British Journal of Pharmacology (1999) 126, 1575–1584

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An investigation into the structural determinants of cannabinoid receptor ligand efficacy

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1 A number of side-chain analogues of Δ^8-THC were tested in GTPγS binding assay in rat cerebellar membranes. O-1125, a saturated side-chain compound stimulated GTPγS binding with an E_{max} of 165.0%, and an EC_{50} of 17.4 nM.

2 O-1236, O-1237 and O-1238, three-enyl derivatives containing a cis carbon-carbon double bond in the side-chain, stimulated GTPγS binding, acting as partial agonists with E_{max} values ranging from 51.3–87.5% and EC_{50} values between 4.4 and 29.7 nM.

3 The stimulatory effects of O-1125, O-1236, O-1237 and O-1238 on GTPγS binding were antagonized by the CB_1 receptor antagonist SR 141716A. The K_B values obtained ranged from 0.11–0.21 mM, suggesting an action at CB_1 receptors.

4 Five-ynyl derivatives (O-584, O-806, O-823, O-1176 and O-1184), each containing a carbon-carbon triple bond in the side-chain, did not stimulate GTPγS binding and were tested as potential cannabinoid receptor antagonists.

5 Each -ynyl compound antagonized the stimulatory effects of four cannabinoid receptor agonists on GTPγS binding. The K_B values obtained, all found to be in the nanomolar range, did not differ between agonists or from cerebellar binding affinity.

6 In conclusion, alterations of the side-chain of the classical cannabinoid structure may exert a large influence on affinity and efficacy at the CB_1 receptor.

7 Furthermore, this study confirms the ability of the GTPγS binding assay to assess discrete differences in ligand efficacies which potentially may not be observed using alternative functional assays, thus providing a unique tool for the assessment of the molecular mechanisms underlying ligand efficacies.

Keywords: Cannabinoid receptors; [^{35}S]-GTPγS binding; G-proteins; rat cerebellum; agonist; partial agonist; antagonist; efficacy

Abbreviations: CP 55,940, (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; Δ^8-THC, delta-8-tetrahydrocannabinol; HU-210, (−)-11-OH-delta-8-tetrahydrocannabinol-dimethylheptyl; O-584, 3-(2-Octynyl)-delta-8-tetrahydrocannabinol; O-689, 2-Methylarachidonyl-(2′-fluoroethyl)amide; O-806, 3-(6-bromo-2-hexynyl)-delta-8-tetrahydrocannabinol; O-823, 3-(6-cyano-2-hexynyl)-delta-8-tetrahydrocannabinol; O-1125, 3-(1,1-dimethyl-6-dimethylcarboxamide)-delta-8-tetrahydrocannabinol; O-1176, 3-(6-isothiocyano-2-hexynyl)-delta-8-tetrahydrocannabinol; O-1184, 3-(6-azido-2′-hexenyl)-delta-8-tetrahydrocannabinol; O-1236, 3-(6-bromo-3-hexenyl)-delta-8-tetrahydrocannabinol; O-1237, 3-(6-cyano-3-hexenyl)-delta-8-tetrahydrocannabinol; O-1238, 3-(6-azido-3-hexenyl)-delta-8-tetrahydrocannabinol; [^{35}S]-GTPγS, guanosine-5′-O-(3-[^{35}S]-thio)-triphosphate; THC, Delta-9-tetrahydrocannabinol; SR 141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride; WIN 55212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone

Introduction

Delta-9-tetrahydrocannabinol (THC) has long been recognized as the major psychoactive component of marijuana. It exerts a diverse range of pharmacological effects in both animals and man, with these effects thought to be largely mediated through two subtypes of cannabinoid receptor, CB_1 and CB_2 (Pertwee, 1997). One consequence of the discovery of receptor subtypes for cannabinoid ligands has been an ongoing attempt to produce subtype-selective ligands, from antagonists through high efficacy, high potency agonists. This continuing synthesis of novel cannabinoid receptor ligands is enabling a gradual understanding of the structural components which confer affinity and efficacy to a ligand as well as specificity for one or other cannabinoid receptor subtype (Showalter *et al*., 1996; Compton *et al*., 1993; Martin *et al*., 1995).

Agonist binding to cannabinoid receptors has previously been demonstrated to stimulate guanosine-5′-O-(3-[^{35}S]-thio)-triphosphate ([^{35}S]-GTPγS) binding in membrane preparations and in brain slices (Sim *et al*., 1995; Selley *et al*., 1996). This technique has been employed for the functional characterization of both cannabinoid receptor agonists and antagonists. We have previously reported the activity of several cannabinoid receptor ligands, agonists and antagonists using this assay (Griffin *et al*., 1998). One notable difference between this assay and other functional models is the activity of THC. Other studies, for example those using smooth muscle preparations, have shown THC to behave as a full agonist (Pertwee & Griffin, 1995) whereas in the [^{35}S]-GTPγS binding assay, THC produces very little stimulation of binding (Sim *et al*., 1996;

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Burkey *et al*., 1997; Griffin *et al*., 1998). The reasons for this apparently lower efficacy of THC in the GTPγS binding assay are yet to be fully understood. THC has also been demonstrated to antagonize the effects of WIN 55212-2, a cannabinoid receptor agonist, in rat brain membrane preparations (Selley *et al*., 1996).

The classical cannabinoid tricyclic structure, for example that of THC, has been extensively studied using molecular modelling and structure-activity relationships with regards to the individual molecular components which contribute towards the overall activity of a compound. These studies have enabled an improved understanding of ligand-receptor coupling, and have led to the development of the three-point model of cannabinoid receptor interaction (for review, see Martin *et al*., 1995). This model, in part, demonstrates the importance of the aliphatic side-chain of the THC molecule. This is further supported by the production of high affinity and high potency cannabinoid compounds such as HU-210 and CP 55,940, which both contain dimethylheptyl side-chains rather than the pentyl side-chain of THC. Previously, it has been reported that 3-(6-cyanohexynyl)-delta-8-tetrahydrocannabinol (O-823), a structural analogue of delta-8-tetrahydrocannabinol (Δ^8-THC) with modifications centred in the side-chain and depicted in Figure 1, acts as a partial agonist at CB_1 receptors and exhibits agonist and/or antagonist activity depending on the model used. In the myenteric-plexus longitudinal muscle preparation of the guinea-pig ileum (MP-LM), O-823 acted as a cannabinoid receptor antagonist with an equilibrium dissociation constant (K_B) value that correlated with its CB_1 binding affinity ($K_i = 0.77 \pm 0.05$ nM). However, in the mouse vas deferens, O-823 acted as a highly potent partial agonist unless the tissues were made tolerant to THC, whereupon O-823 acted as a cannabinoid receptor antagonist, with a K_B comparable to that observed in the MP-LM preparation (Pertwee *et al*., 1996). This high affinity/low efficacy combination is unique in cannabinoid pharmacology to date and may represent the potential for a new class of cannabinoid compounds. The purpose of this study was to further characterize the pharmacological activity of this compound, as well as other novel, structurally similar compounds. Additionally, the effect of structural modifications of the side-chain on the efficacy of cannabinoid receptor ligands at the CB_1 receptor was investigated.

