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Comparison of novel cannabinoid partial agonists and SR141716A in the guinea-pig small intestine

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1 The controversial nature of the CB_1 receptor antagonist, SR141716A, in the guinea-pig small intestine was investigated by comparing it with four analogues of Δ^8-tetrahydrocannabinol (Δ^8-THC): O-1184, O-1238, O-584 and O-1315.

2 These compounds (10–1000 nM) inhibited the electrically-evoked contractions with a rank order of potency of O-1238 > O-1184 > O-584 > O-1315. Log concentration-response curves for O-1238, O-1184 and O-1315 were significantly shifted to the right by SR141716A and the maxima were significantly less than that of the CB_1 agonist, WIN55212-2, an indication of partial agonism.

3 Partial saturation of the triple bond in O-1184 to a *cis* double bond (O-1238) increased its potency as an agonist (pEC_{50} from 6.42 to 7.63) and as an antagonist of WIN55212-2, (pK_B, from 8.36 to 9.49). Substitution of the terminal azide group by an ethyl group (O-584) or removal of the phenolic hydroxyl group (O-1315) had no significant effect on the agonist or antagonist potency. None of these analogues increased the twitch response in a manner resembling that of SR141716A.

4 O-1184 (10 and 100 nM) shifted the log concentration-response curve of WIN55212-2 for inhibition of the twitch responses to the right with pK_B values of 8.29 and 8.38, respectively.

5 We conclude that these Δ^8-THC analogues behave as partial agonists rather than silent antagonists at CB_1 binding sites in this tissue. There was no evidence of antagonism of endocannabinoids thus supporting the hypothesis that, in this tissue, SR141716A is an inverse agonist of constitutively active CB_1 receptors.
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Abbreviations: K_B, dissociation constant; O-1184, 6′-azidohex-2′-yne-Δ^8-tetrahydrocannabinol; O-1238, 6′-azidohex-*cis*-2′-ene-Δ^8-tetrahydrocannabinol; O-584, octyl-2′-yne-Δ^8-tetrahydrocannabinol; O-1315, 1-deoxy-octyl-2′-yne-Δ^8-tetrahydro cannabinol; SR141716A, [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride]; WIN55212-2, {(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)methanone}

Introduction

A significant milestone in cannabinoid pharmacology was the development of SR141716A, a selective antagonist for the CB_1 cannabinoid receptor, having over 100-fold selectivity over the CB_2 subtype and non-cannabinoid receptors and ion channels (Rinaldi-Carmona *et al*., 1994). In the functional isolated myenteric-plexus-longitudinal muscle preparation (MP-LM) of the guinea-pig small intestine, SR141716A was shown to be a potent antagonist (K_B values in the low nanomolar range) of the inhibitory effects of cannabinoid agonists on the contractions evoked by electrical stimulation (Coutts *et al*., 1995; Coutts & Pertwee, 1997). This inhibitory effect of cannabinoid agonists was shown to be a dose-related, stereoselective presynaptic action on nerve terminals of cholinergic parasympathetic fibres resulting in a reduction in evoked acetylcholine (ACh) release (Coutts & Pertwee, 1997). In addition, an unexpected observation was that SR141716A alone, induced a dose-related effect that was opposite to that seen in response to CB_1 cannabinoid receptor agonists, that is, an increase in the twitch response and a corresponding increase in the evoked release of ACh compared with control values (Coutts *et al*., 1995; Coutts & Pertwee, 1997). Similar inverse agonist effects of SR141716A have since been reported in other isolated intact peripheral preparations (Pertwee & Fernando, 1996; Izzo *et al*., 1998), in the central nervous system (Gifford & Ashby, 1996) and *in vivo* (Richardson *et al*., 1997; Gessa *et al*., 1998; Rubino *et al*., 1998; Colombo *et al*., 1998; Strangman *et al*., 1998). For example, SR141716A induces hyperalgesia in a rodent model of thermal pain (Richardson *et al*., 1997). In some studies, this action of SR141716A *per se* is interpreted as evidence of ongoing endocannabinoid release (Santucci *et al*., 1996; Strangman *et al*., 1998). However, other groups (Bouaboula *et al*., 1997; Landsman *et al*., 1997; MacLennan *et al*., 1998) have found that, in Chinese hamster ovary (CHO) cells expressing high levels of transfected human CB_1 receptors, SR141716A significantly inhibits the basal incorporation of [^{35}S]-GTPγS, whereas cannabinol at 100 times its K_i is without effect, suggesting that there is no ongoing activation of CB_1 receptors (MacLennan *et al*., 1998), therefore, they concluded that the behaviour of SR141716A is due to inverse agonism. While this might be true in cell lines, the inverse agonist action of SR141716A may not explain its behaviour in the many studies on intact tissues where receptor expression is more likely to be at natural levels and there is the possibility of ongoing endocannabinoid activity. Therefore, in the MP-LM preparation the conundrum remains as to whether the effect of SR141716A, in augmenting the evoked responses

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of the ileum, is due to antagonism of an endogenous ligand or to preferential binding to precoupled CB_1 receptors.

