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# Comparison of cannabinoid binding sites in guinea-pig forebrain and small intestine

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**1** We have investigated the nature of cannabinoid receptors in guinea-pig small intestine by establishing whether this tissue contains cannabinoid receptors with similar binding properties to those of brain $CB_1$ receptors. The cannabinoids used were the $CB_1$-selective antagonist SR141716A, the $CB_2$-selective antagonist SR144528, the novel cannabinoid receptor ligand, 6′-azidohex-2′-yne-$\Delta^8$-tetrahydrocannabinol (O-1184), and the agonists CP55940, which binds equally well to $CB_1$ and $CB_2$ receptors, and WIN55212-2, which shows marginal $CB_2$ selectivity.

**2** [$^3$H]-CP55940 (1 nM) underwent extensive specific binding both to forebrain membranes (76.3%) and to membranes obtained by sucrose density gradient fractionation of homogenates of myenteric plexus-longitudinal muscle of guinea-pig small intestine (65.2%).

**3** Its binding capacity ($B_{max}$) was higher in forebrain (4281 fmol mg$^{-1}$) than in intestinal membranes (2092 fmol mg$^{-1}$). However, the corresponding $K_D$ values were not significantly different from each other (2.29 and 1.75 nM respectively). Nor did the $K_i$ values for its displacement by CP55940, WIN55212-2, O-1184, SR141716A and SR144528 from forebrain membranes (0.87, 4.15, 2.85, 5.32 and 371.9 respectively) differ significantly from the corresponding $K_i$ values determined in experiments with intestinal membranes (0.99, 5.03, 3.16, 4.95 and 361.5 nM respectively).

**4** The $B_{max}$ values of [$^3$H]-CP55940 and [$^3$H]-SR141716A in forebrain membranes did not differ significantly from each other (4281 and 5658 fmol mg$^{-1}$) but were both greater than the $B_{max}$ of [$^3$H]-WIN55212-2 (2032 fmol mg$^{-1}$).

**5** O-1184 (10 or 100 nM) produced parallel dextral shifts in the log concentration-response curves of WIN55212-2 and CP55940 for inhibition of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation, its $K_D$ values being 0.20 nM (against WIN55212-2) and 0.89 nM (against CP55940).

**6** We conclude that cannabinoid binding sites in guinea-pig small intestine closely resemble $CB_1$ binding sites of guinea-pig brain and that O-1184 behaves as a cannabinoid receptor antagonist in the guinea-pig myenteric plexus-longitudinal muscle preparation.

**Keywords:** Cannabinoid receptors; myenteric plexus; guinea-pig small intestine; guinea-pig brain; 6′-azidohex-2′-yne-$\Delta^8$-tetrahydrocannabinol; O-1184; cannabinoid receptor antagonists

## Introduction

Results from previous experiments have led to the conclusion that post-ganglionic neurones of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine contain cannabinoid $CB_1$ receptors that can mediate inhibition of electrically-evoked contractions when activated (Pertwee, 1997). In particular, certain $CB_1$ receptor agonists demonstrate high potency as inhibitors of such contractions as well as appropriate stereoselectivity (Pertwee *et al*., 1992). Also, $CB_1$ mRNA has been detected in the myenteric plexus-longitudinal muscle preparation (Griffin *et al*., 1997) and the potencies of cannabinoids as inhibitors of electrically-evoked contractions of this preparation correlate well with their potencies for the displacement of [$^3$H]-CP55940 from specific cannabinoid binding sites in rat brain tissue (Herkenham *et al*., 1990; Pertwee *et al*., 1996). In line with their postulated prejunctional site of action, $CB_1$ receptor agonists have an inhibitory effect on acetylcholine release in the myenteric plexus-longitudinal muscle preparation (Pertwee *et al*., 1996) and the selective $CB_1$ receptor antagonist, SR141716A, markedly attenuates cannabinoid-induced inhibition both of the twitch response and of acetylcholine release with a potency that is consistent with its affinity for $CB_1$ receptors (Pertwee *et al*., 1996).

The main aim of this investigation was to seek out further evidence for the presence of $CB_1$ receptors in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine by looking for cannabinoid binding sites in this tissue and then comparing the binding properties of any such sites with those of $CB_1$ receptors in forebrain membranes. The latter were obtained from guinea-pigs to avoid possible species differences. Since there are no previous peer-reviewed accounts of binding assays with guinea-pig tissue, our initial experiments compared the binding properties in guinea-pig forebrain membranes of three established radiolabelled cannabinoid receptor probes, [$^3$H]-CP55940, [$^3$H]-WIN55212-2 and [$^3$H]-SR141716A. As significantly more animals were needed to provide tissue for binding experiments with myenteric plexus-longitudinal muscle membranes than with forebrain membranes, it was decided not to extend this comparative study to myenteric plexus-longitudinal muscle membranes. Instead, binding experiments with such membranes were carried out with just one probe, [$^3$H]-CP55940. This compound was selected as it has been used in previous cannabinoid binding

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assays far more frequently than [$^3$H]-WIN55212-2 or [$^3$H]-SR141716A (Pertwee, 1997).