Methods

Materials

Male Sprague-Dawley rats (150–250 g) were obtained from Harlan (Dublin, VA). GDP and GTPγS were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). [^{35}S]-GTPγS (1000–1200 Ci mmol^{-1} was purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]-SR141716A (55 Ci mmol^{-1}) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Other reagent grade chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Δ^8-THC was obtained from the National Institute on Drug Abuse (NIDA). (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol (CP 55,940) and N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride (SR 141716A) were generously provided by Pfizer Inc., Groton, CT, (−)-11-OH-delta-8-tetrahydrocannabinol-dimethylheptyl (HU-210) was generously provided by Prof Raphael Mechoulam (Hebrew University, Jerusalem, Israel) and (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone (WIN 55212-2) was purchased from Research Biochemicals International (Natick, MA, U.S.A.). 3-(2-Octynyl)-delta-8-tetrahydrocannabinol (O-584), 2-Methylarachidonyl-(2′-fluoroethyl)amide (O-689), 3-(6-bromo-2-hexynyl)-delta-8-tetrahydrocannabinol (O-806), 3-(6-cyano-2-hexynyl)-delta-8-tetrahydrocannabinol (O-823), 3-(1,1-dimethyl-6-dimethylcarboxamide)-delta-8-tetrahydrocannabinol (O-1125), 3-(6-isothiocyano-2-hexynyl)-delta-8-tetrahydrocannabinol (O-1176), 3-(6-azido-2′-hexenyl)-delta-8-tetrahydrocannabinol (O-1184), 3-(6-bromo-3-hexenyl)-delta-8-tetrahydrocannabinol (O-1236), 3-(6-cyano-3-hexenyl)-delta-8-tetrahydrocannabinol (O-1237) and 3-(6-azido-3-hexenyl)-delta-8-tetrahydrocannabinol (O-1238) were synthesized by Dr Raj Razdan (Organix, Inc., Woburn, MA, U.S.A.). All compounds were stored as 1 mg ml^{-1} solutions in ethanol at −20°C.

Membrane preparation

Cerebella were dissected on ice from three freshly decapitated male Sprague-Dawley rats. The tissue was then homogenized in centrifugation buffer (mM; Tris HCl 50, EGTA 1, $MgCl_2$ 3; pH 7.4) and the homogenate centrifuged at 48,000 × *g* for 20 min at 4°C. The pellet was then resuspended in GTPγS assay buffer (mM: Tris HCl 50, $MgCl_2$ 9, EGTA 0.2, NaCl 150; pH 7.4), homogenized, and centrifuged at 48,000 × *g* for 20 min at 4°C. The final pellet was then resuspended in GTPγS assay buffer, homogenized, and diluted to a concentration of approximately 2 μg μl^{-1} with assay buffer. Membrane homogenates were also prepared from the remaining brain regions (whole brain minus the cerebellum) in an identical fashion. Cerebellar membranes to be used for radioligand binding experiments were resuspended in binding buffer A (mM; Tris-HCl 50, EDTA 1, $MgCl_2$ 3, 1 mg ml^{-1} fatty acid bovine serum albumin (BSA), pH 7.4). The protein concentrations of membrane preparations were determined by the method of Bradford (1976). Aliquots were then stored at −80°C.

[^{35}S]-GTPγS binding

The methods for measuring agonist-stimulated [^{35}S]-GTPγS binding were adapted from those of Sim *et al*. (1995). Rat cerebellar membranes (10 μg) were incubated in assay buffer, or in sodium-free assay buffer, containing 0.1% BSA with GDP, [^{35}S]-GTPγS (0.05 nM) and either cannabinoids or an ethanol control in siliconized glass tubes. Two concentrations of GDP were used: 100 μM for all experiments except those using O-1236 and O-1237 (10 μM). Additionally any compound producing no stimulation of GTPγS binding at 100 μM was also tested with the lower GDP concentration (results not shown). This was done as a reduction in the GDP concentration has been previously shown to increase the stimulation of GTPγS binding produced by lower efficacy agonists (Griffin *et al*., 1998). The total assay volume was 0.5 ml which was incubated at 30°C for 30 min, with the exception of experiments using HU-210 which were incubated for 60 min at 30°C. Previous observations have demonstrated this to be optimum for HU-210-stimulation of [^{35}S]-GTPγS binding (Griffin *et al*., 1998). The reaction was terminated by addition of 2 ml ice-cold wash buffer (mM; Tris HCl 50, $MgCl_2$5; pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass-fibre filters using a 12-well sampling manifold. The tubes were washed once with 2 ml of

ice-cold wash buffer, and the filters were washed twice with 4 ml of ice-cold wash buffer. Filters were placed into 7 ml plastic scintillation vials and 5 ml BudgetSolve scintillation fluid added (RPI Corp., Mount Prospect, IL, U.S.A.). After shaking for 1 h, bound radioactivity was determined by liquid scintillation. Non-specific binding was determined using GTPγS (10 μM). Basal binding was assayed in the absence of agonist and in the presence of GDP. The stimulation by agonist was defined as a percentage increase above basal specific binding levels (i.e. [(d.p.m. (agonist) − d.p.m. (no agonist))/d.p.m. (no agonist)] × 100). Experiments with whole brain (minus cerebellum) membrane homogenates were conducted identically to those using cerebellar membranes except 20 μg of protein were used rather than 10 μg.

Radioligand binding

The methods used for radioligand binding were essentially those described by Compton *et al.* (1993) with minor exceptions. Binding was initiated by the addition of 20 μg membrane protein to siliconized tubes containing [^{3}H]-SR 141716A and a sufficient volume of binding buffer A to bring the total volume to 0.5 ml. O-584 or O-1184 (0.01 nM – 1 μM) were also included for competition experiments, which were performed either in binding buffer A or GTPγS assay buffer containing GDP (100 μM) and GTPγS (0.05 nM) identical assay conditions as those used for GTPγS binding experiments. The addition of SR 141716A (1 μM) was used to assess non-specific binding. Following incubation (30°C for 1 h) binding was terminated by the addition of 2 ml of ice-cold binding buffer B (Tris-HCl (50 mM), 1 mg ml^{-1} BSA; pH 7.4) and vacuum filtration through Whatman GF/C filters (pretreated with polyethyleneimine (0.1%) for at least 4 h). Tubes were then rinsed with 2 ml of ice-cold binding buffer B, which was also filtered, and the filters were subsequently rinsed twice with 4 ml of ice-cold binding buffer B. Before radioactivity was quantitated by liquid scintillation spectrophotometry, filters were shaken for 1 h in 5 ml scintillation fluid.