O-1184, a synthetic analogue of the natural cannabinoid, Δ^8-tetrahydrocannabinol (Δ^8-THC), was reported to be a potent CB_1 receptor antagonist when dispersed in Tween 80 and used at relatively low concentrations in the MPLM (Ross *et al*., 1998) and in the [^{35}S]-GTPγS binding assay in rat cerebellar membranes (Griffin *et al*., 1999). Hence, O-1184 seemed to be a useful tool with which to elucidate the problem of the action of SR141716A in the myenteric plexus, since, should the increase in evoked response be due to 'silent' antagonism of an endogenous ligand, for example anandamide (Devane *et al*., 1992) or 2-arachidonoyl glycerol (Mechoulam *et al*., 1995), it would be expected that O-1184 would also increase the evoked contractile response. Subsequent ligand binding assays (Griffin *et al*., 1999) and functional measurements of the effects of O-1184 and its analogues on forskolin-stimulated adenylyl cyclase activity in cells transfected with CB_1 or CB_2 receptors (Ross *et al*., 1999) suggest that these cannabinoids may also have low efficacy agonist activity in isolated membrane preparations. The K_B and K_i values found in these binding studies were significantly lower than the K_B values reported for O-1184 for the mouse vas deferens experiments in which Tween 80 was the vehicle. Therefore, in our studies, the vehicle for SR141716A, O-1184 and its analogues was ethanol, in line with the conditions used in radioligand and [^{35}S]-GTPγS binding (Griffin *et al*., 1999) assays and cyclic AMP assays (Ross *et al*., 1999). The analogues of O-1184 used in our study possess changes in the aliphatic side chain of O-1184 molecule (Figure 1), which is an important feature with respect to the affinity and potency of cannabinoid agonists (Martin *et al*., 1995; Griffin *et al*., 1999).

The aim of the present investigation was 2 fold: firstly, to examine more fully the pharmacological properties of ethanolic solutions of O-1184 and its structural analogues in the MP-LM preparation and thus ascertain the structure-activity relationship of these compounds compared with those found in isolated membranes and secondly, by comparing the effects of these analogues with that of SR141716A, to discover evidence that would help to determine the mechanism by which SR141716A produces its inverse cannabimimetic effects in this tissue. The synthetic ligand, WIN55212-2 has been used as the agonist in this investigation since it has been shown to have stereo-specific agonist activity at CB_1 receptors in this tissue and is a commonly used agonist of choice in many cannabinoid receptor studies (Coutts *et al*., 1995; Coutts & Pertwee, 1997).

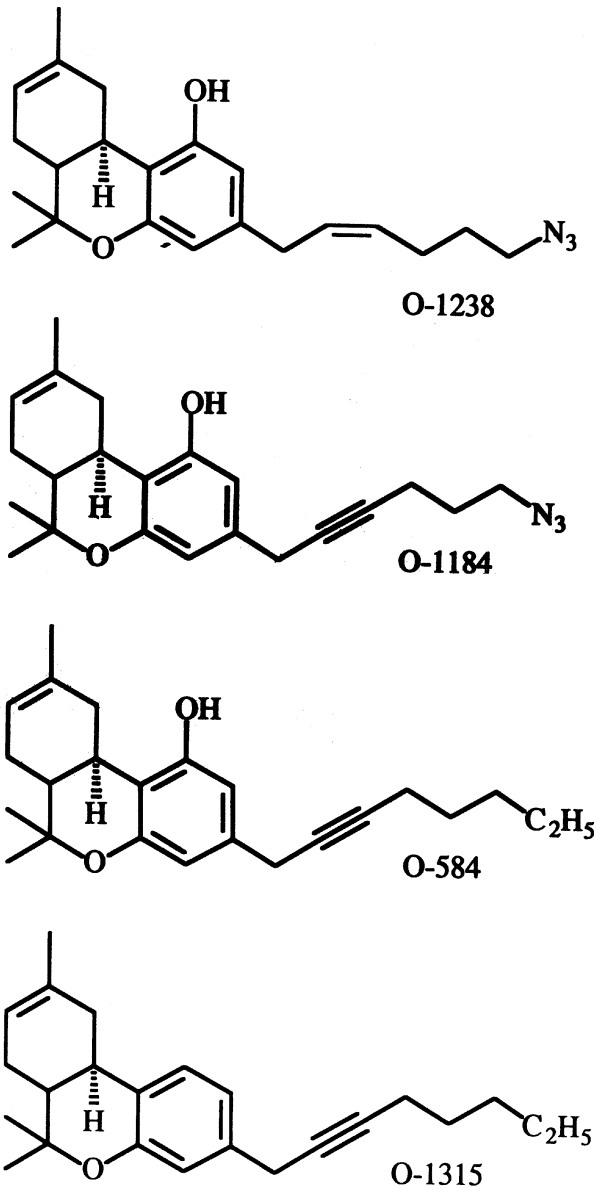

Figure 1 Molecular structures of O-1184, O-1238, O-584 and O-1315.