Both saturation and displacement assays were performed in our experiments with [$^3$H]-CP55940. For the displacement assays, we used WIN55212-2 and CP55940, which are well-established agonists at both $CB_1$ and $CB_2$ receptors, and SR141716A, a $CB_1$-selective antagonist (Pertwee, 1997). Two additional ligands were used. One of these was the potent $CB_2$-selective antagonist, SR144528, which was included in this study because it has much lower affinity for $CB_1$ receptors than any of the other ligands we used (Pertwee, 1997; Rinaldi-Carmona *et al*., 1998). Its inclusion also allowed us to screen for the presence of $CB_2$ receptors in myenteric plexus-longitudinal muscle membranes. The second of these additional ligands was 6′-azidohex-2′-yne-$\Delta^8$-tetrahydrocannabinol (O-1184) that we had found in pilot experiments to be a potent displacer of [$^3$H]-CP55940 from $CB_1$ binding sites on guinea-pig forebrain membranes. Additional experiments were carried out to establish if O-1184 interacts with the myenteric plexus-longitudinal muscle preparation either to inhibit electrically-evoked contractions or to antagonize the ability of the cannabinoid receptor agonists, WIN55212-2 and CP55940, to inhibit such contractions.

## Methods

### *Drugs*

CP55940 {(−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol} was supplied by Pfizer, 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} by Research Biochemicals International and SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] and SR144528 {N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} by Sanofi Recherche. O-1184 was synthesized in Dr Razdan's laboratory. Clonidine HCl and acetylcholine chloride were obtained from Sigma and normorphine HCl from MacFarlan Smith. [$^3$H]-CP55940 and [$^3$H]-WIN55212-2 were supplied by NEN Life Science Products and [$^3$H]-SR141716A by Amersham International.

### *Myenteric-plexus-longitudinal muscle preparation*

Strips of myenteric plexus-longitudinal muscle were dissected from the small intestine of male albino Dunkin-Hartley guinea-pigs (326–619 g) using the method of Paton and Zar (1968). Each tissue was mounted in a 4 ml organ bath at an initial tension of 0.5 g as described by Pertwee *et al*. (1992; 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 (in mM): NaCl 118.2, KCl 4.75, $KH_2PO_4$ 1.19, $NaHCO_3$ 25.0, $MgSO_4.7H_2O$ 1.29, glucose 11.0 and $CaCl_2.6H_2O$ 2.54. In most experiments, contractions were evoked by electrical field stimulation through a platinum electrode attached to the upper end and a stainless steel electrode attached to the lower end of each bath. These were elicited by single bipolar rectangular pulses of 110% maximal voltage, 0.5 ms duration and 0.1 Hz frequency. Stimuli were generated by a Grass S48 stimulator, then amplified (Med-Lab channel attenuator) and divided to yield separate outputs to four organ baths (Med-Lab StimuSplitter). All contractions were monitored by computer (Apple Macintosh LC or Performa 475) using a data recording and analysis system (MacLab) that was linked *via* preamplifiers (Macbridge) to Dynamometer UF1 transducers (Pioden Controls).

Drugs were added only after the twitch amplitude had become constant. Agonists were added cumulatively, normorphine every min, clonidine every 2 min, WIN55212-2 every 15 min and CP55940 every 30 min. When used, O-1184 was administered 30 min before the first addition of an agonist. Once a drug had been added, tissues underwent electrical stimulation for several hours without the fluid in the bath being replaced. It was not possible to reverse the inhibitory effect of cannabinoids on the twitch response by washing them out of the organ bath. Consequently only one WIN55212-2 or CP55940 concentration-response curve was constructed per tissue. Contractions were sometimes induced by acetylcholine in which case non-cumulative concentration-response curves were constructed with this agonist using 5-min dose-cycles. These were initiated 30 min after the addition of O-1184 or its vehicle.

For addition to organ baths, WIN55212-2, CP55940 and O-1184 were each mixed with two parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline) as described previously for $\Delta^9$-tetrahydrocannabinol (Pertwee *et al*., 1992). Clonidine HCl, acetylcholine chloride and normorphine HCl were dissolved in saline. Drug additions were made in a volume of 10 $\mu$l. In control experiments, Tween 80 was added instead of O-1184. The control dose of Tween 80 was the same as the dose added in combination with the highest dose of O-1184 used.

### *Membrane preparations*

Forebrains (15 to 30 g wet weight) and strips of myenteric plexus-longitudinal muscle (0.5 to 1 g wet weight) from three to four adult male Dunkin Hartley guinea-pigs were suspended in 50 mM Tris buffer (pH 7.4). The tissue was homogenized in 20 ml 0.32 M sucrose/50 mM Tris buffer (pH 7.4) with an Ultra-Turrex homogenizer. Myenteric plexus-longitudinal muscle homogenates were centrifuged at 3000 $g_{av}$ for 30 min and the resultant top layer of fatty tissue removed. The pellet was resuspended using a hand-held homogenizer and the homogenate added to test tubes containing three layers of sucrose (0.84 M, 1.06 M and 1.25 M sucrose). The tubes were then centrifuged at 100,000 $g_{av}$ for 2 h in a Beckman SW40 swing-out rotor. The tissue was distributed into three fractions found respectively at the tissue/0.84 M sucrose interface, the 0.84 M/1.06 M sucrose interface and the 1.06 M/1.25 M sucrose interface. Each fraction was placed in a separate tube, diluted with 50 mM Tris buffer (pH 7.4) and centrifuged at 100,000 $g_{av}$ for 1 h in a fixed angle rotor. Experiments were carried out to determine the level of specific binding in each fraction (see Results and Figure 1). For routine saturation and competition assays the method was later modified using only the 0.84 M and 1.06 M sucrose solutions, the membranes being obtained from the 0.84 M/1.06 M interface. Myenteric plexus-longitudinal muscle from three to four guinea-pigs was required for each 24- or 48-tube binding assay. The final pellets of myenteric plexus-longitudinal muscle membranes were resuspended in 1 to 2.5 ml Tris buffer (50 mM, pH 7.4) to yield a protein concentration of 1 mg ml$^{-1}$. Myenteric plexus-longitudinal muscle membranes were either used on the day of preparation or stored at −80°C for less than 1 week. Forebrain homogenates were centrifuged at 100,000 $g_{av}$ for 1 h. The final pellet was resuspended in Tris buffer (50 mM, pH 7.4) to give a protein concentration of 1 mg ml$^{-1}$ and stored at −80°C for up to 1 month. Forebrain tissue from