Data analysis

Data are reported as means ± s.e.mean of 4 – 8 experiments, performed in triplicate. Non-linear regression analysis of concentration-response data was performed using Prism 2.0 software for the Macintosh (GraphPad Software, San Diego, CA, U.S.A.) in order to calculate and compare E_{max} and EC_{50} values. The equilibrium dissociation constant (K_B) for the interaction of the antagonist and the receptor has been calculated from the equation $K_B = [B]/(\text{dose ratio} - 1)$, where [B] is the concentration of the antagonist used in the experiment (Schild, 1949). In experiments involving multiple concentrations of antagonist, the K_B value was calculated from Schild plots of the data (Schild, 1949). K_B and EC_{50} values are presented with 95% confidence limits indicated by parentheses. B_{max} and K_D values obtained from Scatchard analysis of saturation binding curves were determined by the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ, U.S.A.). Displacement IC_{50} values were determined originally by unweighted least-squares non-linear regression of log concentration-percentage of displacement data and then converted to K_i values using the method of Cheng & Prusoff (1973). Students *t*-test, two-tailed (unpaired) was used for comparison of K_i values ($P < 0.05$).

Results

Effects of Δ^8-THC analogues on [^{35}S]-GTPγS binding

Δ^8-THC, O-584, O-806, O-823, O-1125, O-1176, O-1184, O-1236, O-1237 and O-1238 (Figure 1) were tested for their ability to stimulate [^{35}S]-GTPγS binding in rat cerebellar membrane preparations. At a GDP concentration of 100 μM, it was found that only O-1125 and O-1238 produced a concentration-dependent stimulation of [^{35}S]-GTPγS binding (Figure 2A). O-1125 stimulated binding with a maximal stimulation (E_{max}) of 165.0 ± 12.8% and an EC_{50} of 17.4 (12.0 – 26.5) nM (95% confidence limits are indicated by parentheses). O-1238 stimulated binding with an E_{max} of 58.3 ± 8.5% and an EC_{50} of 29.7 (14.2 – 59.0) nM. In order to establish whether an inability to stimulate binding was due to low efficacies of the other compounds, similar experiments were carried out with a lower GDP concentration (10 μM). Under these conditions, O-1236 [E_{max} = 87.5 ± 9.7% and EC_{50} = 16.6 (10.1 – 30.6) nM] and O-1237 [E_{max} = 51.3 ± 5.5% and EC_{50} = 4.4 (2.5 – 7.1) nM] also produced a concentration-

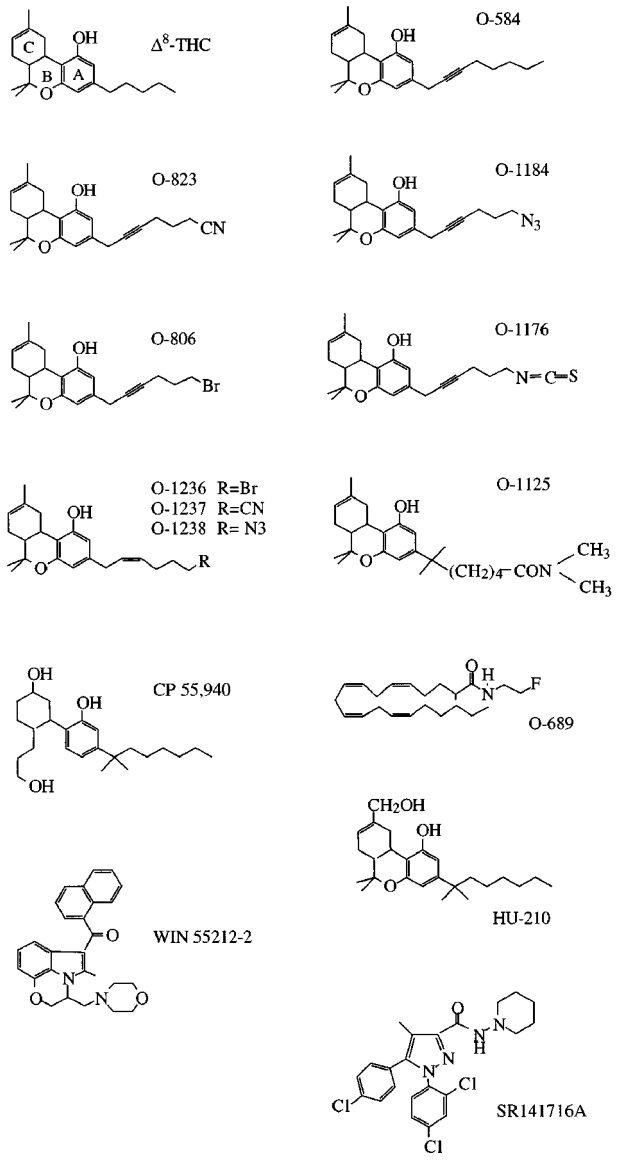

Figure 1 Chemical structures.

dependent stimulation of [^{35}S]-GTPγS binding (Figure 2B). At this GDP concentration, the E_{max} and EC_{50} values of O-1238 were not significantly affected (E_{max} of 52.1 $\pm$ 6.8% and an EC_{50} of 10.6 (2.40 – 46.6) nM). Compounds which still did not produce any stimulation of binding (The-ynyl compounds, O-584, O-806, O-823, O-1176, O-1184 and Δ^8-THC) were then tested in the absence of sodium ions at this lower GDP concentration. None of these compounds stimulated [^{35}S]-GTPγS binding under these conditions (data not shown).

Ability of the CB_1 receptor antagonist, SR 141716A, to attenuate agonist-induced stimulation of [^{35}S]-GTPγS binding

The ability of the CB_1-selective antagonist, SR 141716A (Rinaldi-Carmona *et al*., 1994), to attenuate the effects of O-1125, O-1236, O-1237 and O-1238 was investigated. SR 141716A, at concentrations of 3 nM (experiments with O-1236, O-1237 and O-1238) and 10 nM (O-1125) was found to antagonize the agonist effects of each of these compounds. The equilibrium dissociation constants (K_B values), of SR 141716A calculated in the presence of O-1236, O-1237, O-1238 and O-1125 were calculated to be 0.18 (0.14 – 0.23) nM, 0.11 (0.03 – 0.29) nM, 0.15 (0.08 – 0.29) nM and 0.21 (0.12 – 0.35) nM, respectively. These values do not differ significantly from each other and suggest that each compound is acting *via* the same receptor, which is likely to be CB_1. These values also agree with those previously found in the rat cerebellum using other cannabinoid receptor agonists such as CP 55,940 and WIN 55212-2 (Griffin *et al*., 1998).

