

THEMED ISSUE: CANNABINOIDS

RESEARCH PAPER

Evidence for both inverse agonism at the cannabinoid CB₁ receptor and the lack of an endogenous cannabinoid tone in the rat and guinea-pig isolated ileum myenteric plexus-longitudinal muscle preparationR Makwana¹, A Molleman² and ME Parsons²

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Background and purpose: Cannabinoid receptor agonists reduce intestinal propulsion in rodents through the CB₁ receptor. In addition to its antagonistic activity at this receptor, rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) alone augments intestinal transit. Using rat and guinea-pig ileum MPLM (myenteric plexus-longitudinal muscle) preparations, we investigated whether the latter effect was through inverse agonism or antagonism of endocannabinoid agonist(s).

Experimental approach: Inverse agonism was investigated by comparing the maximal enhancement of electrically evoked contractions of the MPLM by two CB₁ receptor antagonists, AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and O-2050 [(6aR,10aR)-3-(1-methanesulphonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran], with that produced by rimonabant. To reveal ongoing endocannabinoid activity, effects of inhibiting endocannabinoid hydrolysis by fatty acid amide hydrolase (FAAH) using AA-5HT (arachidonyl-5-hydroxytryptamine), PMSF (phenylmethylsulphonyl fluoride) or URB-597 (3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate), or putative uptake using VDM-11 [(5Z,8Z,11Z,14Z)-N-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide] was evaluated.

Key results: The presence of CB₁ receptors was revealed by antagonism of exogenous anandamide, arachidonylethanolamide (AEA) 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] by rimonabant. The rank order of potentiation of contractions was AM 251 > rimonabant > O-2050. Neither the FAAH inhibitors nor VDM-11 affected electrically evoked contractions. Each FAAH inhibitor increased the potency of AEA but not WIN 55,212-2. VDM-11 did not alter the inhibitory effect of AEA.

Conclusions and implications: The different levels of maximal potentiation of contractions by the CB₁ receptor antagonists suggest inverse agonism. The potentiation of the action of AEA by the FAAH inhibitors showed that FAAH was present. The lack of effect of FAAH inhibitors and VDM-11 alone on electrically evoked contractions, and on the potency of exogenous AEA suggests that pharmacologically active endocannabinoids were not released and the endocannabinoid transporter was absent. Thus, the CB₁ receptor antagonists behave as inverse agonists.

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Keywords: cannabinoid; endocannabinoid tone; myenteric plexus; small intestine; fatty acid amide hydrolase; cannabinoid receptor agonists; cannabinoid receptor antagonists; cannabinoid receptor inverse agonists; inverse agonism

Abbreviations: AA-5HT, arachidonyl-5-hydroxytryptamine; AEA, anandamide, arachidonylethanolamide; AM 251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; MPLM, myenteric plexus-longitudinal muscle; O-2050, (6aR,10aR)-3-(1-methanesulphonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6-H-dibenzo[b,d]pyran; PMSF, phenylmethanesulphonyl fluoride; rimonabant, N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; URB-597, 3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate; VDM-11, (5Z,8Z,11Z,14Z)-N-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide; 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

Introduction

Being a potent antagonist of the cannabinoid CB₁ receptor, rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) (pK_B 8.00; Rinaldi-carmona *et al.*, 1994) readily prevents or reverses the action of a range of agonists acting at this receptor in several *in vivo* and *in vitro* functional bioassays (Howlett *et al.*, 2002). However, in addition to its antagonist activity, rimonabant and a number of other CB₁ receptor antagonists, such as AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (Gatley *et al.*, 1997) and LY 320135 (Felder *et al.*, 1998), alone elicit CB₁ receptor-mediated effects that are invariably opposite in direction from those produced by cannabinoid receptor agonists (Pertwee, 2005). For example, while *in vivo* administration of cannabinoid receptor agonists into rodents delays intestinal transit, via a reduction in the propulsive function of the small intestine, rimonabant alone increases intestinal transit (Colombo *et al.*, 1998; Izzo *et al.*, 1999a,b; 2001). Such antagonist-mediated changes have been attributed to the unmasking of a constitutive activity of the CB₁ receptor via inverse agonism, or the antagonism of tonically released endocannabinoid agonists at this receptor. To date, rimonabant has only been conclusively demonstrated to behave as an inverse agonist in recombinant cell-based assays in which the CB₁ receptor was overexpressed (Bouaboula *et al.*, 1997; MacLennan *et al.*, 1998). Therefore, it has appeared unlikely that such activity could occur in intact tissues in which receptor expression was likely to be at a natural physiological level.

Over the last decade, evidence has emerged to indicate that a functional endocannabinoid agonist tone may be present in the intestinal tract. This has been primarily because of the identification of anandamide, arachidonylethanolamide (AEA) and 2-arachidonylglycerol (2-AG, Izzo *et al.*, 2003; Fegley *et al.*, 2005; Guagnini *et al.*, 2006) and the mechanisms for their inactivation by facilitated uptake, via a putative transporter protein (Mascolo *et al.*, 2002), and enzymatic hydrolysis, such as by fatty acid amide hydrolase (FAAH, Katayama *et al.*, 1997). Furthermore, the demonstration that *in vivo* administration of inhibitors of the putative uptake transporter or FAAH into rodents mimics the inhibitory effects of exogenously administered cannabinoid receptor agonists has added weight to a possible physiological inhibitory role of an endocannabinoid agonist tone on intestinal motility (Pinto *et al.*, 2002; Capasso *et al.*, 2005). Nevertheless, the intestinal prokinetic effect of rimonabant may not be attributed unequivocally to the presence of ongoing local

enteric endocannabinoid agonist activity because the existence of a possible constitutive activity of the CB₁ receptor in the small intestine has not been investigated.

Although *in vivo* bioassays have played an important role in characterizing the pharmacology of cannabinoids on intestinal motility, it has been difficult to assess whether locally released endocannabinoid agonists may modulate intestinal motility. To simplify studies, we have used the myenteric plexus-longitudinal muscle (MPLM) preparation of the rat and guinea-pig isolated ileum to elucidate the mechanism by which rimonabant augments small intestinal motility. Consistent with the inhibitory effect of cannabinoid receptor agonists on small intestinal transit *in vivo*, these *in vitro* bioassays exploit the ability of these agonists to attenuate twitch contractions evoked by electrical field stimulation (EFS) of the longitudinal smooth muscle, in a rimonabant-sensitive CB₁ receptor-dependent manner, by reducing the release of acetylcholine (ACh) from the nerve terminals innervating the smooth muscle layer. Moreover, rimonabant alone, by increasing the release of ACh, augments the EFS-evoked twitch contractions of these tissues in a concentration-dependent manner (Coutts and Pertwee, 1997; Mang *et al.*, 2001; Makwana *et al.*, 2006).