three guinea-pigs was sufficient for about 60 binding assays with 48 tubes. All centrifugation procedures were carried out at 4°C.

### *Binding assays*

A filtration procedure was used to measure [$^3$H]-CP55940 binding. This is a modification of the method described by Compton *et al*., (1993). Binding assays were performed with [$^3$H]-CP55940, [$^3$H]-WIN55212-2 or [$^3$H]-SR141716A in 50 mM Tris buffer (pH 7.4) containing 1 mM $MgCl_2$, 1 mM EDTA and 2 mg ml$^{-1}$ bovine serum albumin (BSA). The total assay volume was 500 $\mu$l. Binding was initiated by the addition of forebrain (50–75 $\mu$g) or myenteric plexus-longitudinal muscle membranes (20–40 $\mu$g). Incubations were carried out at 30°C for 90 min before termination by addition of ice-cold wash buffer (50 mM Tris buffer and 1 mg ml$^{-1}$ BSA) and vacuum filtration using a 12-well sampling manifold (Brandel Cell Harvester) and Whatman GF/B glass-fibre filters that had been soaked in wash buffer at 4°C for 24 h. Each reaction tube was washed three times with a 4 ml aliquot of wash buffer. The filters were oven-dried for 60 min and then placed in 5 ml of scintillation fluid (Ultima Gold XR, Packard), and radioactivity quantified by liquid scintillation spectrometry. Specific binding of [$^3$H]-CP55940, [$^3$H]-WIN55212-2 and [$^3$H]-SR141716A was defined as the difference between the binding that occurred in the presence and absence of 1 $\mu$M unlabelled CP55940, WIN55212-2 or SR141716A respectively. Protein assays were performed using a Bio-Rad Dc kit. Unlabelled cannabinoids were each added in a volume of 50 $\mu$l after serial dilution from a 1 mg ml$^{-1}$ ethanolic stock solution using buffer (50 mM Tris buffer containing 10 mg ml$^{-1}$ BSA). Ethanolic stock solutions of radiolabelled cannabinoids were also diluted in this way and added in a 50 $\mu$l volume. In saturation binding assays, the concentrations of the radiolabelled ligands used ranged between 0.1 and 20 nM. In displacement assays, [$^3$H]-CP55940 was used at a concentration of 1 nM.

### *Analysis of data*

Values have been expressed as means and variability as mean$\pm$s.e.mean or as 95% confidence limits. Mean values have been compared using Student's *t*-test (two-tail) or one-way analysis of variance followed by the Newman-Keuls test or Dunnett's test. A $P$ value $<0.05$ was considered to be significant. The dissociation constants of [$^3$H]-CP55940, [$^3$H]-WIN55212-2 and [$^3$H]-SR141716A ($K_D$), the concentration of specific binding sites ($B_{max}$) and the concentration of cannabinoids ($IC_{50}$) that produced a 50% displacement of [$^3$H]-CP55940 from specific binding sites were calculated using GraphPad Prism (GraphPad Software, San Diego). Competitive binding curves were fitted with minimum and maximum values for displacement of [$^3$H]-CP55940 from specific binding sites constrained to 0 and 100% respectively. In organ bath experiments the degree of drug-induced inhibition of evoked contractions has usually been expressed in percentage terms. This was calculated by comparing the amplitude of the contraction immediately before agonist administration with the amplitude of the contraction at various times after agonist administration. Dose response curves of WIN55212-2 and CP55940 were constructed in the presence or absence of O-1184 and $K_D$ values were each calculated by substituting a single dose ratio value into the equation $(x-1)=B/K_D$, where x (the 'dose ratio') is the dose of agonist that produces a particular degree of inhibition in the presence of O-1184 at a concentration, B, divided by the dose of agonist that produces an identical degree of inhibition in the absence of O-1184 (Tallarida *et al*., 1979). Dose ratio values and their 95% confidence limits were determined by symmetrical (2+2) dose parallel line assays (Colquhoun, 1971), using responses to 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.