Antagonism of agonist-stimulated [^{35}S]-GTPγS binding

The aim of these experiments was to investigate the ability of those compounds which did not stimulate [^{35}S]-GTPγS binding to antagonize the ability of CP 55,940, HU-210, WIN 55212-2 and the metabolically stable anandamide analogue, 2-Methylarachidonyl-(2′-fluoroethyl)amide (O-689), to stimulate [^{35}S]-GTPγS binding. O-689 was chosen as anandamide has been demonstrated to produce no significant stimulation of [^{35}S]-GTPγS binding in rat cerebellar membrane preparations whereas O-689 has been shown to significantly stimulate binding (Griffin *et al*., 1998). CP 55,940, WIN 55212-2 and HU-210 were chosen as these compounds are very potent and have been well characterized in several cannabinoid functional assays, including the [^{35}S]-GTPγS binding assay (Griffin *et al*., 1998). Furthermore, the four compounds represent each of the major structural classes of cannabinoid receptor agonist – bicyclics, tricyclics, aminoalkylindoles and eicosanoids (Figure 1). O-584 (Figure 3), O-823 (Figure 4) and O-1184 (Figure 5) all produced a concentration-dependent antagonism of agonist-stimulated GTPγS binding. The nature of the observed antagonism was usually that of parallel rightward shifts of agonist concentration response curves, with no reduction in the E_{max} of the agonist (from non-linear regression analysis). However, as a result of the low potency of WIN 55,212-2, it is not possible to determine whether the E_{max} of the agonist was affected in the presence of O-823 (100 nM) (Figure 4A). Multiple concentrations of O-584, O-823 and O-1184 (30, 100 and 300 nM) were used in the presence of HU-210 in order to construct Schild plots, the slopes of which did not deviate significantly from unity. Furthermore, the K_B values calculated from the Schild plots did not differ between the agonists used (Table 1) or from binding affinity in rat whole brain (B.R. Martin, unpublished results). As it appears that the K_B values of the Δ^8-THC analogues are largely consistent in their action on agonists of different structural classes, in subsequent experiments, WIN 55212-2 was chosen for analysis of antagonism. O-1176, O-806 and Δ^8-THC each produced a parallel rightward shift of WIN 55212-2-stimulation of GTPγS binding, consistent with that observed with a competitive reversible antagonist. The K_B values, calculated from the Schild equation, are shown in Table 1. Using whole brain (minus cerebellum) membranes, O-584 and O-1184 antagonized WIN 55212-2-stimulation of GTPγS binding, with K_B values of 3.23 (1.64 – 5.67) nM (O-584) and 2.23 (1.13 – 3.82) nM (O-1184). These values were not significantly different to those obtained using cerebellar membranes.

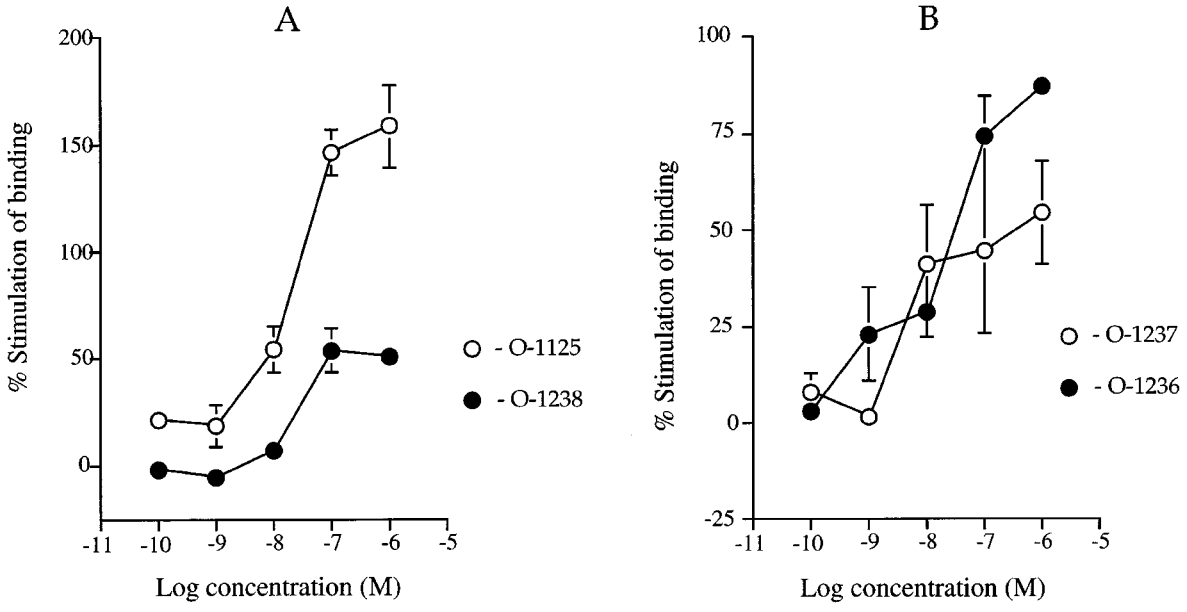

Figure 2 Effect of O-1236, O-1237, O-1238 and O-1125 on [^{35}S]-GTPγS binding. (A) Concentration-response curves of O-1125 and O-1238 constructed in the presence of GDP (100 μM). (B) Concentration-response curves of O-1236 and O-1237 conducted in the presence of GDP (10 μM). Data represent percentage stimulation over basal levels. Results are presented as means $\pm$ s.e.mean for $n = 3 – 4$ experiments.

