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 preparation

R Makwana1, A Molleman2 and ME Parsons2

1The Sackler Institute of Pulmonary Pharmacology, King's College London, Waterloo Campus, London, UK and 2School of Health & Human Sciences, Department of Life Sciences, University of Hertfordshire, College Lane Campus, Hertfordshire, UK

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,7,10a-tetraydro-6,6,9-trimethyl-6-H-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.
Introduction

Being a potent antagonist of the cannabinoid CB1 receptor, rimonabant (N-(piperidino)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; MPLM, myenteric plexus-longitudinal muscle) is the most extensively studied CB1 receptor antagonist (Katayama et al., 2002). However, in addition to its antagonist activity, rimonabant has been shown to increase both intestinal motility (Pinto et al., 2002; Capasso et al., 2005) and gastric emptying (Gatley et al., 1998), alone or in combination with other CB1 receptor antagonists (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 mesylate; VDM-11, (S,2Z,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-benzoaxazin-6-yl)-1-naphthalenylmethane mesylate].

Abbreviations: AA-5HT, arachidonyl-5-hydroxytryptamine; AEA, anandamide, arachidonylethanolamide; AM 251, N-(piperdin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; MPLM, myenteric plexus-longitudinal muscle; O-250, (6aR,10aR)-3-(1-methanesulphonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6-H-dibenzo[b,d]pyran; PMFS, phenylmethylsulphonyl 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, (S,2Z,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-benzoaxazin-6-yl)-1-naphthalenylmethane mesylate.
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 ± 1°C), humidity (55 ± 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 containing KH₂PO₄ 1.2, NaHCO₃ 25, D-glucose 11.1, CaCl₂ 2.5, gassed with 95% O₂ and 5% CO₂ at room temperature (21 ± 5°C). The experiments were carried out at 33°C in a 50 or 10 mL organ bath, and working along and around the circumference of whole ileum. Cotton sutures were tied at both ends of the MPLM and the tissue was suspended in Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂ at 33°C. The experiments were carried out at 33°C in a 50 or 10 mL organ bath, and working along and around the circumference of whole ileum. Cotton sutures were tied at both ends of the MPLM and the tissue was suspended in Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂ at 33°C. The experiments were carried out at 33°C in a 50 or 10 mL organ bath, and working along and around the circumference of whole ileum. Cotton sutures were tied at both ends of the MPLM and the tissue was suspended in Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂ at 33°C. The experiments were carried out at 33°C in a 50 or 10 mL organ bath, and working along and around the circumference of whole ileum. Cotton sutures were tied at both ends of the MPLM and the tissue was suspended in Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂ at 33°C.

Exogenous ACh stimulation

If any drug was found to alter the amplitude of the EFS-evoked contractions to a level nearly equal to that produced by rimonabant. 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 prior to 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.

Cumulative concentration–response curves to the cannabinoid receptor agonists were 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, PMSF, 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 prior to 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.
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);

\[
E = \text{Basal} + \frac{E_{\text{max}} - \text{Basal}}{1 + 10^{\log EC_{50}-\log [A]}}
\]

where \( E \) denotes response, \( \log [A] \) the logarithm of the concentration of an agonist \( A \), \( n \), the midpoint slope of the curve, \( \log EC_{50} \) the logarithm of the midpoint location parameter along the concentration axis, and \( E_{\text{max}} \) and Basal the upper and lower asymptotes respectively.

The concentration–response data were plotted as the mean ± 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\textsubscript{2}) using the Gaddum–Schild equation (Schild, 1949). The pA\textsubscript{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\textsuperscript{-4} 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-SHT and URB-597 were purchased from Axonera Life Sciences, Bingham, Nottingham, UK. All other chemicals were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. Atropine, phystostigmine, 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.

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 \( E_{\text{max}} \). Of the two agonists, WIN 55,212-2 was significantly more potent than AEA on both tissues. Non-linear regression analysis yielded a pEC\textsubscript{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 more potent (\( P < 0.05 \), 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\textsuperscript{-7} or 10\textsuperscript{-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 \( E_{\text{max}} \) 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\textsubscript{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.
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 ± 12.3% (data not shown, $n=6$). Further increases in concentration up to $3 \times 10^{-6}$ 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 $E_{\text{max}}$, the $pEC_{50}$ of physostigmine was 7.69 ± 0.01 (data not shown, $n=6$). Treatment of the MPLM with $3 \times 10^{-6}$ M physostigmine potentiated the EFS-evoked contractions by 48.7 ± 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 $E_{\text{max}}$ 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 $E_{\text{max}}$ 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 $E_{\text{max}}$ values were similar in both tissues that is, AM 251 > rimonabant > O-2050.

Because each set of curves in Figure 4 had different $E_{\text{max}}$, and the slopes were not parallel, a direct comparison between the
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$^{-6}$–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
curve in both the rat and guinea-pig ileum MPLM with no change in the $E_{\text{max}}$ 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 ($3 \times 10^{-8}$ M) had no effect on the location and $E_{\text{max}}$ 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\text{max}) and the ratio of the E\text{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

<table>
<thead>
<tr>
<th>Cannabinoid</th>
<th>Rat MPLM</th>
<th>Guinea-pig MPLM</th>
<th>E\text{max}(rat)/E\text{max}(guinea-pig) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rimonabant</td>
<td>7.46 ± 0.04</td>
<td>12.5 ± 4.1*</td>
<td>6.97 ± 0.01</td>
</tr>
<tr>
<td>AM 251</td>
<td>7.60 ± 0.02</td>
<td>14.6 ± 2.3*</td>
<td>7.07 ± 0.01</td>
</tr>
<tr>
<td>O-2050</td>
<td>7.19 ± 0.01</td>
<td>8.2 ± 2.2*</td>
<td>6.96 ± 0.03</td>
</tr>
</tbody>
</table>

spEC50 and E\text{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 baseline 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-6H-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, 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, AA-5HT (arachidonyl-5-hydroxytryptamine), PMSF (phenylmethylsulphonyl fluoride) or URB-597 (3′-carbamoylbiphenyl-3-yl-cyclohexylocarbamate). 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.
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 (10⁻¹⁰–10⁻⁴ 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 (Coulls and Pertwee, 1997; Mang et al., 2001), are located presynaptically on postganglionic nerves of the myenteric plexus (Lynn and Herkenham, 1994; Coulls 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 10−6 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 phystostigmine 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 Emax 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 Emax 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 pKb 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-SHT, 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., 2003), 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-SHT, 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-SHT, 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 mazzo et al., 2004). In vivo, PMSF (Mcvey et al., 2003) and AA-SHT (Capasso et al., 2005) delay small intestinal uptake transporter, was present and functional in these tissues. 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.
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 synthesise AEA (Randall et al., 1996) and these vessels supply blood to the small intestine. Thus, endocannabinoid agonists synthesised 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). Theoretical ‘two state’ models of receptor/ligand interactions assume that other endocannabinoids may modulate 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.

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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.

References