## Results

### *Binding experiments*

When crude homogenates were used, 1 nM [$^3$H]-CP55940 underwent high specific binding to forebrain tissue (76.3$\pm$3.76%; $n=3$) but not to myenteric plexus-longitudinal muscle tissue (21.9%$\pm$5.2%; $n=3$). However, when the myenteric plexus-longitudinal muscle homogenate was fractionated using a sucrose density gradient (Methods), 1 nM [$^3$H]-CP55940 did undergo high specific binding (65.2$\pm$8.4%; $n=3$) to the tissue found at the interface between 0.84 M and 1.06 M sucrose (fraction 2) (Figure 1). This fraction was used for all subsequent saturation and competition binding assays with myenteric plexus-longitudinal muscle tissue. [$^3$H]-CP55940 underwent saturable binding in both forebrain and myenteric plexus-longitudinal muscle membrane preparations (Figure 2), the binding curves fitting well to a one-site binding hyperbola. The binding capacity ($B_{max}$ value) of [$^3$H]-CP55940 was found to be significantly higher in forebrain membranes than in myenteric plexus-longitudinal muscle membranes, whilst the corresponding $K_D$ values were not significantly different from each other (Table 1).

The dissociation constants ($K_i$ values) for displacement of 1 nM [$^3$H]-CP55940 by CP55940, WIN55212-2, O-1184, SR141716A and SR144528 from specific binding sites on forebrain membranes are not significantly different from the corresponding $K_i$ values determined in experiments with the myenteric plexus-longitudinal muscle membrane preparation (Table 2 and Figure 3).

The ability of [$^3$H]-CP55940 to undergo saturable, specific binding to forebrain membranes was shared by [$^3$H]-WIN55212-2 and [$^3$H]-SR141716A, all the binding curves fitting well to a one-site binding hyperbola (Figures 2 and 4). The $B_{max}$ values of [$^3$H]-CP55940 and [$^3$H]-SR141716A did not

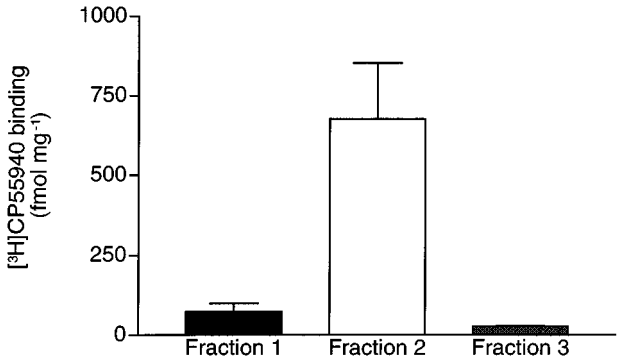


**Figure 1** Concentration of specific cannabinoid binding sites in three fractions of myenteric plexus-longitudinal muscle homogenate found at the tissue/0.84 M sucrose interface (fraction 1), the 0.84 M/1.06 M sucrose interface (fraction 2) and the 1.06 M/1.25 M sucrose interface (fraction 3) following centrifugation in test tubes containing three layers of sucrose.

differ significantly from each other but were both significantly greater than the $B_{max}$ of [$^3$H]-WIN55212-2 (Table 1).

### *Experiments with the myenteric plexus-longitudinal muscle preparation*

Exposure for 30 min to the concentration of Tween 80 used in experiments with 1000 nM O-1184 had no significant effect on the amplitude of the twitch response. Thus, after addition of Tween 80, the mean amplitude fell by 3.72±1.88% ($n=36$; $P>0.05$; paired $t$-test). Corresponding values after 30 min exposure to 10, 100 or 1000 nM O-1184 were 7.6±2.20% ($n=18$), 5.11±3.63% ($n=18$) and 11.45±4.27% ($n=17$) respectively. These changes in amplitude did not differ significantly from the Tween control value shown above (analysis of variance followed by Dunnett's test; $F=1.416$; total degrees of freedom = 88).

At concentrations of 10 or 100 nM, O-1184 produced dextral shifts in the log concentration-response curves of WIN55212-2 and CP55940 that did not deviate significantly from parallelism (Figure 5 and Table 3). The ability of 1 μM WIN55212-2 to inhibit the twitch response was essentially abolished by 1 μM O-1184. WIN55212-2 and CP55940 concentrations above 1 μM were not used in these experiments. This was because at such concentrations, the amount of drug vehicle present is sufficient to inhibit the twitch response of the myenteric plexus-longitudinal muscle preparation in the absence of any other agent. The positions of the log concentration-response curves of clonidine and normorphine were not affected by 1 μM O-1184 (Figure 6). Nor did this concentration of O-1184 affect the contractile potency of acetylcholine (Figure 6).

