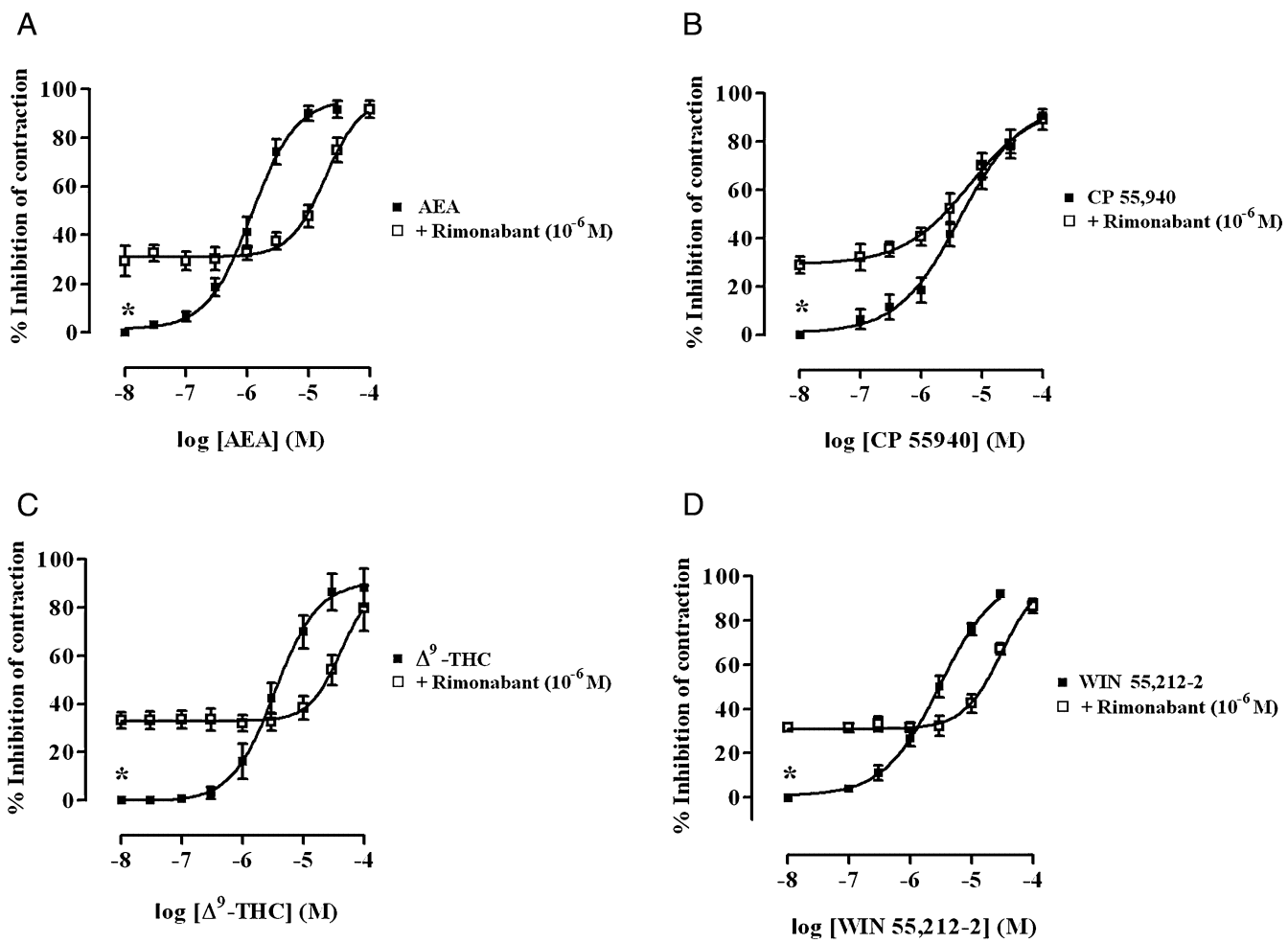

Figure 5 Concentration-response curves for the inhibition of the electrical field stimulation (EFS) (30 Hz frequency, 2 s every min, 0.5 ms duration, 110% supramaximal voltage)-evoked rebound contractions of the rat ileum myenteric plexus-longitudinal muscle by (A) AEA, (B) CP 55,940, (C) Δ^9-THC and (d) WIN 55,212-2 constructed in the presence of vehicle or rimonabant (10^{-6} M, □). Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the twitch response measured immediately before the addition of any drug to the organ bath. Vertical lines indicate SEM, $n \geq 6$ for each curve. Rimonabant or vehicle (ethanol, <1% final concentration) was added 30 min before the first addition of an agonist.