Methods

Measurement of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation

Strips of myenteric plexus-longitudinal muscle were dissected from the small intestine of male albino Dunkin-Hartley guinea-pigs (290–800 g) by the method of Paton & Zar (1968). For the recording of contractile responses to electrical stimulation, tissues were mounted in 4 ml organ baths under an initial tension of 0.5 g with the method described by Pertwee *et al*. (1996). The baths contained Krebs' solution which was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs' solution was (mM): NaCl 118.2, KCl 4.75, KH_2PO_4 1.19, $NaHCO_3$ 25.0, $CaCl_26H_2O$ 2.54, $MgSO_47H_2O$ 1.19 and glucose 11.0. Isometric contractions were evoked by continuous stimulation at 0.1 Hz with pulses of 110% maximal voltage and 0.5 ms pulse duration from Grass 88 stimulators through platinum electrodes attached to the upper and lower ends of each bath. Contractions were recorded *via* Dynamometer UF1 transducers (Ether) linked to a pen oscillograph (Grass Polygraph 7D). No drug additions were made until the control responses to electrical stimulation were constant.

Log concentration-response curves were constructed cumulatively after pretreatment of the preparation for 30 min with either SR141716A (100 nM) or the equivalent concentration of its vehicle, ethanol. For log concentration-response curves for the effect of WIN55212-2 on the twitch response, a 20-min interval was left between consecutive additions of drug. For log concentration-response curves for O-1184 or its analogues, the dose interval was 30 min. Once a cannabinoid receptor agonist or antagonist had been added, tissues were incubated for several hours without replacing the bath fluid. Time control experiments were conducted both in the presence and absence of vehicle alone several times. The evoked responses showed no significant changes over the time course of an experiment. Control experiments were also performed for concentration-

response curves to WIN55212-2 in naive tissues over a similar time course to antagonist-treated tissues to ensure that the sensitivity to WIN55212-2 was not altered over long periods of time.

The antagonist effect of O-1184 on the inhibition of evoked responses due to WIN55212-2 was determined by two methods. In one method, the MP-LM preparation was incubated with O-1184, or the equivalent concentration of its vehicle, ethanol, for 30 min before a log concentration-response curve to WIN55212-2 was constructed. The second method was by a modification of the 'single dose method' of Kosterlitz & Watt (1968). These authors devised this method in order to examine the kinetic parameters of opiate analgesics having dual agonist and antagonist actions, drugs that are also referred to as partial agonists. However, since these drugs were hydrophilic and their actions were reversible, it was possible to construct a standard agonist (morphine) log concentration-response curve in each experiment, before the exposure of the preparation to the partial agonist. In the present study, since the standard agonist drug (WIN55212-2) is essentially unable to be reversed by washing of the tissue, it was necessary to use a standard curve which had been constructed in other preparations for the analysis (Figure 2). The 'single dose method' consists of choosing a dose of partial agonist that will depress the twitch by 20–60% and preferably by 30–40% (Figure 3). The EC_{50} value is then determined by extrapolation by assuming that the slopes of the log concentration-response curves of WIN55212-2 and the partial agonist were not significantly different. Because the slopes of dose-response curves of partial agonists are likely to be less steep than WIN55212-2, the EC_{50} values obtained by this method might be expected to be too low; alternatively, values obtained with the 'multiple-dose method' may be expected to be too high due to the interaction between doses. Complete dose-response curves determined with a single dose per tissue of O-1184, O-1238, O-584 or O-1315 were not constructed due to the small amounts of analogues available and because of the large numbers of animals which would be required. The Kosterlitz & Watt (1968) method is valid only if the interaction between the partial agonist and the standard drug is competitive. We sought to validate this assumption by using the alternative method to demonstrate a parallel rightward shift of the dose-response curve to WIN55212-2 in the presence of O-1184 as described above.

In order to determine the antagonist activity (K_B), of these drugs, a concentration of WIN55212-2 (W_1), which would have been expected to produce the same depressant effect on the twitch as the single dose of partial agonist, was read off the standard agonist dose-response curve (Figure 2). After the preparation had been exposed to the partial agonist for 30 min, WIN55212-2, in a concentration (W_3) was used to depress the twitch further (Figure 3). The agonist concentration (W_2) which would have a depressant effect equal to the combined actions of the partial agonist and the agonist present in the organ bath was read off the standard curve. The concentration of WIN55212-2 (W_2) used in the presence of the antagonist was such that the residual contraction was inhibited by at least 20% and the total contraction was depressed by not greater than 80%. The dose ratio (DR, see 'Analysis of data') was expressed, thus:

$$DR = \frac{W_3}{W_2 - W_1}$$

K_B was calculated from the equation:

$$K_B = \frac{\text{partial agonist concentration}}{DR - 1}$$

The 'effective antagonist potency', (P_a), which takes into account the agonist activity of a partial agonist, was expressed as:

$$P_a = \frac{1}{K_B} / \frac{1}{EC_{50}} = \frac{EC_{50}}{K_B}$$

$P_a = DR - 1$ when the partial agonist is used in the concentration which has an agonist effect equal to its own EC_{50}. Since P_a is calculated from the means of EC_{50} and K_B, the differences between P_a values were not treated statistically. P_a should not be confused with the term, pA_2, which is sometimes used as a measure of antagonist potency only and is the negative logarithm of K_B.