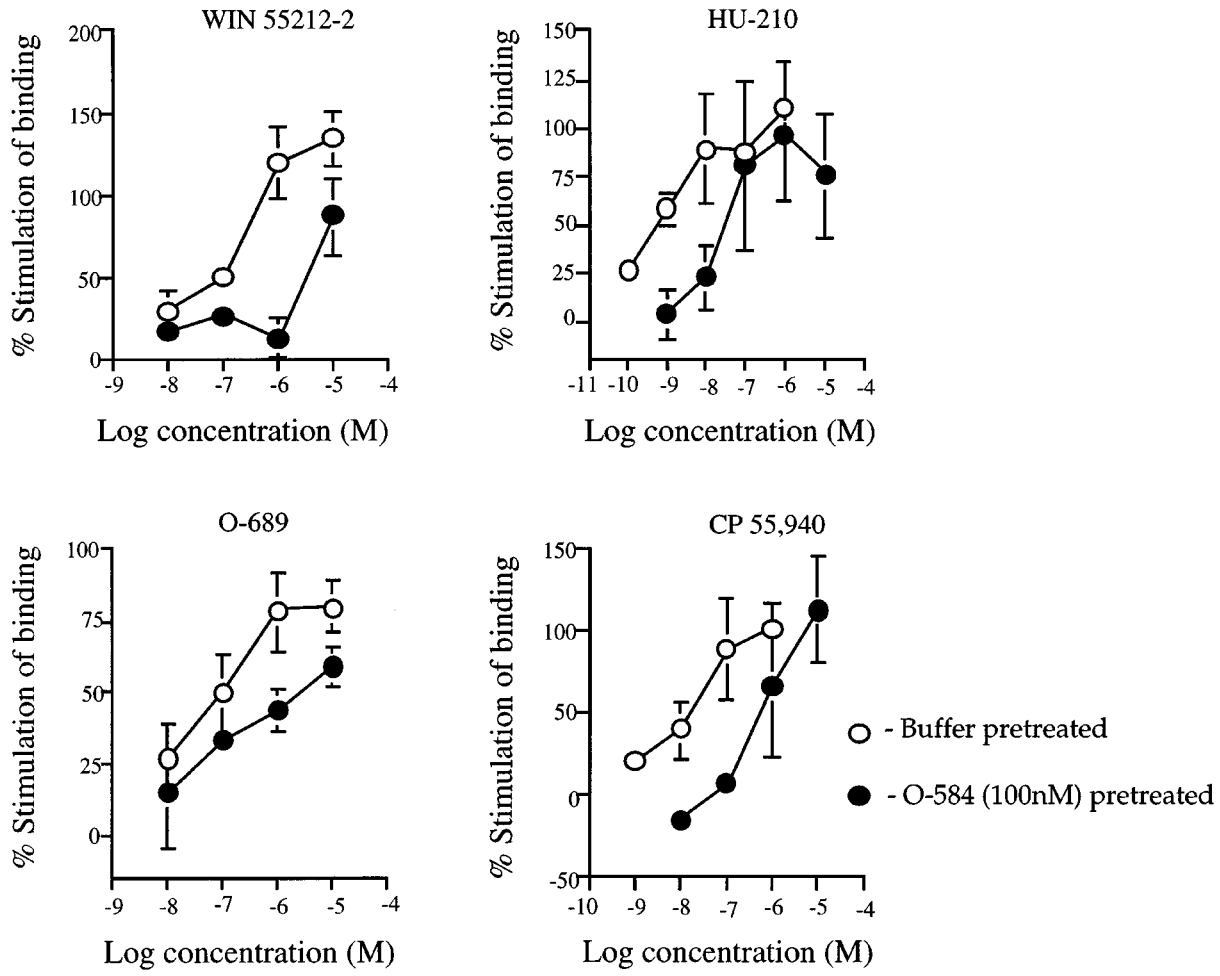

Figure 3 Effect of O-584, at a concentration of 100 nM on the mean concentration-response curves of WIN 55212-2, HU-210, O-689 and CP 55,940. Data represent percentage stimulation over basal levels. Results are presented as means ± s.e.mean for $n = 3-5$ experiments.

Radioligand binding studies

In order to address whether the potencies of the Δ^8-THC analogues correlated with their binding affinity, radioligand binding studies were carried out. Total binding of [^{3}H]-SR 141716A to rat cerebellar membranes displayed a linear relationship at protein concentrations from 10–80 μg 0.5 ml^{-1} (data not shown). Specific binding reached a plateau above 30 μg 0.5 ml^{-1}. Therefore, 20 μg 0.5 ml^{-1} of rat cerebellar membrane was used in all assays. Specific binding to membranes averaged 82% at a radioligand concentration of 0.5 nM. Saturation experiments were conducted with radioligand concentrations of 0.1–5 nM and the K_D value calculated to be 0.36 ± 0.05 nM and a B_{max} of 4.39 ± 0.49 pmol mg^{-1}. The data fitted with a one-site model. These values agree with previously reported data obtained in the cerebellum (Hirst *et al.*, 1996).

As the binding of competitive antagonists such as [^{3}H]-SR 141716A is unaffected by sodium ions and guanine nucleotides (Rinaldi-Carmona *et al.*, 1996), it was of interest to determine the effects of these regulators on the binding of O-584 and O-1184 in order to assess whether they also acted as competitive receptor antagonists. This was achieved by comparing K_i values obtained from experiments conducted in either binding buffer A (sodium-and GDP/GTPγS-free) or in GTPγS assay buffer containing GDP (100 μM) and GTPγS (0.05 nM) (Figure 6). K_i values calculated from experiments using binding buffer A were 5.17 ± 1.19 and 1.98 ± 0.31 nM for O-584 and O-1184 respectively. K_i values calculated from experiments using GTPγS assay buffer were 37.54 ± 2.88 and 9.58 ± 0.37 nM for O-584 and O-1184 respectively. These values are significantly different from those calculated in the absence of sodium and guanine nucleotides (Table 2). The rightward shifts of the displacement curves of O-584 and O-1184 in the presence of GTPγS assay buffer were 7.26 and 4.84 fold respectively. In contrast, K_i values of SR 141716A were not affected by the presence of guanine nucleotides and sodium ions (K_i (Binding buffer A) = 0.46 ± 0.09 nM; K_i (GTPγS assay buffer) = 0.40 ± 0.08 nM) (Table 2).

Discussion

The purpose of this study was to investigate the activities of a range of structural analogues of Δ^8-THC in the [^{35}S]-GTPγS binding assay in rat cerebellar membranes and to evaluate the role of the side-chain of Δ^8-THC in determining receptor efficacy. The structural modifications all centred on the aliphatic side-chain of the Δ^8-THC molecule and included varying the degree of saturation of the side-chain and the addition of various substituent groups to the terminal carbon.