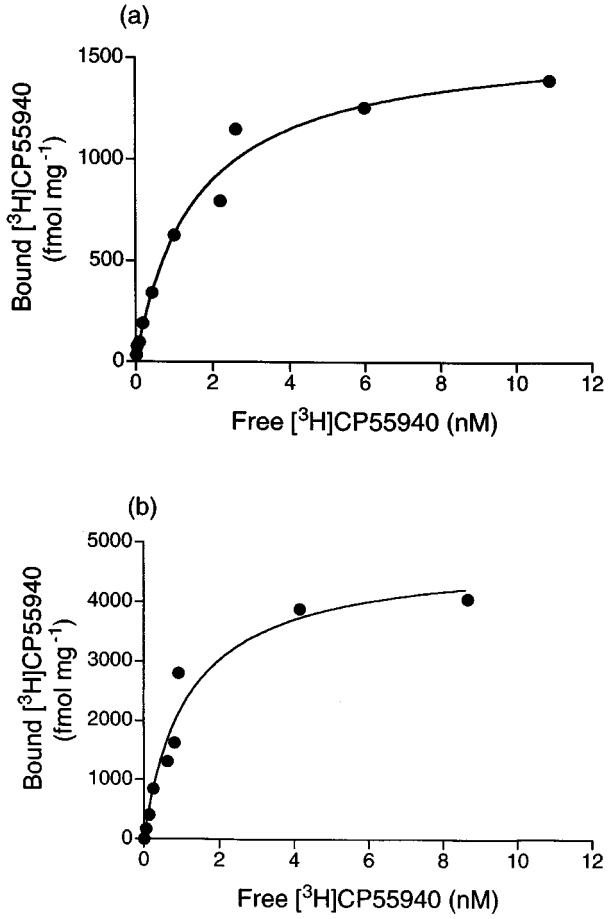


**Figure 2** Representative saturation binding curves for [$^3$H]-CP55940 in (a) myenteric plexus-longitudinal muscle membranes and (b) guinea-pig forebrain membranes.

## Discussion

Our finding that membranes prepared from the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine contain saturable binding sites for the established $CB_1$ receptor probe, [$^3$H]-CP55940, lends further support to the hypothesis that cannabinoid $CB_1$ receptors are present in this tissue (see Introduction). These binding sites were found to resemble $CB_1$ binding sites of the brain in several ways.

**Table 2** Dissociation constant values for displacement of [$^3$H]-CP55940[a] from membranes of guinea-pig forebrain and myenteric plexus-longitudinal muscle membranes

| *Cannabinoid* | *Forebrain* | *MPLM*[b] |
|---|---|---|
| CP55940 | 0.87±0.19 | 0.99±0.26 |
| WIN55212-2 | 4.15±0.91 | 5.03±1.20 |
| SR141716A | 5.32±0.88 | 4.95±1.00 |
| O-1184 | 2.85±0.36 | 3.16±0.62 |
| SR144528 | 371.9±60.3 | 361.5±73.5 |

[a]1 nM [$^3$H]-CP55940. [b]Guinea-pig myenteric plexus-longitudinal muscle membranes. There was no significant difference between the dissociation constant ($K_i$) value of any ligand determined using forebrain membranes and that determined using MPLM membranes ($P>0.05$; $n=5$ for each treatment; Student's $t$-test using log values of $K_i$).

**Table 1** Saturation analysis of [$^3$H]-CP55940, [$^3$H]-WIN55212-2 and [$^3$H]-SR141716A in membranes from guinea-pig forebrain and myenteric plexus-longitudinal muscle

| | *Forebrain* | | *MPLM* | |
|---|---|---|---|---|
| *Cannabinoid* | $B_{max}$ (fmol mg$^{-1}$) | $K_D$ (nM) | $B_{max}$ (fmol mg$^{-1}$) | $K_D$ (nM) |
| [$^3$H]-CP55940 | 4281±403 | 2.29±0.49[a] | 2092±386 | 1.75±0.56 |
| [$^3$H]-WIN55212-2 | 2032±419 | 2.34±0.83 | – | – |
| [$^3$H]-SR141716A | 5658±658 | 1.24±0.21 | – | – |

[a]Not significantly different from the $K_D$ value determined for [$^3$H]-CP55940 in myenteric plexus-longitudinal muscle (MPLM) membranes ($P>0.05$; Student's $t$-test using log values of $K_D$; $n=5$ for each treatment). Analysis of variance followed by the Newman-Keuls test showed the mean $B_{max}$ value for [$^3$H]-WIN55212-2 to be significantly less than the mean $B_{max}$ values for [$^3$H]-CP55940 and [$^3$H]-SR141716A in forebrain membranes, the mean $B_{max}$ values for [$^3$H]-CP55940 and [$^3$H]-SR141716A in forebrain membranes not to be significantly different from each other and the mean $B_{max}$ value for [$^3$H]-CP55940 to be significantly less in MPLM membranes than in forebrain membranes ($F=13.65$; total degrees of freedom = 19; $n=5$ for each treatment).

First, the dissociation constant of [$^3$H]-CP55940 in myenteric plexus-longitudinal muscle membranes approximated closely to the value for its dissociation constant obtained in our [$^3$H]-CP55940 saturation binding assays with guinea-pig forebrain membranes and also to dissociation constant values for [$^3$H]-CP55940 obtained in previous binding studies with other brain preparations (Pertwee, 1997). Second, CP55940, WIN55212-2, SR141716A and SR144528 each showed a similar potency in displacing [$^3$H]-CP55940 from specific binding sites on myenteric plexus-longitudinal muscle membranes as in displacing this radioligand from specific binding sites on guinea-pig forebrain membranes. Third, the dissociation constant of SR141716A for displacement of [$^3$H]-CP55940 from myenteric plexus-longitudinal muscle membranes (3.4 nM) is close to previously reported dissociation constant values of SR141716A calculated by Schild analysis from its ability to antagonize inhibition of electrically-evoked contractions of the guinea-pig myenteric plexus-longitudinal muscle preparation induced by $\Delta^9$-tetrahydrocannabinol (5.47 nM), WIN55212-2 (9.65 nM) or CP55940 (12.07 nM) (Pertwee *et al.*, 1996).