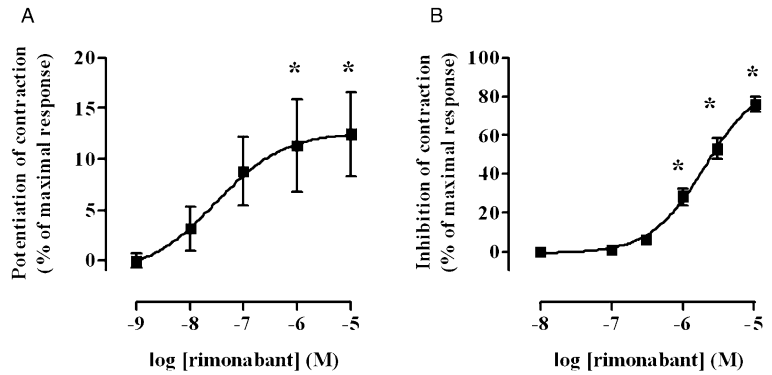

Figure 6 Concentration-response curves for the effect of rimonabant on the electrical field stimulation (EFS)-evoked contractions of the rat ileum myenteric plexus-longitudinal muscle (A) Data for the enhancement of the twitch contractions evoked by EFS at 0.05 Hz frequency, 0.5 ms duration, 110% supramaximal voltage. Each symbol represents the mean value of increase in the contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the addition of rimonabant to the organ bath ($n = 6$). Increasing concentrations of rimonabant were added every 20 min. (B) Data for the inhibition of the rebound contractions evoked by EFS at 30 Hz frequency, 2 s every min, 0.5 ms duration, 110% supramaximal voltage. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage of the amplitude of the contraction measured immediately before the addition of rimonabant to the organ bath ($n = 9$). Increasing concentrations of rimonabant were added every 30 min. Vertical lines indicate SEM. Both curves were fitted by non-linear regression analysis. *Represents a statistical difference ($P < 0.05$, paired *t*-test) compared with amplitude in the absence of rimonabant, that is 0%

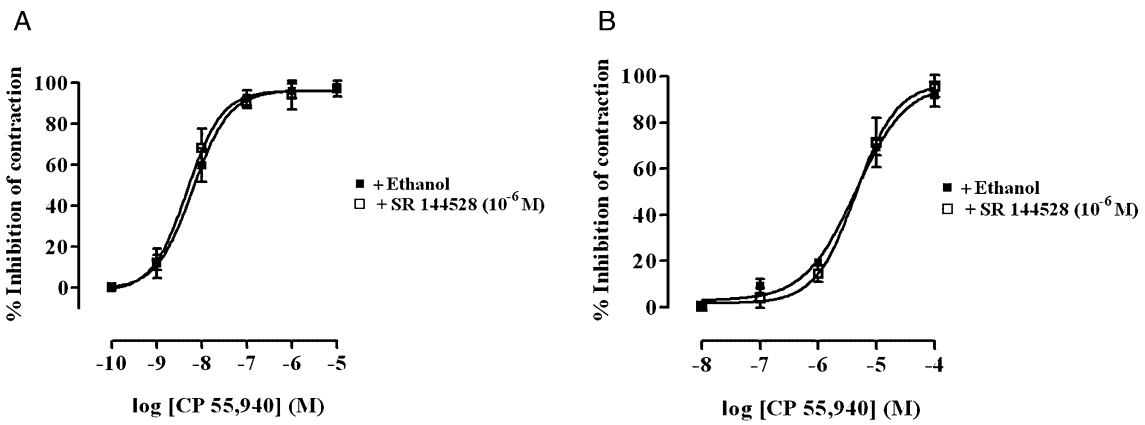

Figure 7 Concentration-response curves for the inhibition of the electrical field stimulation (EFS)-evoked contractions of the rat ileum myenteric plexus-longitudinal muscle by CP 55,940 in the presence of ethanol (vehicle) and SR 144528 (10^{-6} M). (A) Data for the twitch contractions evoked by EFS at 0.05 Hz frequency, 0.5 ms duration, 110% supramaximal voltage. (B) Data for the inhibition of the rebound contractions evoked by EFS at 30 Hz frequency, 2 s every minute, 0.5 ms duration, 110% supramaximal voltage. Each symbol represents the mean value of increase in the contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the addition of SR 144528 (10^{-6} M) to the organ bath. Vertical lines indicate SEM, $n = 6$ for each curve. All curves were fitted by non-linear regression analysis. SR 144528 or vehicle (ethanol, <1% final concentration) was added 20 and 30 min before the first addition of CP 55,940 in (A) and (B) respectively.