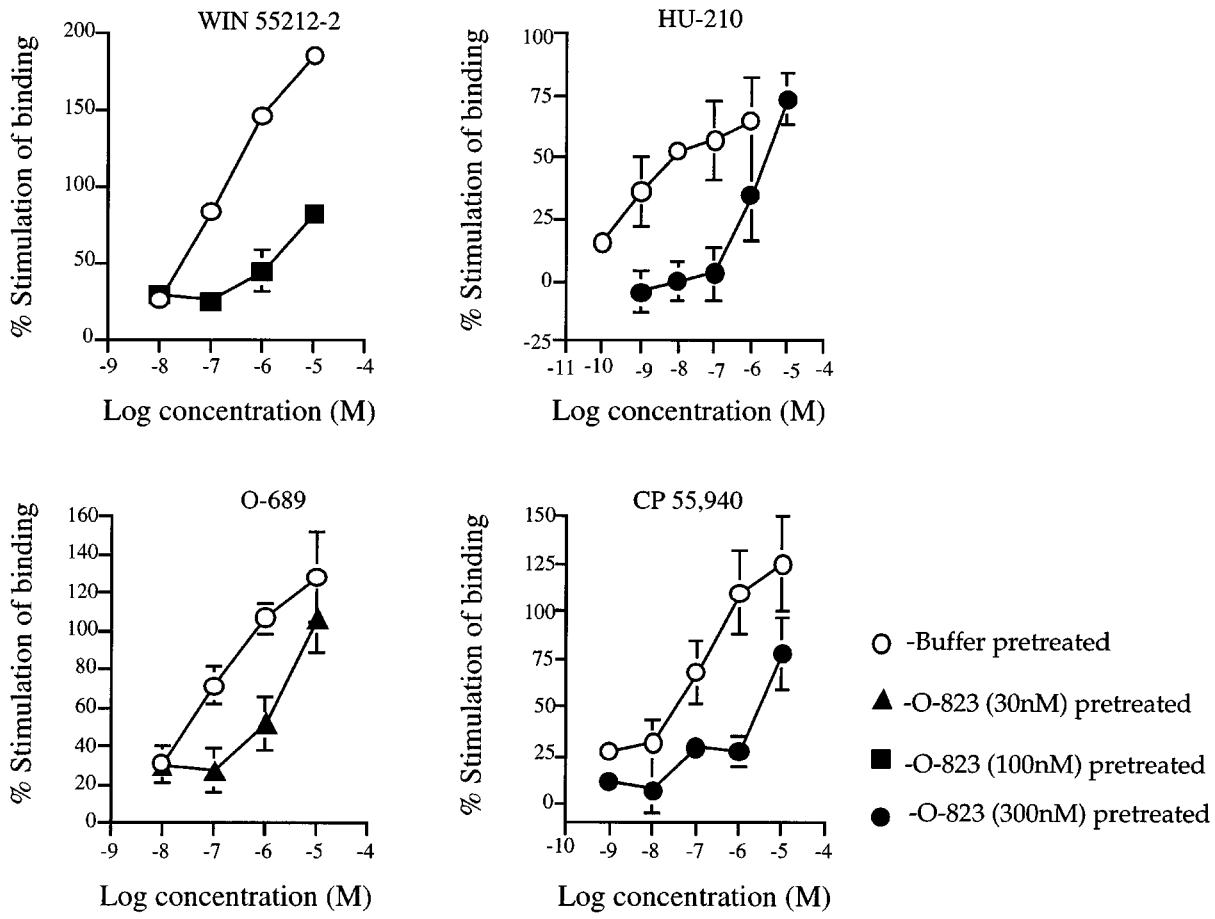

Figure 4 Effect of O-823, at concentrations of 30, 100 and 300 nM on the mean concentration-response curves of WIN 55212-2, HU-210, O-689 and CP 55,940. Data represent percentage stimulation over basal levels. Results are presented as means ± s.e.mean for $n=4$ experiments.

At high GDP concentrations (100 μM), it was found that only two compounds, 3-(1,1-dimethyl-6-dimethylcarboxamide)-Δ^8-THC (O-1125) and 3-(6-azido-2-hexenyl)-Δ^8 – THC (O-1238), produced stimulation of [^{35}S]-GTPγS binding. O-1125 acted as a full potent agonist with an efficacy comparable to other full agonists in the GTPγS binding assay (CP 55,244, HU-210 and WIN 55212-2), and a potency comparable to CP 55,940 (Griffin *et al*., 1998). O-1238 produced a lower maximal stimulation of binding (60% basal levels as opposed to 165% for O-1125). However, when the GDP concentration was reduced to 10 μM, which may favour lower efficacy agonists, as previously shown with THC (Griffin *et al*., 1998), the bromo- (O-1236) and cyano- (O-1237) homologues to O-1238 also stimulated binding, acting as partial agonists. The activity of O-1238 was not affected as a result of this change in GDP concentration. SR 141716A was found to antagonize the effects of O-1125, O-1236, O-1237 and O-1238 with consistent K_B values. These K_B values correlate with those previously found using other cannabinoid receptor agonists in cerebellar membranes (Griffin *et al*., 1998). These findings suggest the likelihood that these compounds act at a single receptor site, CB_1. The remaining compounds were also tested at 10 μM GDP and in the absence of sodium ions. Reducing the concentration of sodium ions in the assay may further increase the stimulatory effect of low efficacy agonists as sodium ions have been shown to modulate the affinity of the receptor for the G-protein, reduce spontaneous receptor/G-protein coupling and to increase the inhibitory influence of GDP on basal levels of GTPγS binding (Kenakin, 1996; Weiland & Jacobs, 1994). A recent study, (Petitet *et al*., 1997), demonstrated that in the absence of sodium, THC and other low efficacy agonists such as cannabinol, produced a significant stimulation of binding. However, removing the sodium ions did not increase the ability of the remaining compounds to stimulate [^{35}S]-GTPγS binding in this study. Due to a lack of detectable agonist effect, each compound was then tested for its ability to antagonize one or more standard cannabinoid receptor agonists including CP 55,940, WIN 55212-2, HU-210 and O-689.

All compounds tested in this way, the -ynyl compounds (O-584, O-806, O-823, O-1176 and O-1184), and Δ^8-THC attenuated the effects of each of the agonists used. O-584, O-823 and O-1184 each acted as surmountable antagonists, confirmed by the use of multiple concentrations of antagonist in the presence of a single agonist. Construction of Schild plots yielded slopes which did not differ from unity. Δ^8-THC, O-806 and O-1176 produced a parallel rightward shift of the WIN 55212-2 concentration-response curve without affecting the maximal response of the agonist, consistent with the possibility of competitive antagonism. The K_B values obtained for O-584 and O-1184 did not differ significantly from the binding affinities in rat cerebellum. Furthermore, radioligand binding experiments conducted in a CB_1-transfected cell line demonstrate that the K_i values for these compounds do not differ significantly from those obtained in the cerebellum (G. Griffin, unpublished results). This suggests that the binding, at