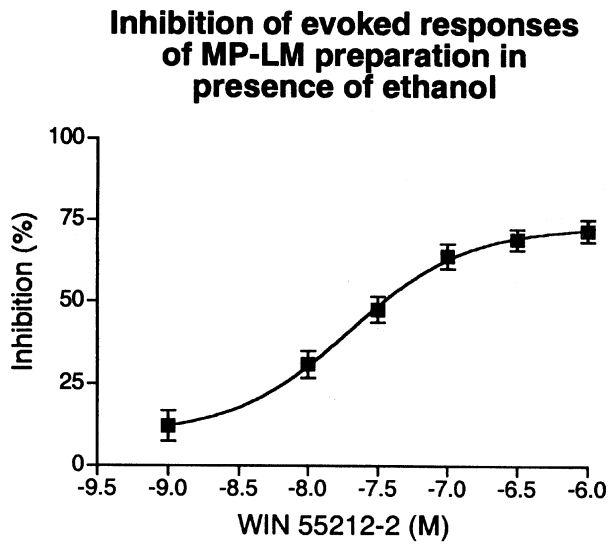

Figure 2 Log concentration curve for the inhibition of the evoked contractile responses of the guinea-pig myenteric plexus-longitudinal muscle preparation by WIN55212-2 in the presence of the vehicle equivalent of SR141716A (100 nM) ($n = 16$). Data points are the mean ± s.e.mean.

Drugs

WIN55,212-2 {(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl) methanone} from Sanofi Winthrop was stored as an ethanolic solution then mixed with two parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline) as described previously (Pertwee *et al.*, 1992). SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamine hydrochloride was obtained from Sanofi. 6′-azidohex-2′-yne-Δ^8-tetrahydrocannabinol (O-1184; Figure 1), 6′-azidohex-*cis*-2′-ene-Δ^8-tetrahydrocannabinol (O1238; Figure 1), octyl-2′-yne-Δ^8-tetrahydrocannabinol (O-584; Figure 1) and 1-deoxy-octyl-2′-yne-Δ^8-tetrahydrocannabinol 6′ (O-1315; Figure 1) were gifts from Dr Razdan, Organix, Inc., Woburn, MA, U.S.A. O-1184 and its analogues were stored in ethanolic solutions of 1 mg/ml at −20°C and diluted in saline as required.

Analysis of data

Values are expressed as means and limits of error as standard errors of the mean or as 95% confidence limits. Inhibition of the electrically-evoked twitch response is expressed in percentage terms and has been calculated by comparing the

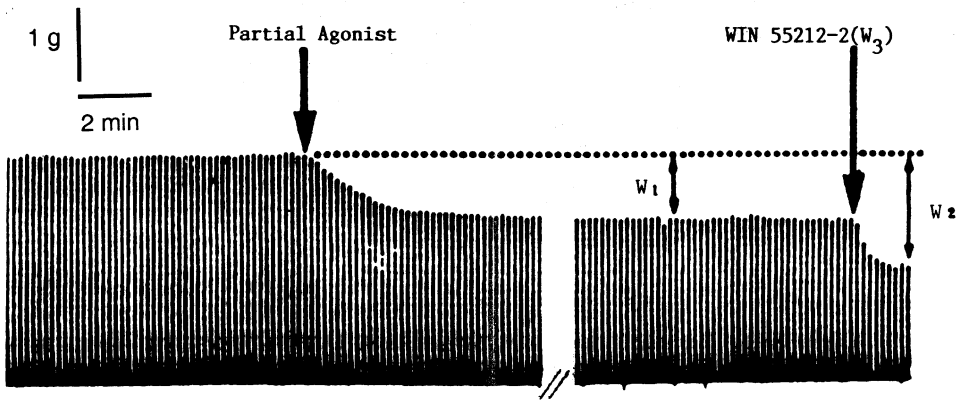

Figure 3 Diagrammatical representation of the measurement of the antagonist activity of a partial agonist by determination of its equilibrium dissociation constant, K_B. Isometric recording of the electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. At the arrow marked Partial Agonist, a single dose of the partial agonist was added to the organ bath and produced an inhibition of the twitch equal to the inhibition caused by WIN55212-2 in a concentration W_1. WIN55212-2 was added 30 min later to give a concentration, W_3. The total depression was equal to a reduction in the height of the twitch due to WIN55212-2 in a concentration, W_2, in the absence of any antagonism. Dose ratio, $DR = W_3/(W_2 - W_1)$.

amplitude of the twitch response after each addition of a twitch inhibitor with its amplitude immediately before the first addition of the inhibitor. Levels of significance for differences between two sets of results were calculated by the paired or unpaired Student's *t*-test as appropriate. The method of determination of dissociation constant (K_B) has been described previously (Pertwee *et al*., 1996). K_B values have been calculated from the equation $(DR-1) = B/K_B$, where DR (dose ratio) is the dose of an agonist that produces a particular degree of inhibition in the presence of an antagonist at a concentration, B, divided by the dose of the same agonist that produces an identical degree of inhibition in the absence of the antagonist. Dose ratios have been calculated from mean EC_{50} values as determined from non-linear regression analysis with GraphPad Prism (Graphpad Software, San Diego, CA, U.S.A.). For the effect of SR141716A on the agonist activity of O-1184 and its analogues, dose ratio values and their 95% confidence limits were determined by symmetrical (2 + 2) dose parallel line assays (Colquhoun, 1971), using pairs of agonist concentrations located on the steepest part of each log concentration-response curve. This method was also used to establish whether any pairs of log concentration-response curves showed significant deviation from parallelism. Maxima of log concentration-response curves for O-1184, O-1238, O-1315 and O-584 were compared by one-way analysis of variance (ANOVA, followed by Newman-Keuls test, GraphPad Prism).