Pretreatment of the rat MPLM with the CB_2 receptor antagonist/inverse agonist SR 144 528 (10^{-6} M) did not cause a shift of the CP 55,940, WIN 55,212-2, AEA or Δ^9-THC concentration-response curves under either EFS conditions. Using the data for CP 55,940 as an example, Figure 7 shows the lack of an effect of SR 144 528 on the inhibitory effect of CP 55,940 under both types of the EFS-evoked contractions. The effect of SR 144 528 at concentrations higher than 10^{-6} M was not investigated. Under both EFS conditions, pretreatment of the tissues with capsazepine (10^{-5} M), a transient receptor potential vanilloid 1 ($TRPV_1$) receptor antagonist, did not attenuate the inhibitory effect of AEA (Figure 8). At this concentration, capsazepine alone significantly ($P < 0.05$) inhibited both the twitch and rebound contractions by 22.3 ± 5.5% ($n = 6$) and 15.3 ± 6.1% ($n = 6$) respectively. Concentrations of capsazepine higher than 10^{-5} M were not used.

Effect of WIN 55,212-2 on the EFS-evoked contractions of the guinea pig MPLM in the absence and presence of rimonabant

WIN 55,212-2 caused a concentration-dependent inhibition of both the EFS-evoked twitch and rebound contractions of the guinea pig ileum MPLM with a similar E_{max} (Figure 9). The pEC_{50} value for the inhibition of twitch and rebound contractions was 8.62 ± 0.04 ($n = 6$) and 6.25 ± 0.03 ($n = 6$) respectively. This cannabinoid receptor agonist was chosen because previous studies have demonstrated its stereospecific action in this tissue (Pertwee *et al.*, 1996; Coutts and Pertwee, 1997).

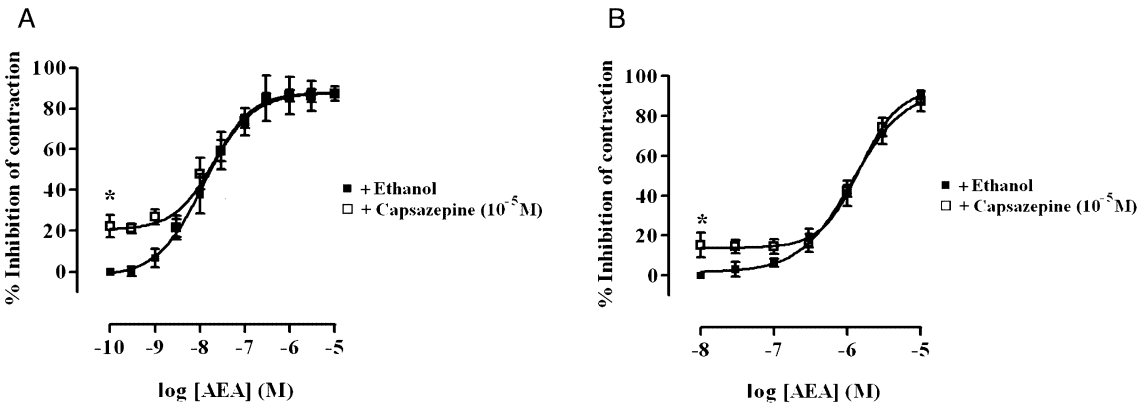

Figure 8 Concentration-response curves for the inhibition of the electrical field stimulation (EFS)-evoked contractions of the rat ileum myenteric plexus-longitudinal muscle by AEA constructed in the presence of ethanol (vehicle) or capsazepine (10^{-5} M, □). (A) Data for the twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110% supramaximal voltage (B) Data for the inhibition of the rebound contractions to EFS at 30 Hz for 2 s every min, 0.5 ms duration and 110% supramaximal voltage. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the contractions measured immediately before the addition of capsazepine to the organ bath ($n = 6$). Vertical lines indicate SEM. Capsazepine or vehicle (ethanol, <1% final concentration) was added 30 min before the first addition of AEA. *Represents a statistical difference ($P < 0.05$, paired *t*-test) in the inhibition of contractions caused by the presence of capsazepine