Recently, a number of ligands that are thought to behave as pure CB₁ receptor antagonists have been developed (Pertwee, 2005). Among these is O-2050 [(6aR,10aR)-3-(1-methanesulphonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6-H-dibenzo[b,d]pyran], a sulphonamide analogue of Δ^9 -tetrahydrocannabinol, which was reported to be devoid of agonist or inverse agonist activity on the EFS-evoked contractions of the mouse isolated vas deferens (Martin *et al.*, 2002). Therefore, the availability of O-2050 offered the opportunity to elucidate the mechanism by which rimonabant alone produced its inherent effects. We also evaluated the effects of three inhibitors of FAAH, AA-5HT (arachidonyl-5-hydroxytryptamine), PMSF (phenylmethanesulphonyl fluoride) and URB-597 (3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate), and an inhibitor of the putative endocannabinoid uptake transporter, VDM-11 [(5Z,8Z,11Z,14Z)-N-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide], as an approach to unmask an inhibitory endocannabinoid tone by limiting the inactivation of endocannabinoid agonists. Additionally, the effect of AA-5HT, PMSF, URB-597 and VDM-11 on the potency of exogenously applied AEA and WIN 55212-2, inhibitors of the EFS-evoked twitch contractions of the tissues, was examined. The latter cannabinoid receptor agonist was used because it is not a substrate for FAAH as it lacks an amide linkage, and in addition to its common use as an agonist, it displays stereospecific

activity at the CB₁ receptor in these tissues (Coutts and Pertwee, 1997; Makwana *et al.*, 2006).

A preliminary account of some of the present data has been communicated to the British Pharmacological Society (Makwana *et al.*, 2007).

Methods

Tissue preparation

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 UK (Margate, Kent, UK) and Harlan UK (Bicester, Oxford, UK) respectively. The animals were housed in rooms with a controlled temperature ($22 \pm 1^\circ\text{C}$), humidity ($55 \pm 10\%$) and 12 h light/dark cycle. Food and water were available *ad libitum*. Rats were killed by carbon dioxide (CO₂) asphyxiation, while guinea-pigs were killed by cervical dislocation followed by exsanguination. All animal care and killing was conducted in accordance with requirements of the Animals (Scientific Procedures) Act 1986 and the University of Hertfordshire ethics committee.

A 15 cm ileum segment was excised from the small intestine of each animal and immersed in Krebs-Henseleit solution of the composition (in mM): NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, D-glucose 11.1, CaCl₂ 2.5, gassed with 95% O₂ and 5% CO₂ at room temperature ($21 \pm 4^\circ\text{C}$). A maximum of four MPLM strips were dissected from the ileal segment by the method of Paton and Zar (1968) after the first 5 cm length closest to the ileo-caecal junction had been discarded. 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 the mesentery had been trimmed, the longitudinal muscle with the adhered 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 5 or 10 mL organ bath containing Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂ at 33°C . The experiments were carried out at 33°C in order to dampen down the irregular spontaneous activity of the tissues, particularly of the rat MPLM, to enable the evoked responses to be quantified easily.

Changes in tension of the MPLM strips was recorded isometrically in millinewtons (mN) 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, www.apple.com/uk) or MX216 or MultiTrace-4 chart recorders (Lectromed, Hertfordshire, UK).

EFS experiments

Each MPLM strip was placed between a pair of platinum prong (10 mm length and 5 mm apart) electrodes connected

to either Grass S11 or S88 stimulators (Grass Instruments, Slough, Berkshire, UK) or a Multistim D330 stimulator (Digi-timer, Welwyn Garden City, Hertfordshire, UK).

After the tissue had been placed in the organ bath for 10 min, the Krebs-Henseleit solution was renewed once and each tissue was stretched by 5.0 mN and allowed to equilibrate for a further 50 min until EFS was commenced. The guinea-pig and rat MPLM strips were subjected to EFS over the entire duration of the experiment with single pulses of 0.5 ms duration repeated at a frequency of 0.1 and 0.05 Hz respectively. Each pulse was delivered at a voltage that was 10% greater than the voltage required to elicit maximal contractions.

No drugs were added until the amplitude of EFS-evoked contractions had become consistent for at least 30 min. The time taken for the amplitude of the contractions to become uniform was about 3–4 h. It was also noted that the amplitude of the contractions remained stable for a further 4 h without the need to renew the bathing Krebs-Henseleit solution. Cumulative concentration–response curves to the cannabinoid receptor agonists were constructed with a 20 min dosing interval. Only one concentration–response curve was constructed per tissue because of difficulties in washing the drug from the tissue with drug-free Krebs-Henseleit solution.

On each day, each MPLM strip from an animal was subjected to a different drug treatment, and the drug treatments were randomized between the organ baths. When competition studies were performed, rimonabant was added 20 min prior to the addition of a cannabinoid agonist whereas AA-5HT, PMSE, URB-597 or VDM-11 was added 15 min prior to the addition of the cannabinoid agonist. To exclude the possibility that rimonabant antagonized the inhibitory effect of the cannabinoid receptor agonists by virtue of its ability to enhance the EFS-evoked contractions, that is, through functional antagonism, the inhibitory effect of 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] was compared after the contractions had been augmented with physostigmine, an acetyl cholinesterase inhibitor. This experiment was only performed on the guinea-pig MPLM because both rimonabant and physostigmine produced large and clearly defined enhancement of contractions in this tissue. Physostigmine was administered 20 min before the addition of WIN 55,212-2 at a concentration that potentiated the EFS-evoked contractions to a level nearly equal to that produced by rimonabant. All experiments were performed in parallel with vehicle-treated and time-matched controls.

Exogenous ACh stimulation

If any drug was found to alter the amplitude of the EFS-evoked contractions, its ability to produce a similar effect on contractions induced by exogenously applied ACh was investigated. A single high concentration of the drug, which had a maximally effect on the amplitude of EFS-evoked contractions, was administered between two ACh (10^{-10} – 10^{-5} M) cumulative concentration–response curves constructed 30 min apart. Each tissue acted as its own control. All experiments were performed in parallel with relevant vehicle-treated and time-matched 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 after the addition of the agonist compared with the amplitude immediately before the first addition of the competing ligand or its vehicle. The enhancement of the contractions induced by certain ligands was quantified in percentage terms by calculating the increase in amplitude after each addition of the ligand compared with 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);

$$E = \text{Basal} + \frac{E_{\max} - \text{Basal}}{1 + 10^{(\text{LogEC}_{50} - \text{Log}[A])^{1/n_H}}} \quad (\text{Equation 1})$$

where E denotes response, $\text{Log}[A]$ the logarithm of the concentration of an agonist A , n_H the midpoint slope of the curve, LogEC_{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 \pm SEM, and n represents the number of preparations from different animals. Shifts of the agonist concentration–response curve by the presence of a competing ligand were quantified as the ratio of the agonist concentrations corresponding to the 50% equieffective response level. The concentration ratio for the rightward shift of the agonist curve was used to calculate the antagonist potency (pA_2) using the Gaddum–Schild equation (Schild, 1949). The pA_2 represents the negative logarithm of the concentration of the antagonist that 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 is expressed as a percentage of the maximal contraction to ACh (10^{-5} M), obtained from the initial ACh concentration–response curve constructed on each tissue. Where appropriate, shifts of an agonist concentration–response curve by the presence of a competing ligand were compared by a one-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons or Student's unpaired *t*-test for comparisons of individual means. The probability $P < 0.05$ was taken to be statistically significant.

Drug and molecular target nomenclature conforms to *British Journal of Pharmacology* Guide to Receptors and Channels (Alexander *et al.*, 2008).

Drugs and chemicals

ACh (acetylcholine chloride), atropine sulphate, physostigmine hemisulphate, hexamethonium bromide, PMSF were purchased from Sigma-Aldrich (Poole, Dorset, UK). AEA, AM 251, O-2050, TTX (tetrodotoxin), VDM-11 and WIN 55,212-2 were purchased from Tocris Biosciences (Avon, Bristol, UK).