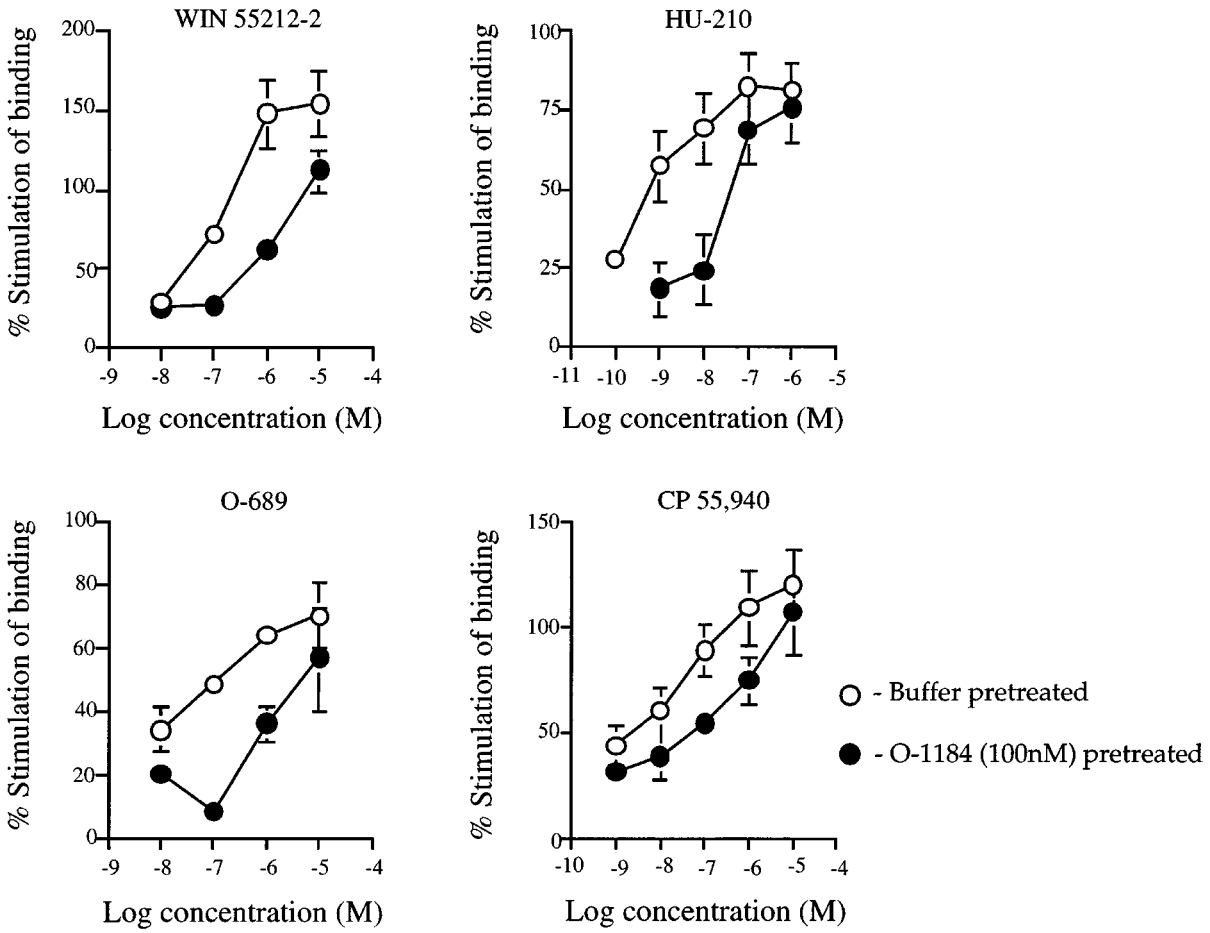

Figure 5 Effect of O-1184, at a concentration of 100 nM on the mean concentration-response curves of WIN 55212-2, HU-210, O-689 and CP 55,940. Data represent percentage stimulation over basal levels. Results are presented as means ± s.e.mean for $n = 4-8$ experiments.

Table 1 Equilibrium dissociation constants (K_B values) of O-584, O-823, O-1184, O-806, O-1176 and Δ^8-THC calculated in the presence of four cannabinoid receptor agonists

Analogue	*WIN 55212-2* (nM)	*HU-210* (nM)	*Fluoromethanadamide* (nM)	*CP 55,940* (nM)
O-584	3.40 (2.16–5.08)	3.17 (1.09–7.06)	2.58 (0.93–5.51)	3.21 (1.01–7.52)
O-823	0.97 (0.46–1.71)	0.25 (0.05–0.66)	1.82 (1.14–2.83)	4.85 (4.41–5.38)
O-1184	4.47 (3.04–6.36)	1.62 (1.44–1.83)	2.98 (0.61–8.53)	2.97 (1.02–10.78)
Δ^8-THC	70.67 (41.29–118.48)	N.D.	N.D.	N.D.
O-1176	11.93 (8.03–18.15)	N.D.	N.D.	N.D.
O-806	0.63 (0.39–1.01)	N.D.	N.D.	N.D.

The data are expressed as nM with the 95% confidence limits indicated by parentheses. $n = 5-7$ experiments. The concentrations of antagonist used in these experiments ranged from 30–300 nM. N.D., not determined.

least for these two compounds, reflects an activity solely at CB_1 receptors. In general, it was found that O-584, O-823 and O-1184 were equally effective in attenuating the stimulatory effects on GTPγS binding of WIN 55212-2, CP 55,940, HU-210 and O-689.

The results of the experiments with this series of compounds demonstrates the importance, and a possible role, of the aliphatic side-chain of Δ^8-THC, and by inference, other classical cannabinoid structures such as THC and HU-210. Previously, extending the length of the side chain has been shown to increase the affinity and potency of cannabinoid receptor ligands for the CB_1 receptor and was postulated to be one of the three key points of THC for receptor interaction (Martin *et al.*, 1995). An example is the extension of the pentyl Δ^8-THC side-chain to a dimethylheptyl. This alteration results in a 10–30 fold potency increase *in vivo* and a 60 fold increase in affinity of the molecule (Martin *et al.*, 1995). Furthermore, an identical side-chain is found on other high-potency, high-affinity cannabinoid receptor ligands such as HU-210, CP 55,940 and CP 55,244. This study investigated two aspects of this side-chain. Firstly, the importance of the degree of saturation of the side chain was examined (Δ^8-THC and O-1125 have saturated side-chains; O-1236, O-1237 and O-1238 all contain a cis-double bond within the side chain and the remaining compounds all contain a triple bond). The lengthening of the Δ^8-THC side-chain, in the cases of O-584 and O-1184, increased the affinity of the ligand for the CB_1 receptor over the parent molecule (O-584 – 5.17 nM, O-1184 –