Results

Agonist activity of O-1184, O-1238, O-584 and O-1315

Log concentration-response curves for the inhibition of evoked responses by O-1184, O-1238, O-584 and O-1315 were constructed after pretreatment of the MP-LM preparation with either SR141716A (100 nM) or the equivalent concentration of its vehicle (Figure 4a–d). These curves indicate that all these compounds display some degree of dose-related agonist activity in the MP-LM preparation. Compared with the log concentration-response curve to the standard cannabinoid receptor agonist, WIN55212-2 (Figure 2), the slopes and maxima of these curves are significantly reduced (for differences in maxima, *P* values ranged from <0.0005 for O-584 to <0.013 for O-1238, unpaired *t*-test), indicative of their low efficacy and partial agonism. The slopes of the curves for O-1184 appear to be steep, but it is not possible from these data alone, to be sure where the maxima lie. Higher concentrations of O-1184 were not used due to lack of availability of drugs and also to avoid non-specific effects due to high concentrations of vehicle. There was no significant difference between the maxima of the log concentration response curves for the various analogues over the concentration range that was used (ANOVA, $P<0.05$). For O-1184, O-1238 and O-1315, the log concentration curves were significantly shifted to the right by SR141716A (100 nM) ($P<0.05$; 2 + 2 symmetrical parallel line assay). The corresponding dose ratios were: for O-1184, 4.50 (95% C.I 0.38 and 81.4), for O-1238, 5.41 (2.55 and 12.55) and for O-1315, 2.49 (1.37 and 8.20). The log concentration-response curve for O-584, though dose-related, did not appear to be sigmoid in shape and the slope seemed to be increased in the presence of SR141716A although in these experiments the changes were not statistically significant. This was the only analogue for which the maximum response, over the concentration range that was investigated, did not exceed 50% inhibition of the twitch.

In three out of six experiments, on completion of the concentration-response curve for O-1184, when the bath concentration of O-1184 was 1 μM, SR141716A (100 nM) was added. In each experiment SR141716A caused a complete reversal of the inhibitory agonist effect of O-1184 to at least the control response. This reversal effect of SR141716A supports the observation that the concentration-response curve to O-1184 is shifted significantly to the right by SR141716A. A similar reversal by SR141716A of the agonist effect was also seen in tissues that had been exposed to O-1238 up to 1 μM ($n = 6$) (data not shown).

The results with O-1184, O-1238 and O-1315 suggest that the inhibition of evoked responses by these compounds is mediated by CB_1 receptor activation. At no concentration tested did they display any sign of inverse agonism. This contrasts with the effect of SR141716A (100 nM) which elicits an increase in the electrically-evoked responses (Figure 5).

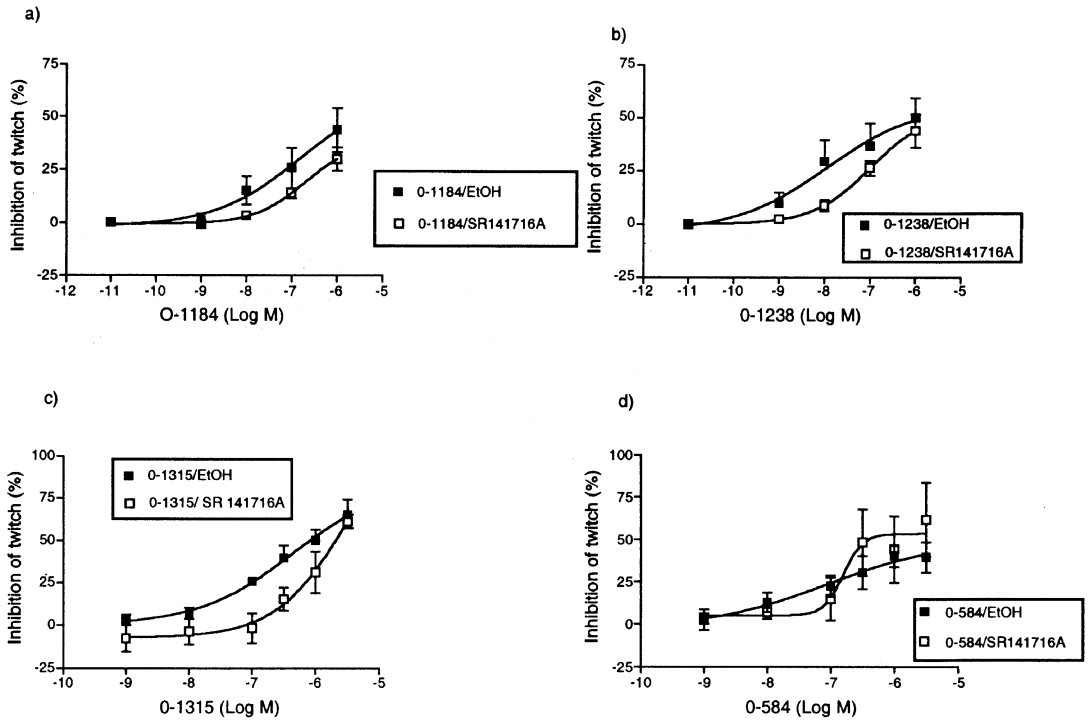

Figure 4 Log concentration response curves for the inhibition of the evoked contractile responses of the myenteric plexus - longitudinal muscle preparation by O-1184 ($n=5$), O-1238 ($n=6$), O-1315 ($n=6$) and O-584 ($n=4$) in the presence of SR141716A (100 nM) or its vehicle control, ethanol (Prism, GraphPad). Data points are the mean ± s.e.mean. There was a significant rightward shift ($P<0.05$) of the curves to O-1184, O-1315 and O-1238 in the presence of SR141716A compared with curves determined in the presence of ethanol (2 + 2 parallel line assay).