Rimonabant was a generous gift from Sanofi-Recherche, Montpellier, France. AA-5HT and URB-597 were purchased from Axxora Life Sciences, Bingham, Nottingham, UK. All other chemicals were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. Atropine, physostigmine, hexamethonium, nicotine and TTX were dissolved in distilled water. 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

The EFS responses of the rat and guinea-pig MPLM

Electrical field stimulation (EFS) of the MPLM from both species elicited a monophasic transient twitch contraction during each electrical pulse. The amplitudes of EFS-evoked contractions of the rat and guinea-pig MPLM before the addition of any drug were 4.02 ± 2.16 mN ($n = 100$) and 17.0 ± 7.45 mN ($n = 100$) respectively. The contractions of both MPLM tissues were abolished by treatment with either the voltage-gated Na⁺ channel blocker TTX (10^{-6} M, data not shown, $n = 6$) or the muscarinic ACh receptor antagonist atropine (10^{-6} M, data not shown, $n = 6$) but not with the nicotinic ACh receptor antagonist hexamethonium (10^{-4} M, data not shown, $n = 6$).

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

Cumulative additions of AEA (Figure 1A and B) or WIN 55,212-2 (Figure 1C and D) resulted in a concentration-related reduction of the EFS-evoked contractions of both MPLM tissues with a similar E_{\max} . Of the two agonists, WIN 55,212-2 was significantly ($P < 0.05$, unpaired *t*-test) more potent than AEA on both tissues. Non-linear regression analysis yielded a pEC_{50} of 7.67 ± 0.05 ($n = 6$) and 8.27 ± 0.06 ($n = 6$) for AEA and WIN 55,212-2 on the rat MPLM, respectively, and 6.81 ± 0.02 ($n = 6$) and 8.52 ± 0.03 ($n = 6$) for AEA and WIN 55,212-2 on the guinea-pig MPLM respectively. Unlike WIN 55,212-2, which showed similar potency on both MPLM tissues, AEA was significantly ($P < 0.05$, unpaired *t*-test) more potent, one logarithmic unit, on the rat MPLM. The inhibition of the EFS-evoked contractions of both tissues by each agonist was slow in onset with the maximal effect being achieved within 15 min of administration.

A 20 min pretreatment of both tissues with rimonabant (10^{-7} or 10^{-6} M) resulted in a significant ($P < 0.05$, ANOVA and Dunnett's test) dextral shift of the concentration–response curve of both agonists with no reduction in their E_{\max} values (Figure 1). Figure 2 shows representative traces of the WIN 55,212-2-induced inhibition of the EFS-evoked contractions of the guinea-pig MPLM in the absence and presence of rimonabant. The pA_2 values for rimonabant against AEA and WIN 55,212-2 were 7.68 and 8.21, respectively, on the rat MPLM and 7.90 and 7.97, respectively, on the guinea-pig MPLM.

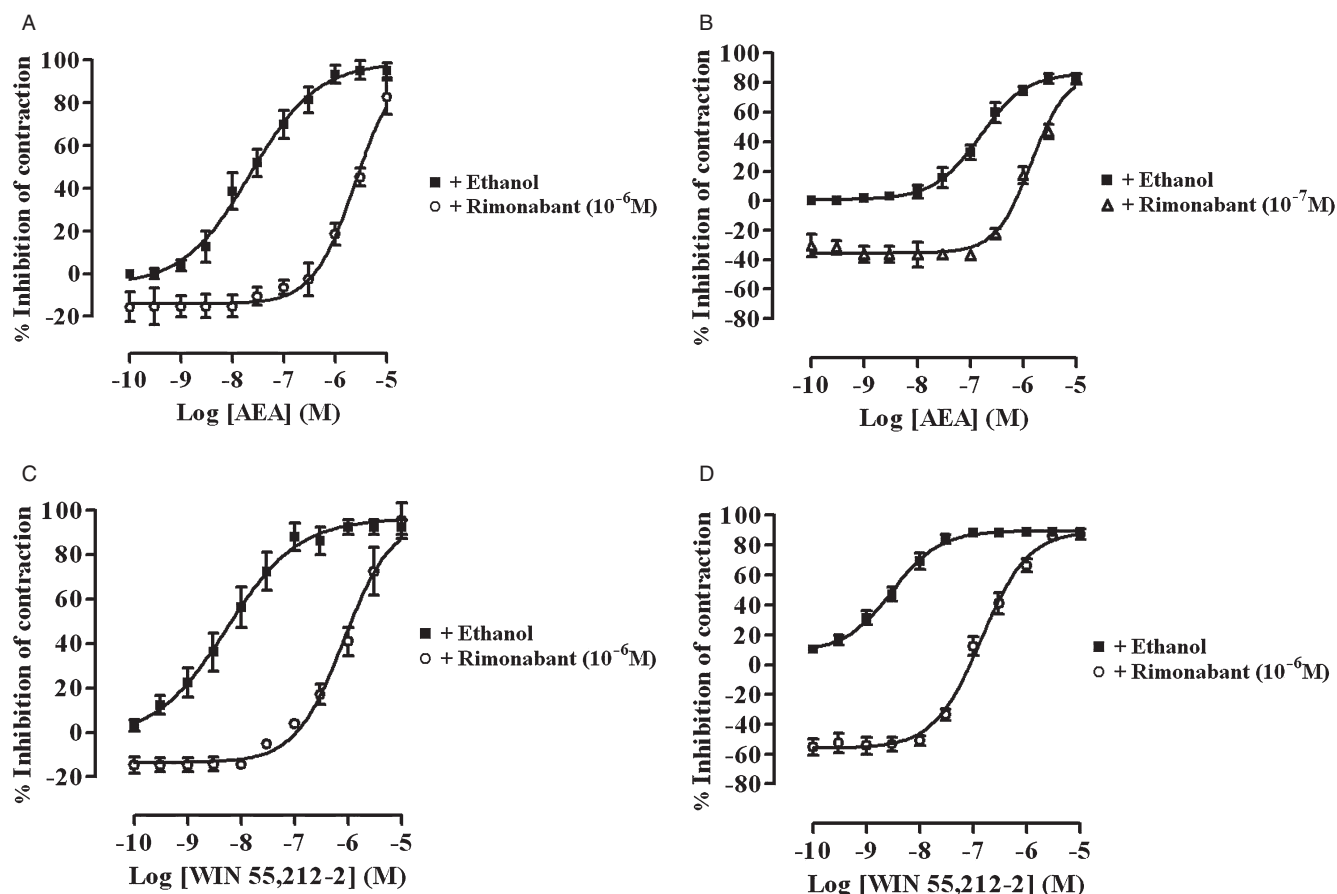


Figure 1 Concentration–response curves for the inhibition of the electrical field stimulation-evoked contractions of the rat (A and C) and guinea-pig (B and D) myenteric plexus-longitudinal muscle (MPLM) by anandamide, arachidonylethanolamide (AEA) (A and B) and WIN 55,212-2 [(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate] (C and D) constructed in the presence of ethanol or rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide). The rat and guinea-pig MPLM were subjected to single electrical pulses of 0.5 ms duration, 110% supramaximal voltage at a frequency of 0.05 and 0.1 Hz respectively. 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 any drug to the organ bath. Vertical lines indicate SEM, $n = 6$ for each curve. Rimonabant or ethanol was added 20 min before the first addition of an agonist.