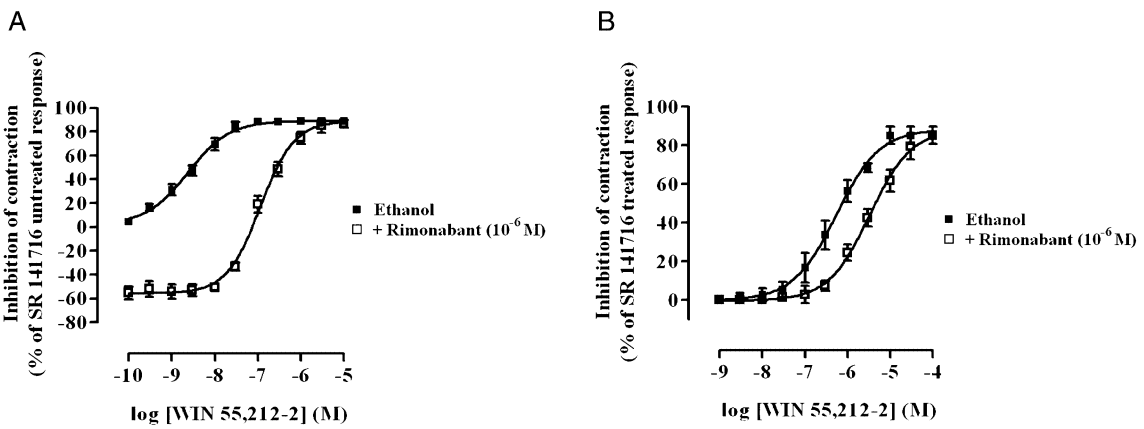

Figure 9 Concentration-response curves for the inhibition of the electrical field stimulation (EFS)-evoked contractions of the guinea pig ileum myenteric plexus-longitudinal muscle by WIN 55,212-2 in the absence and presence of ethanol (vehicle) or rimonabant (10^{-6} M). (A) Data for the twitch contractions to EFS at 0.1 Hz frequency, 0.5 ms duration and 110% supramaximal voltage (B) Data for the rebound contractions to EFS at 30 Hz for 2 s every min, 0.5 ms duration and 110% supramaximal voltage. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of EFS-evoked contractions expressed as a percentage reduction of the amplitude of contractions measured immediately before the addition of any drug to the organ bath ($n = 6$). Rimonabant or vehicle (ethanol, <1% final concentration) was added 20 and 30 min before the first addition of WIN 55,212-2 on tissues subjected to EFS at 0.1 Hz and 30 Hz respectively.

The twitch contractions of this tissue were attenuated more readily by significantly ($P < 0.05$, unpaired *t*-test) lower concentrations of the agonist than the rebound contractions. The pEC_{50} values of WIN 55,212-2 for the inhibition of the twitch contractions of the guinea pig MPLM and rat MPLM were very similar. In contrast, WIN 55,212-2 was a 0.75 logarithmic unit more potent in the guinea pig MPLM than in the rat MPLM for the inhibition of the rebound contractions.

The onset and duration of action of WIN 55,212-2 at each concentration was similar to that on rat MPLM under the respective EFS conditions (data not shown). Pre-exposure of the guinea pig MPLM to rimonabant (10^{-6} M) resulted in a significant ($P < 0.05$, ANOVA and Dunnett's test) parallel dextral shift of the WIN 55,212-2 concentration-response curve without a reduction in the mean E_{max} under either EFS conditions (Figure 9). The pA_2 value of rimonabant for the antagonism of the WIN 55,212-2-induced inhibition of the twitch and rebound contractions was 7.97 and 6.71 respectively. Rimonabant (10^{-6} M) alone caused a significant ($P < 0.05$, paired *t*-test) 55.3 ± 5.5% ($n = 6$) increase in the amplitude of the twitch contractions. By contrast, this concentration of rimonabant had no effect on the amplitude of the rebound contractions of the tissue.