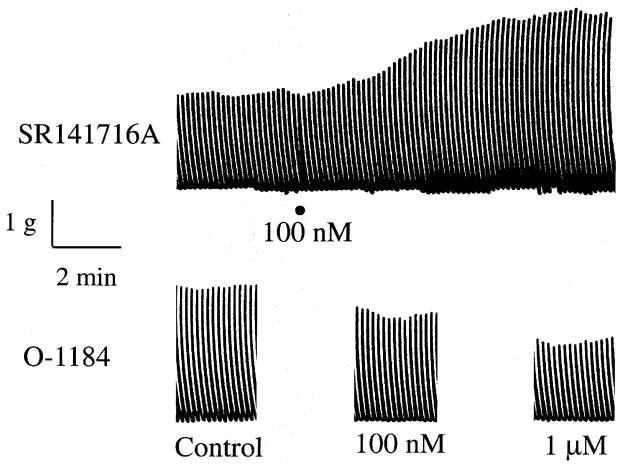

Figure 5 Changes in evoked contractile responses of the guinea-pig myenteric plexus-longitudinal muscle preparation due to SR141716A or O-1184. Upper trace: SR141716A was added at the dot (●) to give a final bath concentration of 100 nM. The lower trace shows the evoked responses following 30 min incubation of preparations with sequential treatments of O-1184, 100 nM and 1 μM. Both preparations were taken from the same animal.

The agonist and antagonist activity and effective antagonist potencies (P_a) of O-1184, O-1238, O-584 and O-1315

Since O1184 and its analogues appear to be partial agonists, the agonist and antagonist properties of these ligands were determined by the 'single dose method' of Kosterlitz & Watt (1968) as described in 'Methods.' In this procedure, the inhibitory response on the evoked contractile responses of the ileum due to a single dose of WIN55212-2 was determined after pretreatment of the preparation with a single addition of the partial agonist. The results of these experiments are outlined in Table 1. For both the EC_{50} (agonist activity) and K_B values (antagonist activity), the rank order of potencies was the same. The replacement of the carbon-carbon triple bond in the aliphatic side-chain of O-1184 with a more flexible double bond with a *cis* configuration (O-1238) increased the potency as both agonist ($P<0.05$; unpaired *t*-test) and antagonist ($P<0.0001$; unpaired *t*-test), however, the effective antagonist potency (P_a) remained essentially the same as the parent compound. Substitution of the azide group of the terminal carbon of O-1184 with an ethyl group (O-584) or loss of the phenolic hydroxyl (O-1315) had no statistically significant effect on the agonist and antagonist potencies of O-1184. For these two compounds, there was a tendency for the antagonist potency to be reduced more than the agonist potency which resulted in a reduction in the P_a values indicating around 50% reduction in their ability to act as antagonists at the CB_1 receptor.

The effects of O-1184 and SR141716A on log concentration-response curves to WIN55212-2

Log concentration-response curves for the inhibition of evoked responses by WIN55212-2 were constructed after pretreatment of the MP-LM preparation with O-1184 at concentrations of either 10 or 100 nM. These curves were compared with those constructed after pretreatment with the selective CB_1 receptor antagonist SR141716A (100 nM) or the equivalent concentration of the vehicle, ethanol, alone (Figure 6). In this series of experiments, the addition of O-1184 (10 nM) had a small but insignificant effect on the twitch, but at the higher dose

Table 1 Kinetic parameters of O-1184 and its analogues as measured by the 'single dose method' of Kosterlitz & Watt (1968)

Cannabinoid	EC_{50} (nM)	pEC_{50}	K_B (nM)	pK_B	P_a
O-1315	767±214.3 (6)	6.18±0.14	25.9±13.6	7.89±0.23	45.5
O-584	537.5±170 (4)	6.34±0.15	9.16±3.97	81.9±0.22	58.7
O-1238*	41.4±18.0 (5)	7.63±0.25	0.39±0.13	9.49±0.13	106.2
O-1184	482±166.4 (5)	6.42±0.15	4.43±0.39	8.36±0.04	108.8

Means and s.e.means of agonist activity (EC_{50}) and antagonists activity (K_B). Number of experiments are given in parenthesis. The values of effective antagonists potency, P_a were calculated from the means of EC_{50} and K_B. Cannabinoid concentration was 100 nM. *Due to potent agonist activity, concentration of 0-1238 was 5 nM.