Effect of WIN 55,212-2 on the EFS-evoked contraction of the guinea-pig MPLM in the absence and presence of physostigmine
Physostigmine alone caused a concentration-related enhancement of the contractions of the guinea-pig MPLM up to 10^{-7} M; the enhancement induced by 10^{-7} M was $66.3 \pm 12.3\%$ (data not shown, $n = 6$). Further increases in concentration up to 3×10^{-6} M yielded a complex response, which consisted of a simultaneous progressive contraction of the muscle and an inhibition of the EFS-evoked contractions (recording not shown, $n = 6$). These responses were difficult to quantify. Using the enhancement to 10^{-7} M as the E_{\max} , the pEC_{50} of physostigmine was 7.69 ± 0.01 (data not shown, $n = 6$). Treatment of the MPLM with 3×10^{-8} M physostigmine potentiated the EFS-evoked contractions by $48.7 \pm 11.0\%$ ($n = 6$), a level comparable to that produced by rimonabant (10^{-6} M). The presence of physostigmine at this concentration caused a small but non-significant ($P > 0.05$, ANOVA and Dunnett's test) dextral shift of the WIN 55,212-2 concentration–response curve with no reduction in the E_{\max} values (Figure 3).

Effect of rimonabant, AM 251 or O-2050 alone on the EFS-evoked contractions

Figure 4 shows that rimonabant, AM 251 and O-2050 all caused a concentration-dependent enhancement of the EFS-evoked contractions of both the rat and guinea-pig MPLM. The EFS-evoked contractions in the presence of these cannabinoids were abolished by application of either TTX (10^{-6} M) or atropine (10^{-6} M). Figure 5 illustrates representative traces of the enhancement of the EFS-evoked contractions of both tissues by rimonabant and the inhibitory effect of atropine. All three cannabinoids produced different degrees of maximal enhancement of the EFS-evoked contractions in both tissues. The E_{\max} values of all three antagonists were over 20 times ($P < 0.05$, unpaired t -test) greater in the guinea-pig MPLM than in the rat MPLM (Table 1). Nevertheless, the rank order of the E_{\max} values were similar in both tissues that is, AM 251 > rimonabant > O-2050.

Because each set of curves in Figure 4 had different E_{\max} , and the slopes were not parallel, a direct comparison between the

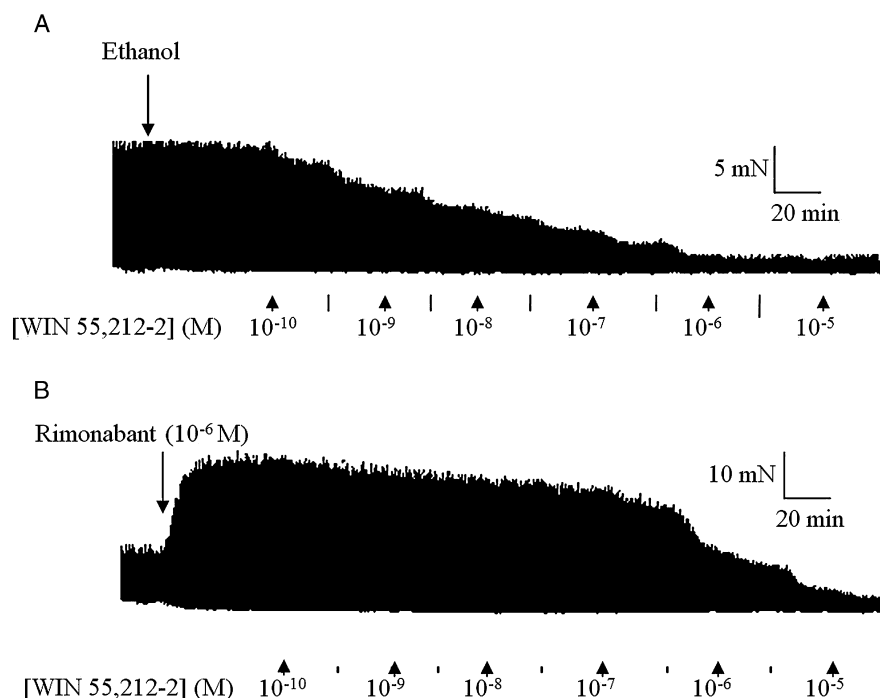


Figure 2 Representative traces of the inhibition of the electrical field stimulation (0.1 Hz frequency, 0.5 ms duration and 110% supramaximal voltage)-evoked contractions of the guinea-pig myenteric plexus-longitudinal muscle by WIN 55,212-2 [(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate] in the presence of (A) ethanol and (B) rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) (10^{-6} M). WIN 55,212-2 was added in half-logarithmic unit increments. Lines represent intermediate concentrations.

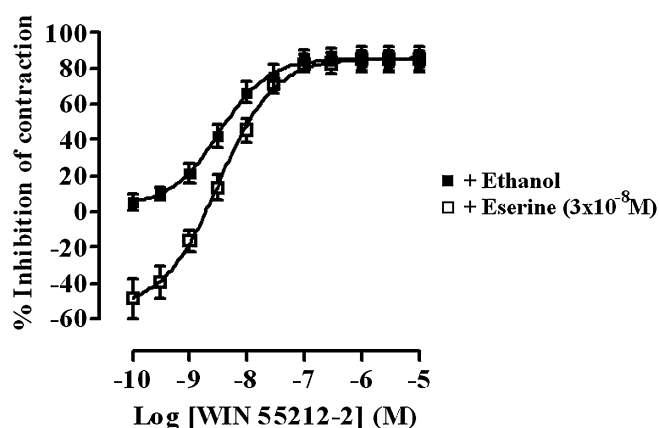


Figure 3 Concentration-response curves for the inhibition of the electrical field stimulation-evoked contractions of the guinea-pig myenteric plexus-longitudinal muscle (MPLM) by WIN 55,212-2 [(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate] constructed in the absence and presence of physostigmine (eserine). The guinea-pig MPLM was subjected to single electrical pulses of 0.5 ms duration, 110% supramaximal voltage at a frequency of 0.05 and 0.1 Hz respectively. 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 any drug to the organ bath. Vertical lines indicate SEM, $n = 6$ for each curve. Physostigmine was added 20 min before the first addition of WIN 55,212-2.

pEC_{50} values of the three cannabinoids was not possible. However, a comparison between the concentrations corresponding to the 5% and 150% level of enhancement of the contractions of the rat and guinea-pig MPLM curves, respectively, indicated that the rank order of potency of the three antagonists on both tissues was similar that is, AM 251 > rimonabant > O-2050.

The time taken for each cannabinoid to enhance the EFS-evoked contractions was slow in both tissues, with the maximal enhancement at each concentration being achieved within 20 min of administration. Half-maximal potentiation of contraction by AM 251, O-2050 and rimonabant at 10^{-6} M was achieved after 4, 3.5 and 5.5 min, respectively, in the rat MPLM and 4, 4 and 5 min, respectively, in the guinea-pig MPLM.