Effect of the cannabinoids on contractions of the rat and guinea pig MPLM evoked by exogenously applied ACh

AEA (10^{-4} M), CP 55,940 (10^{-4} M), Δ^9-THC (10^{-4} M) and WIN 55,212-2 (10^{-4} M), which are the concentrations that produced a marked inhibition of both types of the EFS-evoked contractions of the rat MPLM, had no effect on basal tension and contractions elicited by both single and cumulative

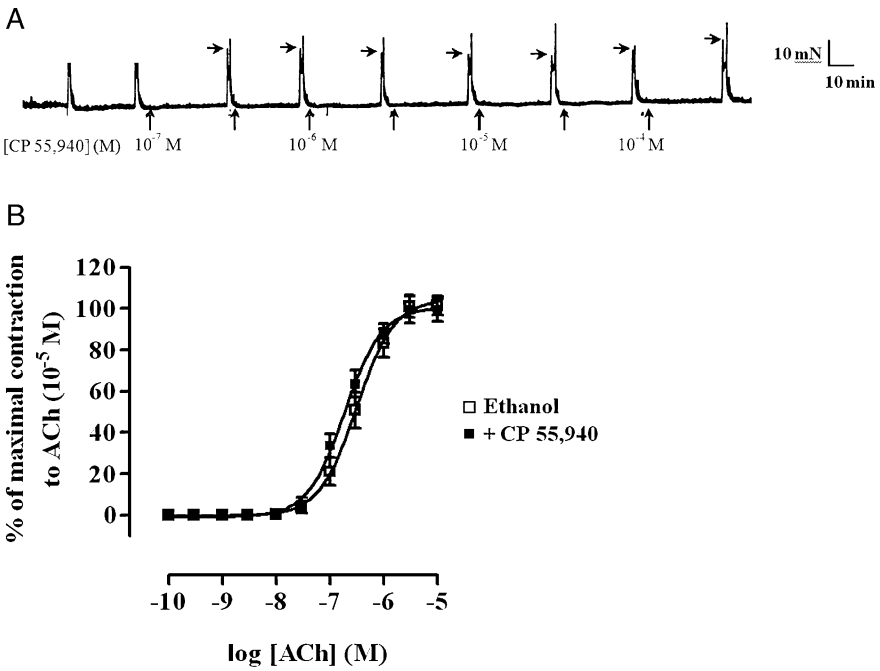

Figure 10 The lack of effect of CP 55,940 on the ACh-evoked contractions of the rat myenteric plexus-longitudinal muscle. (A) Trace illustrating the contraction to single doses of ACh (10^{-6} M) in the presence of CP 55,940 added in half-logarithmic unit increments. Unlabelled arrows represent intermediate concentrations of CP 55,940. → represents the peak of the ACh-evoked contraction. Concentration-response curves to exogenously applied ACh constructed in the presence of ethanol (vehicle) and CP 55,940 (10^{-4} M). Each curve was fitted by non-linear regression analysis. The amplitude of the contractions to cumulative additions of ACh in the presence of ethanol or CP 55,940 were expressed as a percentage of the maximal contraction to ACh obtained at 10^{-5} M from an initial ACh concentration-response curve constructed on each tissue. CP 55,940 or vehicle (ethanol, <1% final concentration) was added 30 min before constructing the second ACh concentration-response curve. $n = 6$ for all curves. All values represent mean ± SEM.

additions of exogenously applied ACh (10^{-6} M). These observations extended to rimonabant (10^{-5} M). Using the data of CP 55,940 as an example, Figure 10 shows the lack of effect of this cannabinoid on the contractions to ACh on the rat MPLM. The presence of WIN 55,212-2 (10^{-4} M) and rimonabant (10^{-4} M) was also without effect on both the basal tension and the amplitude of the ACh-evoked contractions of the guinea pig ileum MPLM (data not shown, n = 6 each).

Discussion and conclusions

In the rat MPLM, the pEC_{50} values of all four agonists to inhibit the twitch contractions and their rank order of pEC_{50} values were consistent with those previously reported at the guinea pig, human, mouse and rat CB_1, but not CB_2 receptor (Pertwee and Fernando, 1996; Pertwee *et al.*, 1996; Showalter *et al.*, 1996; Rinaldi-Carmona *et al.*, 1998; Martin *et al.*, 2000). These findings, together with the demonstration that rimonabant caused similar concentration-dependent, but agonist-independent, parallel and surmountable dextral shifts of the agonist concentration-response curves with pK_B values within its reported range of pK_B/pK_i values at the CB_1 receptor (7.93 to 8.74; Felder *et al.*, 1995; Rinaldi-Carmona *et al.*, 1994; Showalter *et al.*, 1996), suggested that the inhibition produced by each agonist was mediated by the activation of the CB_1 receptor.