Experiments with O-1184 confirmed it to have a high affinity for $CB_1$ receptors as measured by its ability to displace [$^3$H]-CP55940 from specific binding sites in guinea-pig

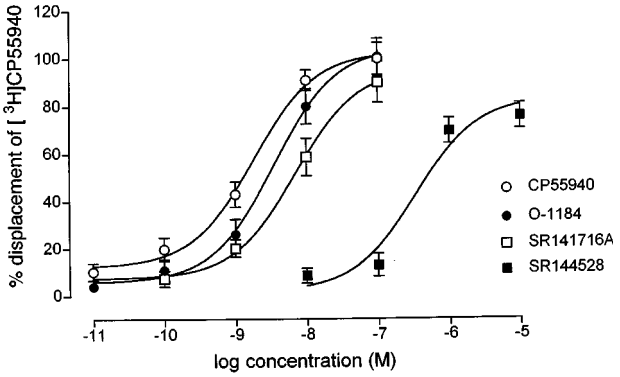


**Figure 3** Displacement of bound [$^3$H]-CP55940 (1 nM) by CP55940, O-1184, SR141716A and SR144528 in myenteric plexus-longitudinal muscle membranes. Each symbol represents the mean % displacement ±s.e.mean ($n$=three or four). There was considerable overlap between the displacement curves of CP55940, O-1184 and SR141716A shown above and the curve for displacement of bound [$^3$H]-CP55940 by WIN55212-2 (not shown for the sake of clarity).

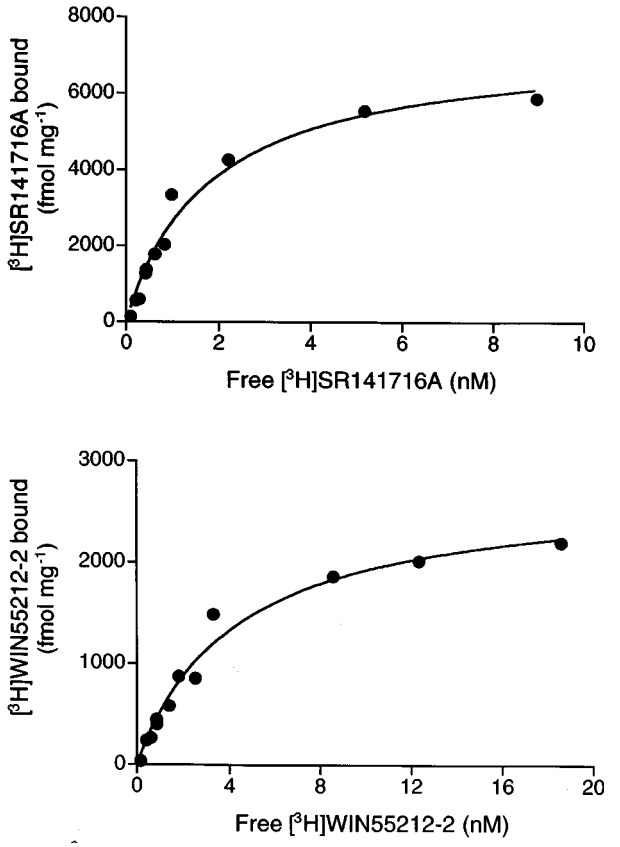


**Figure 4** Representative saturation binding curves for [$^3$H]-SR141716A and [$^3$H]-WIN55212-2 in guinea-pig forebrain membranes.

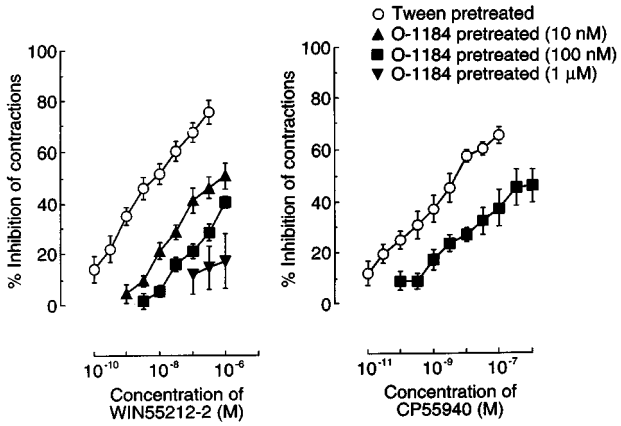


**Figure 5** Effect of pretreatment with O-1184 on mean concentration-response curves for WIN55212-2 and CP55940 in the myenteric plexus-longitudinal muscle preparation. Each symbol represents the mean value ±s.e.mean of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of an agonist to the organ bath ($n$=6 different preparations). O-1184 and Tween 80 were added 30 min before the first addition of WIN55212-2 or CP55940.

**Table 3** Dissociation constant values of O-1184 for antagonism of WIN55212-2 or CP55940 in the myenteric plexus-longitudinal muscle preparation

| *Agonist* | *O-1184 concentration* (nM) | *Mean* $K_D$ *value* (nM) | *95% confidence limits* |
|---|---|---|---|
| WIN55212-2 | 10 | 0.20 | 0.07 and 0.52 |
| WIN55212-2 | 100 | 0.20 | 0.07 and 0.44 |
| CP55940 | 100 | 0.89 | 0.13 and 5.11 |

Dissociation constant ($K_D$) values of O-1184 were determined from its ability to attenuate inhibition of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine induced by WIN55212-2 or CP55940. These values were calculated by substituting a dose ratio value into the equation $(x-1)=B/K_D$, where x (the 'dose ratio') is the dose of agonist that produces a particular degree of inhibition in the presence of O-1184 at a concentration, B, divided by the dose of agonist that produces an identical degree of inhibition in the absence of O-1184 (Tallarida *et al.*, 1979). Dose ratio values and their 95% confidence limits were determined by symmetrical (2+2) dose parallel line assays.