Effect of AA-5HT, PMSF and URB-597 alone and on the inhibitory potency of AEA and WIN 55,212-2 on EFS-evoked contractions

Cumulative addition of AA-5HT (10^{-9} – 10^{-5} M), PMSF (10^{-6} – 10^{-4} M) or URB-597 (10^{-9} – 10^{-5} M) every 15 min had no effect on the basal tension or the EFS-evoked contractions of either the rat or guinea-pig MPLM (data not shown, $n = 6$ each). However, pre-incubation of both tissues for 15 min with AA-5HT (10^{-6} M), PMSF (10^{-4} M) or URB-597 (3×10^{-8} M) resulted in a significant ($P < 0.05$, ANOVA and Dunnett's test) parallel leftward shift of the AEA concentration-response

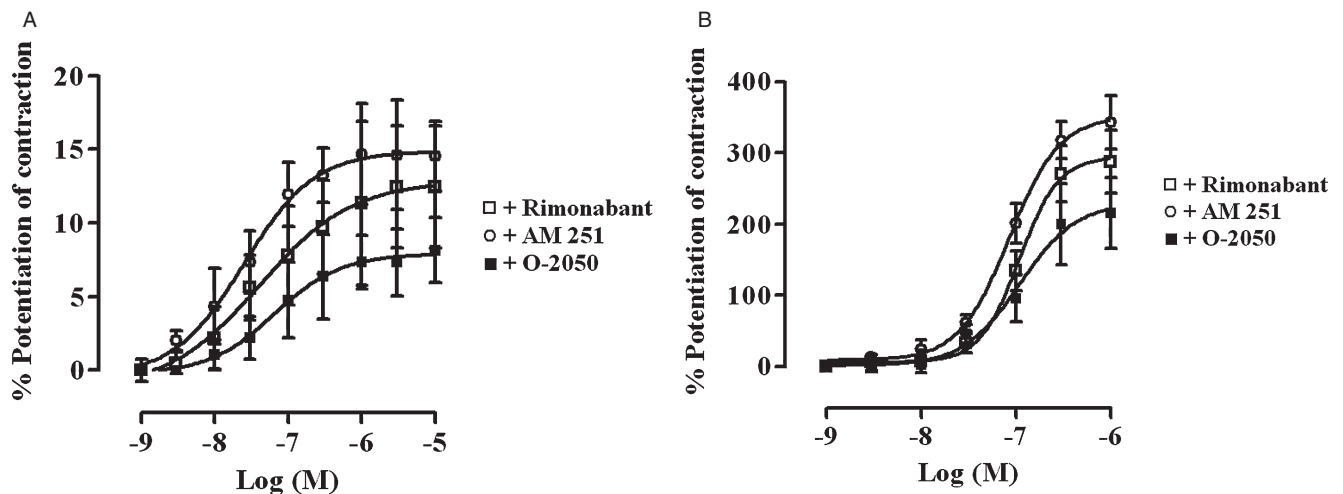


Figure 4 Concentration–response curves for the enhancement of the electrical field stimulation (EFS)-evoked contractions of the (A) rat and (B) guinea-pig myenteric plexus-longitudinal muscle (MPLM) by rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide), AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and O-2050 [(6aR,10aR)-3-(1-methanesulphonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6-H-dibenzo[b,d]pyran]. The rat and guinea-pig MPLM were subjected to single electrical pulses of 0.5 ms duration, 110% supramaximal voltage at a frequency of 0.05 and 0.1 Hz respectively. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value for the increase in the EFS-evoked contractions expressed as a percentage of the amplitude of the contractions measured immediately before the addition of the same cannabinoid to the organ bath ($n = 6$). Vertical lines indicate the SEM.

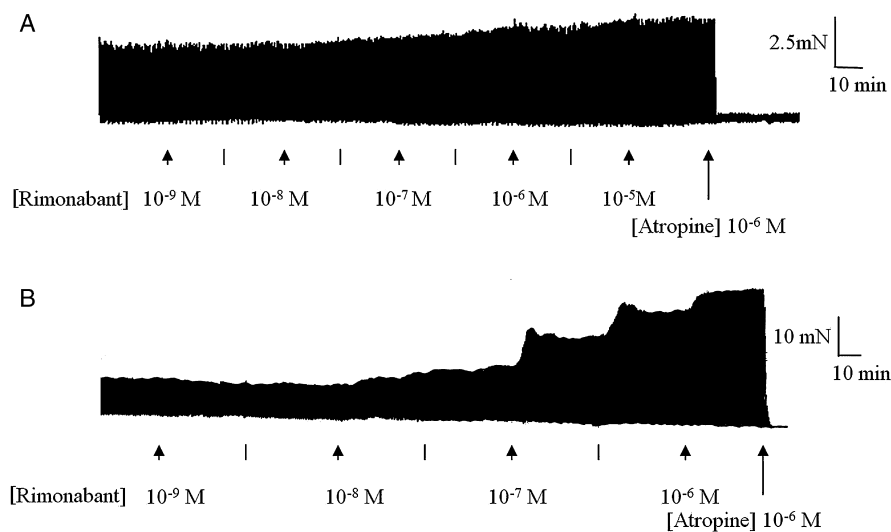


Figure 5 Representative traces of the electrical field stimulation (EFS)-evoked contractions of the (A) rat and (B) guinea-pig myenteric plexus-longitudinal muscle (MPLM) in the absence and presence of rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide), added in half-logarithmic unit increments. Lines represent intermediate concentrations. The rat and guinea-pig MPLM were subjected to single electrical pulses of 0.5 ms duration, 110% supramaximal voltage at a frequency of 0.05 and 0.1 Hz respectively. Note the inhibition of the rimonabant-enhanced EFS-evoked contractions by atropine (10^{-6} M).

curve in both the rat and guinea-pig ileum MPLM with no change in the E_{\max} or slope (Figure 6). The presence of these concentrations of AA-5HT, PMSF and URB-597 increased the potency of AEA by 2.0, 22.1 and 20.2 times, respectively, on the rat ileum MPLM and by 3.2, 32.4 and 48.8 times, respectively, on the guinea-pig ileum MPLM. By contrast, pretreatment of both MPLM tissues for 15 min with AA-5HT (10^{-6} M), PMSF (10^{-4} M) or URB-597 (3×10^{-8} M) had no effect on the location and E_{\max} of the WIN 55,212-2 concentration–response curve (Figure 6).

Effect of VDM-11 alone and on the inhibitory potency of AEA on the EFS-evoked contractions

Cumulative addition of VDM-11 (10^{-9} – 10^{-5} M) every 15 min had no effect on basal tension or EFS-induced contractions of rat and guinea-pig ileum MPLM (data not shown, $n = 6$). Concentrations of VDM-11 higher than 10^{-5} M were not investigated. Pre-incubation of the tissues for 15 min with VDM-11 (10^{-5} M) did not cause a shift in the AEA concentration–response curve on either tissues (Figure 7).

Table 1 A comparison of the potency (pEC₅₀) and tissue maximal response (E_{max}) and the ratio of the E_{max} values of the enhancement of the EFS-evoked contractions of the rat and guinea-pig ileum MPLM by the CB₁ receptor antagonist/inverse agonists rimonabant, AM 251 and O-2050

Cannabinoid	Rat MPLM		Guinea-pig MPLM		$\frac{E_{max(\text{guinea-pig})}(\%)}{E_{max(\text{rat})}(\%)}$
	pEC ₅₀	E _{max} (%)	pEC ₅₀	E _{max} (%)	
Rimonabant	7.46 ± 0.04	12.5 ± 4.1*	6.97 ± 0.01	287.8 ± 43.9*	23.1
AM 251	7.60 ± 0.02	14.6 ± 2.3*	7.07 ± 0.01	343.0 ± 37.5*	23.5
O-2050	7.19 ± 0.01	8.2 ± 2.2*	6.96 ± 0.03	215.5 ± 50.2*	26.4

spEC₅₀ and E_{max} values were derived by non-linear regression analysis for the rat and guinea-pig MPLM strips stimulated electrically with single pulses of 0.5 ms duration, 110% supramaximal voltage at 0.05 and 0.1 Hz frequency respectively.