Schild analysis of the interaction between each of the four agonists and rimonabant yielded Schild plots with slopes of less than unity. This revealed that rimonabant was not a simple competitive CB_1 receptor antagonist in the rat MPLM. However, a re-fit of each plot with a unit slope yielded estimates of the pK_B value of rimonabant that were again both independent of the agonist that was used and within the pK_B value range reported at the CB_1 receptor. These data suggested all four agonists were full agonists at the CB_1 receptor, and that their interaction with rimonabant could be simple competition. These results supported previous immunohistochemical and *in vivo* data describing the presence of the CB_1 receptor in this tissue and its functional role in delaying small intestinal transit *in vivo* respectively (Izzo *et al.*, 1999; Coutts *et al.*, 2002).

In contrast to the CB_1 receptor pharmacology of all four agonists for the inhibition of the twitch contractions, the pEC_{50} values of each agonist and the rank order of their pEC_{50} values for the inhibition of the rebound contractions were not consistent with activity at either the CB_1 or CB_2 receptors (Showalter *et al.*, 1996; Rinaldi-Carmona *et al.*, 1998). This suggested that the inhibition of the rebound contractions was unlikely to be mediated by the CB_1 or CB_2 receptor. An unexpected but consistent finding was that rimonabant only antagonized the ability of AEA, Δ^9-THC and WIN 55,212-2, but not CP 55,940, to inhibit the rebound contractions of the rat MPLM. Moreover, the pA_2 values of rimonabant calculated against each of the former three agonists, albeit being similar, were around 2 logarithmic units lower than its estimated pK_B value at the CB_1 receptor. These data suggested that the rebound contractions were inhibited by AEA, Δ^9-THC and WIN 55,212-2 through a common putative receptor that was

distinct from the CB_1 receptor, and that CP 55,940 did not compete with rimonabant for a common receptor.

The weak inhibitory effects of WIN 55,212-3 relative to those of WIN 55,212-2 suggested that the effects of the latter agonist were stereospecific and supportive of receptor-mediated actions under both EFS conditions. Indeed, WIN 55,212-2 has been demonstrated to show far greater affinity and intrinsic efficacy than its enantiomer in several cannabinoid *in vivo* and *in vitro* assays (Compton *et al*., 1992; Izzo *et al*., 1999; Pacheco *et al*., 1991). Although both WIN 55,212-2 and WIN 55,212-3 have been shown to be equipotent and equi-efficacious at relaxing rat isolated mesenteric arteries, this non-sterospecific action was neither CB_1 nor CB_2 receptor mediated (Ho and Hiley, 2003).

It is well established that all four agonists used in the present study do not show marked selectivity for the CB_1 and CB_2 receptor, and that AEA can also activate the $TRPV_1$ receptor (Zygmunt *et al*., 1999; Smart *et al*., 2000). The inability of SR 144528 to antagonize the inhibitory effects of each of the four agonists, and of capsazepine to block the effect of AEA under both EFS conditions, suggested that neither the CB_2 nor $TRPV_1$ receptor mediated the inhibition of both types of the EFS-evoked contractions. This result was consistent with previous data from similar *in vitro* contraction studies on the guinea pig and human ileal MPLM (Croci *et al*., 1998; Mang *et al*. 2001; Manara *et al*., 1998), and *in vivo* studies evaluating the inhibitory effect of these cannabinoids on small intestinal transit in both the rat and mouse (Izzo *et al*., 1999; 2000; 2003).

Recent studies have described the presence of the CB_2 receptor mRNA and the CB_2 receptor protein in the rat ileal myenteric plexus (Storr *et al*., 2002; Duncan *et al*., 2008). However, the ability of cannabinoid receptor agonists to delay small intestinal transit of the rat *in vivo* through this receptor has been reported to occur only after inflammation of the small intestine with the bacterial endotoxin lipopolysaccharide (Mathison *et al*., 2004), and not other inflammatory agents (Izzo *et al*., 1999). Therefore, in the present study, it is improbable that the CB_2 receptor mediated the inhibitory effects of all four agonists under both EFS conditions, because the MPLM strips were prepared from healthy rats. This conclusion is also supported by the lack of antagonism by SR 144 528. It was unlikely that the $TRPV_1$ receptor was not present in this tissue, because both the mRNA and immunoreactivity for this receptor have been identified in the rat MPLM (Anavi-Goffer *et al*., 2002). Moreover, in the present study, capsazepine alone caused an inhibition of the both types of EFS-evoked contractions of this tissue, possibly through a post-junctional $TRPV_1$ receptor-mediated relaxation of the longitudinal muscle, as reported by Nocerino *et al*. (2002).