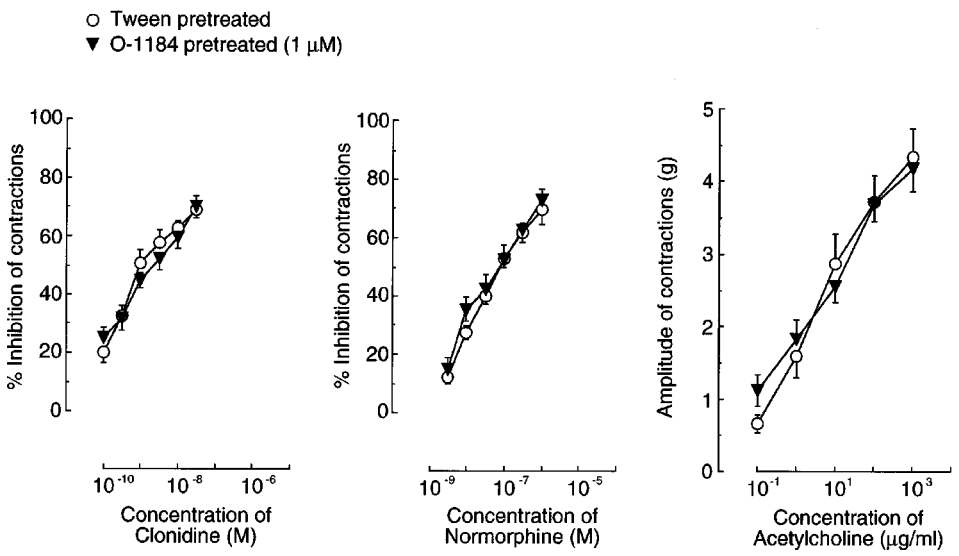


**Figure 6** Effect of pretreatment with 1 $\mu$M O-1184 on mean concentration-response curves for clonidine, normorphine and acetylcholine in the myenteric plexus-longitudinal muscle preparation. Each symbol in the right hand panel represents the mean value ± s.e.mean of the amplitude of contractions produced by acetylcholine ($n = 6$ different preparations). Each symbol in the other two panels represents the mean value ± s.e.mean of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of clonidine or normorphine to the organ bath ($n = 6$ different preparations). O-1184 and Tween 80 were added 30 min before the first addition of clonidine, normorphine or acetylcholine.

forebrain membranes. Like the other cannabinoid receptor ligands we investigated, the dissociation constant of O-1184 for displacement of [$^3$H]-CP55940 was essentially the same in myenteric plexus-longitudinal muscle membranes as in forebrain membranes. At concentrations of up to 1 $\mu$M, O-1184 failed to reduce the amplitude of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation, indicating it to lack detectable cannabinoid receptor agonist activity in this tissue. Instead, experiments with WIN55212-2 and CP55940 showed O-1184 to behave as a cannabinoid receptor antagonist. The dissociation constant of O-1184 (0.89 nM; Table 3), calculated from its ability to antagonize CP55940, is not significantly different from its dissociation constant for the displacement of [$^3$H]-CP55940 from specific binding sites in forebrain (2.85 nM) and myenteric plexus-longitudinal muscle membranes (3.16 nM), demonstrating that O-1184 exhibits activity as a cannabinoid antagonist at concentrations similar to those at which it displaces [$^3$H]-CP55940 from specific binding sites. The contractile potency of acetylcholine in the myenteric plexus-longitudinal muscle preparation was not affected by O-1184 at the highest of the three concentrations used to antagonize WIN55212-2. Nor did this concentration of O-1184 alter the ability of normorphine or clonidine to inhibit electrically-evoked contractions of this preparation. These findings suggest that O-1184 possesses significant selectivity for cannabinoid receptors. In addition, the results obtained in the experiments with acetylcholine point to a prejunctional site of action for O-1184. This finding supports the hypothesis that this agent antagonizes cannabinoids in the guinea-pig myenteric plexus-longitudinal muscle preparation by binding to cannabinoid receptors as the location of cannabinoid receptors in this preparation that mediate inhibition of the twitch response is also thought to be prejunctional.

Our saturation binding experiments with forebrain membranes show the $B_{max}$ value of [$^3$H]-WIN55212-2 to be significantly less than that of either [$^3$H]-CP55940 or [$^3$H]-SR141716A (Table 1). A similar difference can be discerned if one compares previously reported $B_{max}$ values of [$^3$H]-WIN55212-2 (1.15 pmol mg$^{-1}$), [$^3$H]-CP55940 (2.5 pmol mg$^{-1}$) and [$^3$H]-SR141716A (3.86 pmol mg$^{-1}$) determined in rat cerebellar membranes, albeit each in a different laboratory under different conditions (Felder *et al.*, 1992; Kuster *et al.*, 1993; Petitet *et al.*, 1996). It could be, therefore, that [$^3$H]-WIN55212-2 binds to a different population of central cannabinoid receptors than [$^3$H]-CP55940 or [$^3$H]-SR141716A. In line with this possibility is the observation by Song & Bonner (1996) that the introduction of a lysine to alanine mutation at the 192 position in the third transmembrane domain of the $CB_1$ receptor abolishes the ability of CP55940 and certain other cannabinoids to compete with [$^3$H]-WIN55212-2 for specific binding sites without affecting the binding affinity of WIN55212-2 itself. Further evidence for a difference between the binding to $CB_1$ receptors of WIN55212-2 and that of other cannabinoid receptor ligands has been obtained by Petitet *et al.* (1996). They observed WIN55212-2 to be markedly less potent in displacing [$^3$H]-SR141716A than [$^3$H]-WIN55212-2 from specific binding sites on rat cerebellar membranes whereas other cannabinoids, including SR141716A, anandamide and $\Delta^9$-tetrahydrocannabinol showed no such potency difference. A second possibility is that guinea-pig forebrain contains a subpopulation of cannabinoid receptors with high affinity for [$^3$H]-CP55940 and [$^3$H]-SR141716A but not for [$^3$H]-WIN55212-2. However, our other binding data fail to support the presence of more than one population of binding sites for CP55940 or SR141716A in the brain, the saturation data for all three radiolabelled ligands fitting well to a 1-site model.