The asterisk represents a significant ($P < 0.05$, unpaired *t*-test) enhancement of the EFS-evoked contractions over base line contraction level in the absence of the cannabinoids. Where appropriate, values represent the mean ± SEM.

AM 251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; EFS, electrical field stimulation; MPLM, myenteric plexus-longitudinal muscle; O-2050, (6aR,10aR)-3-(1-methanesulphonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6-H-dibenzo[b,d]pyran; rimonabant, N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide.

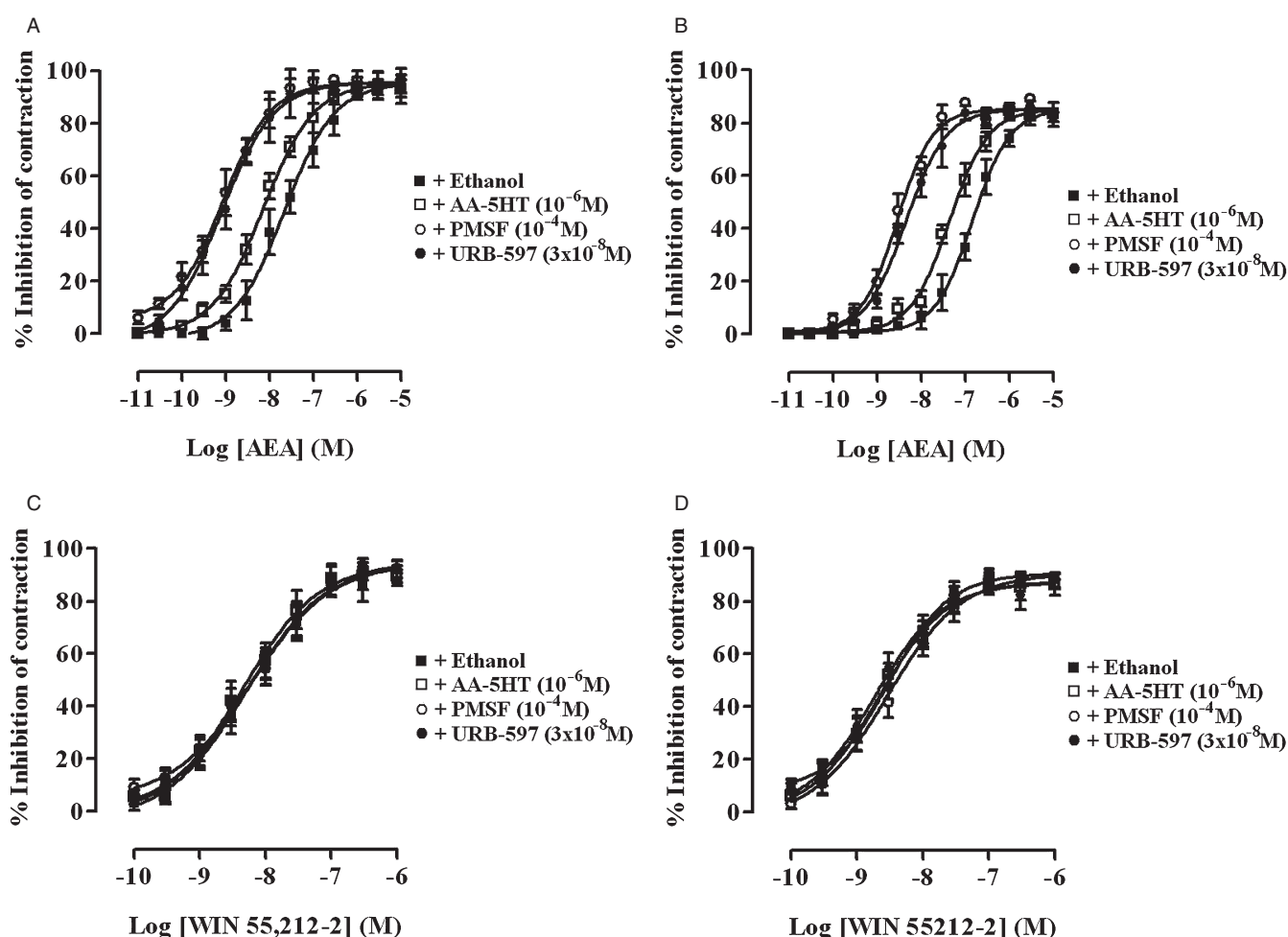


Figure 6 Concentration–response curves for the inhibition of the electrical field stimulation-evoked contractions of the rat (A and C) and guinea-pig (B and D) myenteric plexus-longitudinal muscle (MPLM) by anandamide, arachidonyl ethanolamide (AEA) (A and B) and WIN 55,212-2 [(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate] (C and D), constructed in the presence of ethanol, AA-5HT (arachidonyl-5-hydroxytryptamine), PMSF (phenylmethylsulphonyl fluoride) or URB-597 (3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate). The rat and guinea-pig MPLM were subjected to single electrical pulses of 0.5 ms duration, 110% supramaximal voltage at a frequency of 0.05 and 0.1 Hz respectively. 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 contraction measured immediately before the addition of any drug to the organ bath. Vertical lines indicate SEM, $n = 6$ for each curve. AA-5HT, PMSF or URB-597 or ethanol was added 15 min before the first addition of AEA and WIN 55,212-2.

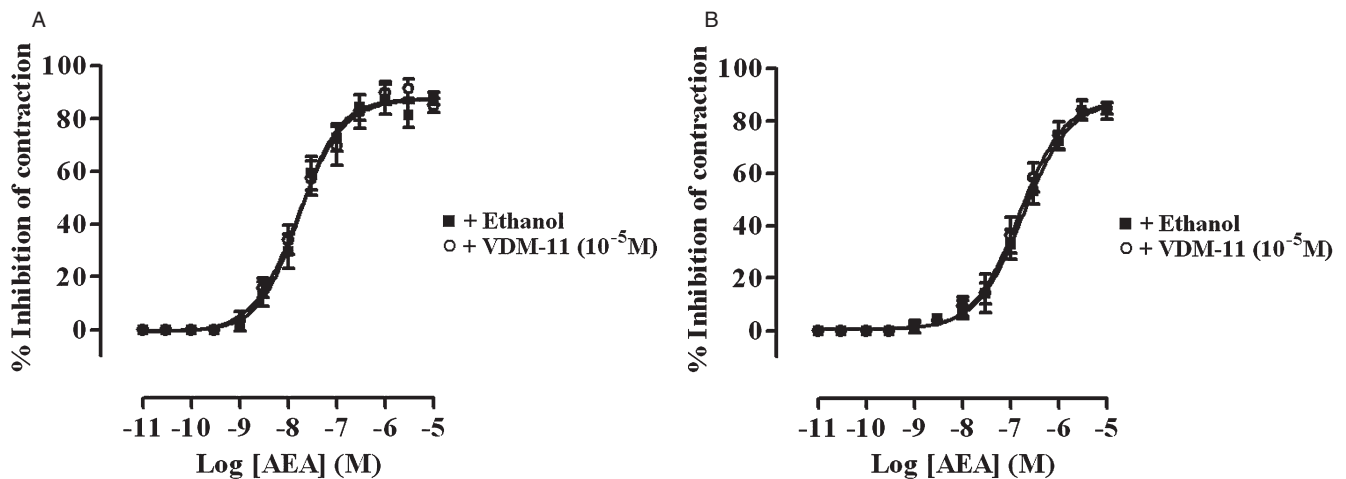


Figure 7 Concentration–response curves for the inhibition of the electrical field stimulation-evoked contractions of the rat (A) and guinea-pig (B) ileum myenteric plexus-longitudinal muscle (MPLM) by anandamide, arachidonylethanolamide (AEA) in the presence of ethanol or VDM-11 [(5Z,8Z,11Z,14Z)-N-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide]. The rat and guinea-pig MPLM were subjected to single electrical pulses of 0.5 ms duration, 110% supramaximal voltage at a frequency of 0.05 and 0.1 Hz respectively. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of contractions expressed as a percentage of the amplitude of the contraction measured immediately before the addition of any drug to the organ bath ($n = 6$). Vertical lines indicate the SEM. VDM-11 or ethanol was added 15 min before the first addition of AEA.