This study also demonstrated an inhibitory effect of WIN 55,212-2 on both the twitch and rebound contractions of the guinea pig MPLM. The pEC_{50} value of this agonist for inhibiting the twitch contractions was similar to that reported previously at the CB_1 receptor in this tissue (Pertwee *et al*., 1996; Coutts and Pertwee, 1997), and to that obtained in the rat MPLM for the inhibition of the twitch contractions. Consistent with data from previous studies, this inhibitory effect of WIN 55,212-2 was confirmed to be mediated by the CB_1 receptor because it was antagonized by rimonabant with a pA_2 value, in agreement with its pK_B value at the CB_1 receptor. However, as was observed in the rat MPLM, the WIN 55,212-2-induced inhibition of the rebound contractions of the guinea pig MPLM was unlikely to be mediated by the CB_1 receptor for two reasons. First, the pEC_{50} value of WIN 55,212-2 was much higher than that commonly obtained at CB_1 receptors. Second, the pA_2 value of rimonabant against WIN 55,212-2 was over a logarithmic unit less than its pK_B value at the CB_1 receptor. Recently, Guagnini *et al*. (2006) showed that the ACh-mediated contractions of the guinea pig MPLM elicited by EFS with 10 s trains of pulses at 20 Hz were inhibited by WIN 55,212-2 with a pEC_{50} value of 8.32. Moreover, rimonabant alone increased the amplitude of these contractions, and its interaction with WIN 55,212-2 yielded a Schild plot with a unit slope and pK_B value of 8.4. Although the EFS parameters we used for eliciting the rebound contractions were almost identical to those used by Guagnini *et al*. (2006), the interaction of WIN 55,212-2 with rimonabant yielded data that were similar to that observed under the twitch contractions conditions. The low pEC_{50} value reported by Guagnini *et al*. (2006) may be attributed to the use of an isotonic recording technique and submaximal voltage for EFS, both of which yield steeper curves that are shifted towards the left (Barlow *et al*., 2001a,b; Cowie *et al*. 1978).

In our rebound contractions experiments, the similarity between the pA_2 value of rimonabant obtained against WIN 55,212-2 in the guinea pig MPLM to that obtained against the same agonist, AEA and Δ^9-THC in the rat MPLM suggested that the MPLM of both species contained the same putative non-CB_1 receptor in addition to the CB_1 receptor. Moreover, in addition to the presence of these two receptors in the rat MPLM, an additional putative non-CB_1-non-CB_2 receptor that mediated the inhibitory effect of CP 55,940 was likely to be present.

The inhibition of twitch and rebound contractions of both MPLM tissues by TTX or atropine but not hexamethonium suggested that EFS indirectly provoked contractions of the longitudinal smooth muscle layer, through the muscarinic ACh receptor, by stimulating the release of ACh from post-ganglionic nerve terminals of the myenteric plexus. The lack of effect of the cannabinoid agonists on the EFS-evoked relaxations of the rat MPLM suggested that the inhibition of the rebound contractions was not through functional antagonism by a facilitation of the release of NO from the myenteric plexus. The failure of EFS to elicit relaxations of the guinea pig ileum MPLM suggested that either a relaxant transmitter was not released by EFS, or that the quanta released was not sufficient to produce a relaxation of the muscle.

None of the four agonists or rimonabant altered the resting tone or contractions of the rat MPLM to exogenously applied ACh. These observations also extended to WIN 55,212-2 and rimonabant on the guinea pig MPLM. Therefore, in agreement with previous studies (Coutts and Pertwee, 1997; Mang *et al*., 2001), the effects of the cannabinoids on the EFS-evoked contractions of both tissues may be ascribed to a pre-synaptic modulation of ACh release from post-ganglionic nerve terminals of the myenteric plexus, and not an interaction with the muscarinic ACh receptor on the smooth muscle or an interference with the hydrolysis of ACh by ACh-esterase.