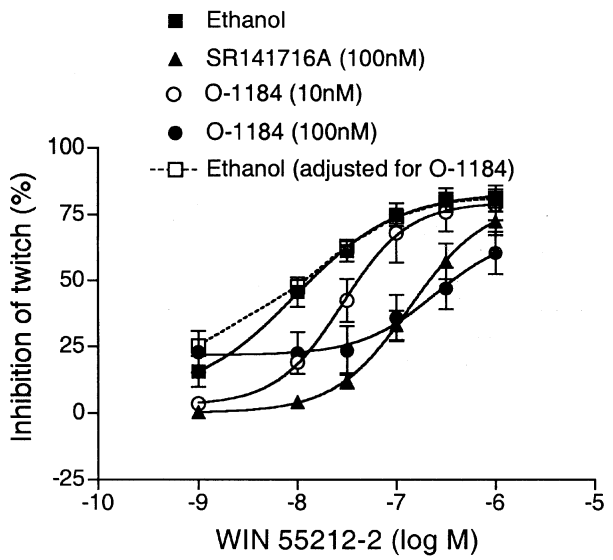

Figure 6 Log concentration-response curves for the inhibition of the evoked contractile responses of the guinea-pig myenteric plexus-longitudinal muscle preparation by WIN55212-2 in the presence of SR141716A (100 nM; $n=6$), its vehicle equivalent ($n=6$) and O-1184 (10 nM; $n=3$ and 100 nM; $n=8$). In order to take into account the agonist activity due to O-1184 (100 nM), the values of inhibition due to WIN55212-2 plus the WIN55212-2 equivalent of 100 nM O-1184 were determined from the control curve for WIN55212-2 alone to generate another curve (dotted line) 'Ethanol (adjusted for O-1184)' with which the O-1184 curve can be directly compared. Data points are the mean±s.e.mean.

Table 2 Effects of CB_1 antagonists on log dose-response curves to WIN55212-2 on myenteric plexus-longitudinal muscle preparations from the guinea-pig ileum

Conditions	EC_{50} (nM)	*DR*	K_B (nM)	pK_B
Ethanol	9.78±1.23* (6)	–	–	–
Ethanol adjusted for O-1184	12.89±1.04 (6)	–	–	–
SR141716A (100 nM)	138.4±1.04 (6)	14.15	7.6	8.119
O-1184 (10 nM)	28.88±1.06 (3)	2.96	5.12	8.291
O-1184 (100 nM)	246.2±1.55 (8)	19.1	5.52	8.258

EC_{50} values (means±s.e.mean) were calculated from Prism (GraphPad). Number of experiments are given in parenthesis. *This value is not significantly different ($P>0.2$) from the EC_{50} obtained for WIN55212-2 after incubation in Kreb's solution alone (10.81±1.26 nM, $n=7$).

(100 nM) inhibited the twitch by 20.24±6.93% (mean±s.e.mean; $n=8$). This value corresponds almost exactly to that obtained for 100 nM O-1184 (20.61±6.85%; $n=9$) when the cumulative log concentration-response curve for the agonist action of this drug was constructed (see above). In order to adjust for this effect, the concentration-response curve for WIN55212-2 in the presence of O-1184 (100 nM) was compared with a different vehicle control curve (ethanol adjusted for O-1184). In this curve, the WIN55212-2-equivalent of the agonist action of O-1184 (100 nM) was added to each point on the control curve constructed in the presence of ethanol. In the presence of SR141716A and O-1184 (10 nM), that have little or no agonist action, the responses to low concentrations of WIN55212-2 were completely abolished and the curve was significantly shifted in parallel to the right. When the curve that was constructed in the presence of O-1184 (100 nM) was compared with the ethanol curve adjusted for the agonist activity of this drug, the steep part of this curve was shifted even further to the right than those with SR141716A and O-1184 (10 nM), indicating its greater antagonist action and higher dose ratio. It would be expected that this concentration of O-1184 would also completely abolish the responses to the lowest concentrations of WIN55212-2. Therefore, it is concluded that the twitch inhibition observed at these lower concentrations of WIN55212-2 are due to the intrinsic agonist activity of O-1184 itself at this concentration. The EC_{50} values and corresponding dose ratios, K_B values and pK_B values were calculated and are given in Table 2. A comparison of K_B values indicates that O-1184 is at least as potent as SR141716A as a CB_1 receptor antagonist in this tissue, that the K_B value is similar irrespective of the concentration of O-1184 used and is very similar to the value obtained with the 'single dose' method.

Discussion

The purpose of this study was an attempt to elucidate the mechanism by which SR141716A increases the evoked responses of the myenteric plexus-longitudinal muscle preparation by comparing its effects with those of a novel series of Δ^8-THC analogues. The CB_1 receptor ligand, O-1184, which had previously been reported to be a potent CB_1 receptor antagonist, was found to have weak agonist activity in inhibiting adenylate cyclase activity of brain isolated membranes (Ross *et al*., 1999).

In this study, we have demonstrated that O-1184 has both agonist and antagonist properties in the MP-LM preparation of the guinea-pig small intestine. We also investigated the effects of certain changes in the aliphatic side chain of this molecule, an important site for structure-activity relationships, on the pharmacological profile of this compound. By comparing the activities of this series of analogues with the activity of SR141716A, we endeavoured to find evidence supporting the hypothesis that augmentation in the evoked responses of this tissue due to SR141716A is due to antagonism of an endogenous ligand for CB_1 receptors.