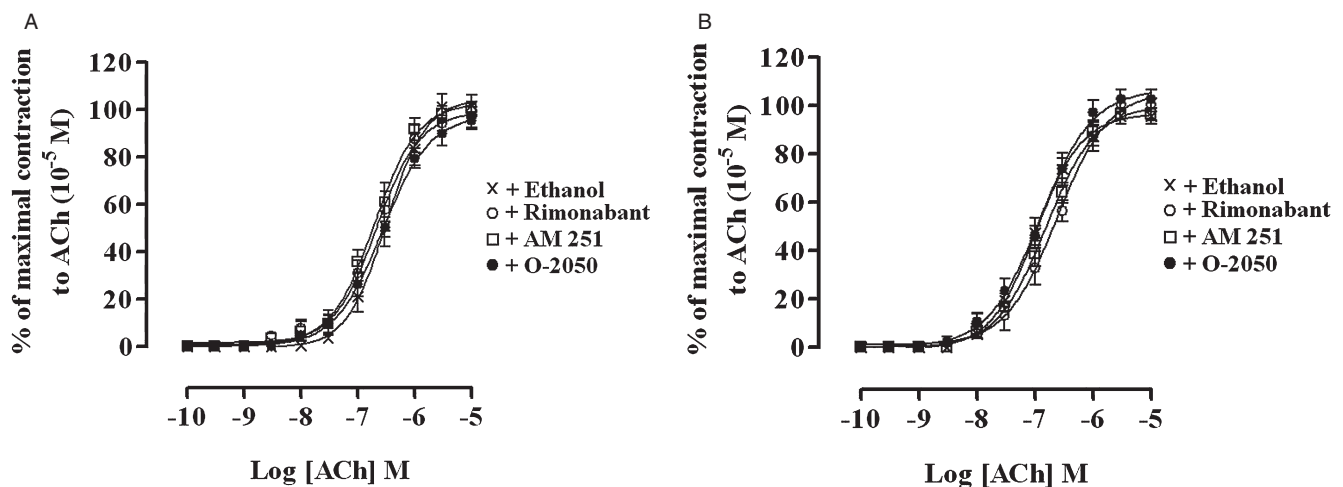


Figure 8 Concentration–response curves for the ACh-evoked contractions of the rat (A) and guinea-pig (B) ileum myenteric plexus-longitudinal muscle in the presence of ethanol, AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (10^{-5} M), rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) (10^{-5} M) or O-2050 [(6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6-H-dibenzo[b,d]pyran] (10^{-5} M). Each curve was fitted by non-linear regression analysis. The amplitude of the contractions to ACh in the presence of ethanol or a cannabinoid was 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. Ethanol or the cannabinoid was added 30 min before constructing the second ACh concentration–response curve; $n = 6$ for all curves. All values represent mean \pm SEM.

Effect of the cannabinoids on the basal tone and on contractions evoked by exogenously applied ACh

The basal tension and amplitude of the contractions of both the rat and guinea-pig MPLM elicited by cumulative addition of ACh (10^{-10} – 10^{-5} M) was not altered in the presence of AEA (10^{-5} M), WIN 55,212-2 (10^{-5} M), rimonabant (10^{-5} M), AM 251 (10^{-5} M) or O-2050 (10^{-5} M). Figure 8 shows the lack of effect of rimonabant (10^{-5} M), AM 251 (10^{-5} M) or O-2050 (10^{-5} M) on ACh-evoked contractions of the rat and guinea-pig MPLM.

Discussion

Consistent with data from previous studies on these tissues (Coutts and Pertwee, 1997; Makwana *et al.*, 2006), rimonabant antagonized the inhibitory effect of both AEA and WIN 55,212-2, and by itself potentiated the amplitude of the EFS-evoked contractions. These effects were shown to be mediated by the CB₁ receptor; the pEC₅₀ values of both agonists and their rank of order are in agreement with those reported previously and the surmountable antagonism by rimonabant

yielded pA₂ values, which are within the range reported at this receptor. There is also evidence that the CB₁ receptors, through which both agonists probably reduce ACh release and so inhibit the EFS-evoked contractions (Coutts and Pertwee, 1997; Mang *et al.*, 2001), are located presynaptically on postganglionic nerves of the myenteric plexus (Lynn and Herkenham, 1994; Coutts *et al.*, 2002). The EFS-evoked contractions were inhibited after blockade of axonal conductance or the muscarinic ACh receptor, but not the nicotinic ACh receptor, and neither agonist had an effect on contractions to exogenous ACh.

The sensitivity of the EFS-evoked contractions augmented by rimonabant, AM 251 and O-2050 to TTX and atropine, and the lack of effect of these cannabinoids on contractions to exogenous ACh suggests that their effect was due to a presynaptic CB₁ receptor-mediated increase in ACh release and not induced by sensitization of the muscle mACh receptor or an anti-ACh-esterase action. Although rimonabant up to 10⁻⁵ M appeared to act selectively through the CB₁ receptor in the MPLM, it can cause non-CB₁ receptor-mediated actions in the micromolar concentration range (White and Hiley, 1998). Although unlikely, it is possible that rimonabant also increases the amount of ACh released, and hence contraction, by blocking inhibitory autoreceptors of other mediators modulating ACh release.

Rimonabant produced an equal rightward shift of the WIN 55,212-2 curve but an unequal downward shift of the basal asymptote on both MPLM tissues. This implies that the antagonist activity of rimonabant is independent of its ability to enhance the EFS-evoked contractions, and therefore, the pA₂ values were not overestimated by the latter property. This reasoning is supported by the finding that physostigmine increased the EFS-evoked contractions, albeit through a different mechanism involving an inhibition of ACh hydrolysis, but did not reduce the potency of WIN 55,212-2.

That all three cannabinoid antagonists were more potent but less efficacious on the rat MPLM could be ascribed to tissue-dependent factors, such as a low stimulus–response coupling capacity, a low tonic release of endocannabinoids (assuming the presence of an endocannabinoid tone) or a lower constitutive activity of the CB₁ receptor (assuming inverse agonism).

The concentration–response curves for all three antagonists had different *E*_{max} values with respect to each other presumably because they behaved as inverse agonists in these tissues as the CB₁ receptor was constitutively active. This is because the *E*_{max} of all three concentration–response curves should have been equal if the potentiation of the EFS-evoked contractions by each cannabinoid were due to a displacement of endocannabinoid agonists from the CB₁ receptor, assuming that the amount of displacement was equal.