The slow onset of inhibition of both EFS-evoked contractions of the MPLM tissues could be attributed to the low aqueous solubility and high lipophilicity of the agonists as reflected by the good correlation between the rank order of the onset of inhibition, that is WIN 55,212-2 > CP 55,940 > Δ^9-THC > AEA and their logarithmic octanol-water partition coefficients, that is 3.4, 4.6, 5.5 and 6.3 respectively (Valiveti *et al*., 2004; Lovinger, 2007). Therefore, it is possible that the difference in the onset of inhibition of both types of contractions was because of the activation of distinct receptors, and not because of differences in the diffusion of the agonists to the receptors.

In the absence of other drugs, the ability of rimonabant to enhance the twitch contractions of both the rat and guinea pig MPLM may be attributed to an antagonism of the tonic activation of CB_1 receptors by endogenously released agonists. Levels of both AEA and 2-AG high enough to cause the activation of CB_1 receptors have been measured in both rat and guinea pig MPLM (Katayama *et al*., 1997; Valenti *et al*., 2005; Guagnini *et al*., 2006). Alternatively, the augmentation of the twitch contractions may be through an unmasking of a constitutive activity of the CB_1 receptor through inverse agonism (Pertwee, 2005).

In the twitch contraction experiments, the presence of rimonabant (10^{-6} M) produced a similar rightward shift of the WIN 55,212-2 curve on both MPLM tissues despite causing a larger downward shift of the basal asymptote of the curve in the guinea pig MPLM. This suggested that the ability of rimonabant to behave as an antagonist was independent of its ability to augment the twitch contractions, and, therefore, the latter property did not induce an error in estimating its pK_B value at the CB_1 receptor. Pharmacological analysis with a silent CB_1 receptor antagonist may substantiate this interpretation. Whether the dual agonist–antagonist activity of rimonabant on the rebound contractions of the rat MPLM was through partial agonism at the putative receptor not activated by CP 55,940 remains to be established. The lack of effect of rimonabant on the rebound contractions of the guinea pig MPLM could be ascribed to a low stimulus-response coupling capacity or density of the putative receptor. This observation also added weight to the idea that the rimonabant-induced inhibition of the rebound contractions of the rat MPLM was not non-specific.

In addition to the CB_1, CB_2 and $TRPV_1$ receptors, several lines of evidence have emerged for the existence of novel cannabinoid pharmacological targets (Pertwee, 2001; 2004; 2005). Among these is the orphan GPR 55 receptor, which has been implicated in mediating a number of non-CB_1-non-CB_2-non-$TRPV_1$ receptor actions of several cannabinoids (Brown, 2007; Ryberg *et al*., 2007). Although the mRNA transcripts of this receptor have been detected in the rat ileum (Baker *et al*., 2006), this receptor is unlikely to mediate the inhibition of the rebound contractions of the MPLM, because WIN 55,212-2 lacks affinity for this receptor, and the rank order of the pEC_{50} values of the other three agonists does not correlate with the activity reported at this receptor (Ryberg *et al*., 2007).

Taken together, evidence was provided for the viability of rat MPLM as a novel *in vitro* ileal cannabinoid receptor bioassay. Representatives of the four main classes of structurally different cannabinoid receptor agonists attenuated the EFS-evoked contractions of this tissue in a concentration-dependent, pre-synaptic and stereo-specific manner. Evidence was also provided for a functional role of the CB_1 receptor and at least two non-CB_1-non-CB_2-non-$TRPV_1$ receptors, of which one was weakly sensitive to rimonabant in mediating the inhibition of the twitch and rebound contractions of the rat MPLM respectively. As demonstrated with WIN 55,212-2, these observations also extended to the guinea pig MPLM subjected to similar EFS conditions. The ability of rimonabant alone to augment the twitch contractions was suggestive of either the presence of an endocannabinoid tone or a constitutively active CB_1 receptor, whereas the inhibition of the rebound contractions of the rat MPLM was suggestive of partial agonism.

The cannabinoids used in the present study represented a small fraction of cannabinoids that have been assayed *in vivo*. Little is known about the pharmacology of the many other endogenous cannabinoids and phytocannabinoids. Therefore, the rat MPLM may prove useful in delineating the pharmacology of these cannabinoids under both physiological and pathophysiological conditions.

Conflict of interest

None.

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