Log concentration-response curves for O-1184, and its analogues demonstrated a dose-related inhibition of the electrically-evoked contractions in this tissue. Due to the limited range of concentrations of these drugs that could be used, it is not possible from these data alone, to be sure where the maxima lie. However, the apparent significant reduction of the maxima for all these curves compared with that of WIN55212-2 supports the conclusion that these drugs behave as partial agonists at the CB_1 receptors (Ross *et al*., 1999) rather than 'silent' antagonists (Ross *et al*., 1998). The agonist effects for all analogues except O-584 were significantly and competitively antagonized, by SR141716A, indicative of a CB_1 receptor site of action. The results with O-584 were somewhat variable, but the number of observations was limited by availability of the compound. This group of data supports similar findings for the antagonism of O-1238 by SR141716A in the [^{35}S]-GTPγS binding assay in rat cerebellar membranes (Griffin *et al*., 1999) and extends them to include O-1184, O-584 and O-1315. Increasing the flexibility of the aliphatic side-chain, by partial saturation of the triple carbon-carbon bond to a *cis*-double bond (O-1238), caused a 10-fold increase in the potency of the molecule both as an agonist and antagonist. These data confirm earlier findings in neuronal membranes (Griffin *et al*., 1999; Ross *et al*., 1999) that the greater flexibility of this side-chain confers a more favourable conformation that may be integral to the intrinsic efficacy rather than the affinity for the receptor. The inhibitory effects of O-1184 and O-1238 were reversed by the subsequent treatment with SR141716A. Assuming the K_B value for O-1184 of 4.4 nM to be correct, then, from the Schild equation, we would expect the dose ratio due to the antagonist action of O-1184 (1 μM) to be of the order of 230. Therefore it is likely that any endocannabinoids present under these conditions would be completely antagonized by O-1184, before the addition of SR141716A.

Removal of the phenolic hydroxyl group (O-1315) or replacement of the azide group with an ethyl group (O-584) did not significantly increase the potency of O-1184, hence the rank order of potency as either agonists or antagonists was the same for both characteristics and supports previous findings for these ligands in binding studies. In this study, the concentration-response curve for WIN55212-2 in the presence of O-1184 (100 nM) was compared with an adjusted control curve in the presence of vehicle alone, i.e. one in which the response is corrected for the agonist effect of O-1184. Here, the responses to low concentrations of WIN55212-2, that are abolished in curves constructed in the presence of SR141716A or O-1184 (10 nM) appear to have been replaced by inhibition due to the agonist activity due to the higher concentration of the partial agonist. At the same time, the antagonism by O-1184 (100 nM) shifts the curve even further to the right. The K_B values measured in this study for the ability of O-1184 to antagonize WIN55212-2 at the CB_1 receptor showed it to be at least as potent as SR141716A and were consistent irrespective of the concentration of O-1184 or the method used in this study; furthermore, they agreed well with that reported for ethanolic solutions of O-1184 in the [^{35}S]-GTPγS binding assay (equilibrium dissociation constant, K_B 3.40 nM, Griffin *et al*., 1999). The internal consistency of the K_B values for O-1184, and compliance of these results with those of other studies supports the validity of the application of the 'single-dose' method (Kosterlitz & Watt, 1968) to assess the agonist and antagonist potencies of partial agonists to cannabinoid drugs in this tissue. The use of this technique extends the findings of the [^{35}S]-GTPγS binding method, which is poor at detecting weak agonist activity under the reported conditions of the assay and hence classifies the triple-bond analogues (O-1184 and O-584) as antagonists rather than partial agonists. The 'single-dose' method also extends the findings from the cyclic AMP assay (Ross *et al*., 1999) in which it was not possible to determine antagonist potency of O-1184 or O-1238 due to their agonist activity. The disadvantage of the 'single dose' method, is that it cannot be used to evaluate the E_{max} of partial agonists, therefore relative efficacies of these ligands in intact isolated tissues must be determined from the maxima of log concentration-response curves. However, a recent review suggests that measurements of maximal effects of partial agonists are of limited value as these measurements are dependent on receptor density, since these ligands behave as antagonists where the receptor density is low or if the method of measuring receptor function is insensitive (Clark *et al*., 1999).

In this study, the ability of SR141716A to antagonize the inhibitory actions of WIN55212-2 in this tissue (Coutts *et al*., 1995; Coutts & Pertwee, 1997) was confirmed and the pK_B values support those in the literature. Whether it is valid to express the antagonist potency of inverse agonists (SR141716A), or partial agonists (O-1184 and its analogues) in the same terms as those used for pure antagonists is an arguable point and is beyond the scope of the present discussion. However, the ability of SR141716A to increase the evoked contractile responses was not mimicked by O-1184 or any of the other analogues at any concentration. Thus, this study did not produce any evidence for the release of endogenous cannabinoid receptor agonists in this tissue under the conditions in which SR141716A shows its facilitatory effect. It is possible that ongoing endocannabinoid release in this tissue exists and the effects of its antagonism are counteracted by the opposing agonist activity of this series of partial agonists.

Our results extend and clarify earlier findings where O-1184 dispersed in Tween 80 appeared to produce pure antagonism of CB_1 receptors in this tissue (Ross *et al*., 1998). These results also confirm and extend the findings of other groups working with hCB_1 receptors in CHO cells (Landsman *et al*., 1997; Bouaboula *et al*., 1997; MacLennan *et al*., 1998) who deduced that the constitutive activity of this receptor, that was unmasked by SR141716A, could not be attributed to an endogenous agonist. We await the discovery of a potent silent CB_1 receptor antagonist to elucidate this problem further.

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