As the concentrations of all three antagonists were about three logarithmic units higher than their respective pK_B values at the CB₁ receptor, it is unlikely that tonically released endocannabinoid agonists bound to the CB₁ receptor already occupied by the antagonist.

It is noteworthy that O-2050 also potentiated the EFS-evoked contractions of both MPLM tissues at concentrations previously shown to be devoid of activity on the EFS-evoked contractions of the mouse vas deferens (Martin *et al.*, 2002).

Although the reason for this discrepancy is not clear, this finding could be attributed to differences in the tissue, animal species, neurotransmitter released, stimulus–response coupling mechanism or a lower basal constitutive activity of the CB₁ receptor in the MPLM. Previous studies have reported differences in the pharmacology of a number of cannabinoids in different bioassays. For example, AM 630, an analogue of WIN 55,212-2, behaved as an agonist in the guinea-pig MPLM (Pertwee *et al.*, 1996), a competitive antagonist in the mouse vas deferens (Pertwee *et al.*, 1995), an inverse agonist at the human CB₁ receptor in CHO cells (Landsman *et al.*, 1997), but was without inherent activity in the mouse urinary bladder (Pertwee and Fernando, 1996).

The termination of endocannabinoid signalling is regarded as a two-step process, involving the translocation of endocannabinoids released from the extracellular space into cells via a putative uptake transporter, followed by enzymatic hydrolysis, predominantly by FAAH. High levels of a number of endocannabinoids including AEA and 2-AG were detected in both the rat and guinea-pig ileum (Katayama *et al.*, 1997; Valenti *et al.*, 2005; Guagnini *et al.*, 2006). Additionally, their protection from inactivation by cellular uptake or degradation by FAAH inhibited intestinal transit in mice (Pinto *et al.*, 2002; Capasso *et al.*, 2005). Therefore, an attempt was made to unmask the presence of a functional inhibitory endocannabinoid agonist tone in the MPLM using VDM-11, AA-5HT, PMSF and URB-597. VDM-11 up to a concentration almost equal to its pEC₅₀, determined in the RBL-2H3 basophilic and C6 glioma cells (De petrocellis *et al.*, 2000), did not inhibit the EFS-evoked contractions. Similarly, application of all three FAAH inhibitors by themselves, up to concentrations that completely inactivate FAAH and lack affinity for the CB₁ receptor *in vitro* (Deutsch *et al.*, 1997; Bisogno *et al.*, 1998; Kathuria *et al.*, 2003), were also without effect on the EFS-evoked contractions. While these results confirmed that VDM-11, AA-5HT, PMSF and URB-597 did not possess cannabinoid activity in these tissues, they suggested that pharmacologically active endocannabinoids or substrates of the putative uptake transporter and FAAH were not released in response to EFS. These findings support the notion that the CB₁ receptor is constitutively active and that rimonabant, AM 251 and O-2050 are inverse agonists.

Because AA-5HT, PMSF and URB-597, but not VDM-11, potentiated the action of exogenous AEA in both MPLM tissues, it is possible that FAAH, unlike the putative uptake transporter, was present and functional in these tissues. The similar concentration ratios for the increase in potency of AEA by a given FAAH inhibitor in both MPLM preparations implies the presence of the same isoform of FAAH. The lack of an effect of all three FAAH inhibitors on the potency of WIN 55,212-2 confirms that this agonist is not a substrate of FAAH. This supports the deduction that FAAH was present in both tissues and the increase in potency of AEA was specific. These experiments also indicate that the FAAH inhibitors did not augment the sensitivity of the CB₁ receptor to AEA.

Endocannabinoids, including AEA and 2-AG, are known not to be stored in vesicles in nerves following synthesis, but are synthesized and released on-demand in response to nerve stimuli (Di marzo *et al.*, 2004). *In vivo*, PMSF (Mcvey *et al.*, 2003) and AA-5HT (Capasso *et al.*, 2005) delay small intestinal

transit in rats and mice respectively. This effect is thought to be due to a potentiation of the inhibition induced by tonically released AEA and 2-AG because levels of these endocannabinoids were elevated in the small intestine. High levels of AEA and 2-AG were also measured in the guinea-pig MPLM (Guagnini *et al.*, 2006). Hence, the levels of these endocannabinoids would be anticipated to be high in the MPLM during EFS. However, the lack of an inhibitory effect of the FAAH inhibitors on contractions to EFS suggests that the endogenous levels may not be sufficient to maintain a sustained activation of the CB₁ receptor and may explain the antagonist-mediated potentiation of the contractions.

The effect of URB-597 on intestinal transit of rodents *in vivo* has not been studied, but this FAAH inhibitor, at concentrations that abolished FAAH activity, selectively elevated AEA levels in the brain without affecting the levels in the rat duodenum (Fegley *et al.*, 2005). This suggests that the hydrolysis of AEA in the rat may occur by at least two enzymes, and that other endocannabinoids may modulate small intestinal transit in this species. It is possible that different enzymes might exist in different places, with FAAH not being strategically located to modulate endocannabinoid function at the cholinergic junction. Several enzymes including monoacylglycerol lipase and *N*-palmitoylethanolamine hydrolase have been implicated in inactivating endocannabinoids in the small intestine (Ueda *et al.*, 2000; van der Stelt and Di Marzo, 2004). Because FAAH is capable of hydrolysing a broad spectrum of established and putative endocannabinoids, including 2-AG and palmitoylethanolamide, it is unlikely that these cannabinoids tonically activate the CB₁ receptor in the MPLM.

Although the data available indicate that rimonabant augments intestinal motility by acting as an inverse agonist, it is conceivable that by using an isolated preparation, an endocannabinoid agonist tone was not detected because endocannabinoids may behave as hormones *in vivo*. For instance, endothelial cells of the mesenteric vasculature can synthesize AEA (Randall *et al.*, 1996) and these vessels supply blood to the small intestine. Thus, endocannabinoid agonists synthesized in the mesenteric vasculature may tonically activate the CB₁ receptor to inhibit enteric transmission via an endocrine hormonal effect. While these speculations may be valid *in vivo*, they do not appear to apply to the MPLM *in vitro*.

If it is assumed that rimonabant, AM 251 and O-2050 are inverse agonists in the MPLM, as the CB₁ receptor was constitutively active, their ability to enhance EFS-evoked contractions may be explained using the theoretical 'two state' receptor model (Leff, 1995; Kenakin and Onaran, 2002). According to this model, the CB₁ receptor exists in at least two interchangeable states, an active state, in which the receptor is coupled to the effector pathways to suppress ACh release, and an inactive state, in which the receptor is not coupled to the effector pathways and thereby would increase ACh release. In the absence of any ligand, both conformations would exist at equilibrium; therefore, the CB₁ receptor would be inhibiting ACh release. However, as inverse agonists, rimonabant AM 251 and O-2050 having preferential affinity for the inactive state would bind to this state and shift the equilibrium to reduce the number of receptors in the active conformation, thereby reversing the suppression of the ACh release.

Taken together, our results suggest that the ability of rimonabant, AM 251 and O-2050 to potentiate the EFS-evoked contraction of the rat and guinea-pig MPLM is through inverse agonism at the CB₁ receptor and not due to antagonism of tonically released endocannabinoid agonists.

Conflict of interest

The authors have no conflict of interest.

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