In conclusion, we have obtained further evidence for the presence of cannabinoid $CB_1$ receptors in the guinea-pig myenteric plexus-longitudinal muscle preparation by showing that this tissue contains cannabinoid binding sites and that

these closely resemble $CB_1$ binding sites of guinea-pig brain. We have also found that O-1184 has high affinity for $CB_1$ receptors and obtained evidence that it behaves as a potent cannabinoid receptor antagonist, at least in the guinea-pig myenteric plexus-longitudinal muscle preparation. Further experiments are required to characterize the pharmacological properties of O-1184 more fully both *in vitro* and *in vivo*. Additional experiments are also required to establish why there appear to be fewer binding sites in guinea-pig brain for WIN55212-2 than for either CP55940 or SR141716A.

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## References

COLQUHOUN, D. (1971). *Lectures on Biostatistics*. Oxford: Oxford University Press.

COMPTON, D.R., RICE, K.C., DE COSTA, B.R., RAZDAN, R.K., MELVIN, L.S., JOHNSON, M.R. & MARTIN, B.R. (1993). Cannabinoid structure-activity relationships: correlation of receptor binding and *in vivo* activities. *J. Pharmacol. Exp. Ther.,* **265,** 218–226.

FELDER, C.C., VELUZ, J.S., WILLIAMS, H.L., BRILEY, E.M. & MATSUDA, L.A. (1992). Cannabinoid agonists stimulate both receptor- and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol. Pharmacol.,* **42,** 838–845.

GRIFFIN, G., FERNANDO, S.R., ROSS, R.A., MACKAY, N.G., ASHFORD, M.L.J., SHIRE, D., HUFFMAN, J.W., YU, S., LAINTON, J.A.H. & PERTWEE, R.G. (1997). Evidence for the presence of $CB_2$-like cannabinoid receptors on peripheral nerve terminals. *Eur. J. Pharmacol.,* **339,** 53–61.

HERKENHAM, M., LYNN, A.B., LITTLE, M.D., JOHNSON, M.R., MELVIN, L.S., DE COSTA, B.R. & RICE, K.C. (1990). Cannabinoid receptor localization in brain. *Proc. Nat. Acad. Sci. U.S.A.,* **87,** 1932–1936.

KUSTER, J.E., STEVENSON, J.I., WARD, S.J., D'AMBRA, T.E. & HAYCOCK, D.A. (1993). Aminoalkylindole binding in rat cerebellum: selective displacement by natural and synthetic cannabinoids. *J. Pharmacol. Exp. Ther.,* **264,** 1352–1363.

PATON, W.D.M. & ZAR, M.A. (1968). The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *J. Physiol.,* **194,** 13–33.

PERTWEE, R.G. (1997). Pharmacology of cannabinoid $CB_1$ and $CB_2$ receptors. *Pharmacol. Ther.,* **74,** 129–180.

PERTWEE, R.G., FERNANDO, S.R., NASH, J.E. & COUTTS, A.A. (1996). Further evidence for the presence of cannabinoid $CB_1$ receptors in guinea-pig small intestine. *Br. J. Pharmacol.,* **118,** 2199–2205.

PERTWEE, R.G., STEVENSON, L.A., ELRICK, D.B., MECHOULAM, R. & CORBETT, A.D. (1992). Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br. J. Pharmacol.,* **105,** 980–984.

PETITET, F., MARIN, L. & DOBLE, A. (1996). Biochemical and pharmacological characterization of cannabinoid binding sites using [$^3$H]-SR141716A. *Neuroreport,* **7,** 789–792.

RINALDI-CARMONA, M., BARTH, F., MILLAN, J., DEROCQ, J.-M., CASELLAS, P., CONGY, C., OUSTRIC, D., SARRAN, M., BOUABOULA, M., CALANDRA, B., PORTIER, M., SHIRE, D., BRELIERE, J.-C. & LE FUR, G. (1998). SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.,* **284,** 644–650.

SONG, Z.-H. & BONNER, T.I. (1996). A lysine residue of the cannabinoid receptor is critical for receptor recognition by several agonists but not WIN55212-2. *Mol. Pharmacol.,* **49,** 891–896.

TALLARIDA, R.J., COWAN, A. & ADLER, M.W. (1979). $pA_2$ and receptor differentiation: a statistical analysis of competitive antagonism. *Life Sci.,* **25,** 637–654.

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