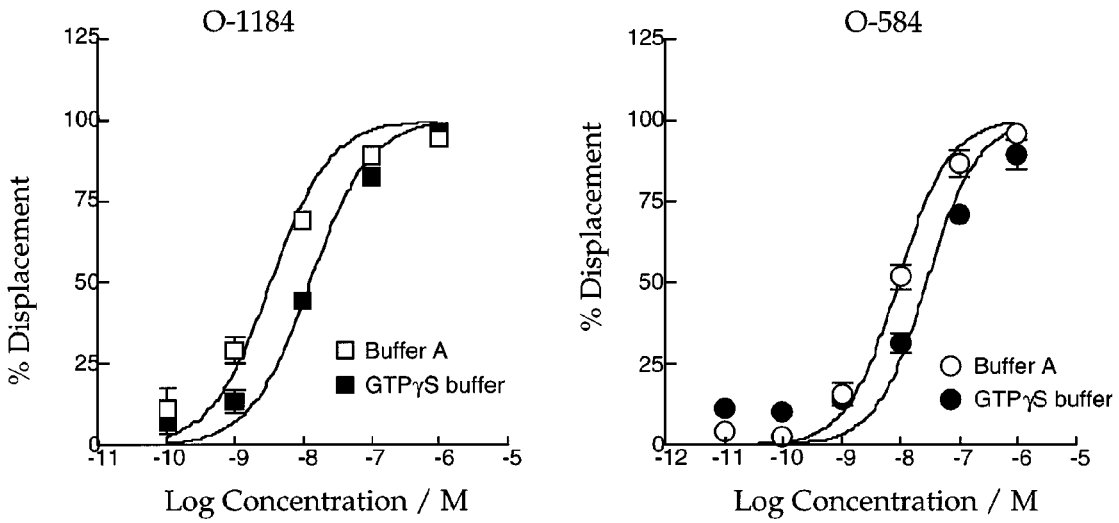

Figure 6 Displacement of bound [^{3}H]-SR 141716A from cerebellar membranes by O-1184 and O-584 in the presence of binding buffer A or GTPγS assay buffer. The data are presented as percentage of displacement of specific binding; 0.35 nM [^{3}H]-SR 141716A was the concentration of radioligand used. Non-specific binding was measured in the presence of SR 1417167A (1 μM). Data points are the means$\pm$s.e.mean of three experiments performed in triplicate.

Table 2 Comparison of K_i values of SR 141716A, O-584, and O-1184 in the rat cerebellum in the presence and absence of sodium ions (150 mM) and guanine nucleotides (GDP 100 μM and 0.05 nM GTPγS)

Analogue	*Rat cerebellum* (nM)	*Rat cerebellum (in presence of Na^+ and guanine nucleotides)* (nM)
O-584	5.17$\pm$1.19	37.5$\pm$2.88***
O-1184	1.98$\pm$0.31	9.58$\pm$0.37***
SR 141716A	0.46$\pm$0.09	0.40$\pm$0.08

***$P<0.0001$, unpaired Student's t-test, two-tailed. The data are expressed as nM$\pm$s.e.mean for $n=4-5$ experiments.

1.98 nM compared to Δ^8-THC $K_i=295$ nM (Hirst *et al*., 1996)). Similarly, the other compounds in the series also display an increased affinity over Δ^8-THC in whole rat brain (B.R. Martin, unpublished results). However, despite the high affinity exhibited by all of these compounds (in the low nanomolar range), there was a distinct pattern of efficacies observed. The saturated side-chain analogue, O-1125, was a potent, high efficacy agonist; the double bond (-enyl) compounds were all partial agonists and the triple bond (-ynyl) compounds antagonists. This trend suggests that although lengthening the Δ^8-THC side-chain increases a compound's affinity for the CB_1 receptor, irrespective of the substituent group used (at least with the compounds used in this study), the efficacy of the compound is decreased as the degree of unsaturation of the aliphatic side-chain increases. The presence of single bonds throughout the side chain would likely confer a very flexible nature to this part of the molecule whereas the presence of cis-double bonds would increase its rigidity, particularly around the double bond. Similarly, the presence of a triple bond may increase this rigidity even farther, across four carbon atoms and also in a more linear conformation. It is possible, therefore, that the steric conformation of the side-chain may be integral to the intrinsic efficacy, rather than the affinity of, the cannabinoid receptor ligand at the CB_1 receptor. Further structural modifications are required to fully test this hypothesis. The second aspect of the study was to investigate how substituent groups on the terminal carbon of the side-chain may affect the activity of the compound. Of the substitutions examined in this study (Br-, CN- and N_3- in the double bond series and H-, Br-, CN-, N_3- and NCS- in the triple bond series) there were no dramatic alterations in potency (double bond compounds) or K_B values (triple bond compounds).

An important point raised by both this study, and our previous one (Griffin *et al*., 1998) is the ability of the GTPγS binding assay to predict how a cannabinoid receptor ligand will behave in other functional assays. This was not an aim of this particular project, due to the assay conditions used, but certain comparisons are worth noting. Preliminary studies using the mouse tetrad model have demonstrated the compounds used in this study to behave as a mixture of agonists (O-1125, O-1236, O-1237, O-1238 and O-584), partial agonists (O-1184) and inactive compounds (O-806, O-1176 and O-823) (B.R. Martin, unpublished results). For high and medium efficacy agonists such as O-1236, O-1237, O-1238 and O-1125, activities between different functional assays appears relatively straightforward, as it does with compounds of very low efficacy or pure antagonists (for example, O-806, O-823, O-1176 and SR 141716A). However, with compounds such as O-584 and O-1184 the relationship between ability to stimulate GTPγS binding and agonist activity in other functional assays is less direct. The reasons for this are not immediately obvious and several possibilities exist. There may be a different population of receptors involved in the whole brain (mouse tetrad model) and the cerebellum (GTPγS binding). However, O-584 and O-1184 behaved almost identically in GTPγS binding experiments using either whole brain (minus cerebellum) or cerebellar membranes suggesting that the receptors involved were identical between the two models.

It is also possible that in an assay which measures a variable at the end of the signal transduction cascade rather than at the level of receptor-G-protein coupling, there is sufficient signal amplification through the signal transduction cascade to produce a measureable response. This would be more likely to affect lower efficacy agonists than those of higher efficacies. This differentiation has been seen previously with the GTPγS assay using THC as an example, a compound relatively inactive in the GTPγS binding assay, but a full agonist in other assays, such as in smooth muscle models (Sim *et al*., 1996; Pertwee & Griffin, 1995). We have previously discussed the

bias of our experimental conditions towards high-efficacy compounds such as CP 55,940 and WIN 55212-2, maximizing the stimulation obtained with these compounds, and concurrently reducing the stimulation obtained from lower efficacy compounds such as THC (Griffin *et al*., 1998). Changing the experimental conditions, for example by reducing the GDP concentration may allow efficacy agonists to displace GDP from the G-protein and thus enable a stimulation of [^{35}S]-GTPγS binding not seen at higher GDP concentrations. This relationship has been previously demonstrated with other G-protein coupled receptors such as μ-opioid receptors (Selley *et al*., 1997) and has also been observed in this study with the compounds O-1236 and O-1237. It is possible that O-584 and O-1184 in particular, but also O-823, may have even lower efficacies than these compounds and therefore they may not be able to stimulate significant GTPγS binding under these conditions. In contrast, in a model such as an *in vivo* paradigm, what may be a very low G-protein signal is potentially amplified by the signal transduction cascade sufficiently to produce significant agonism. In an attempt to test this hypothesis, radioligand binding was carried out in the cerebellum using [^{3}H]-SR 141716A. It has previously been shown with both opioid and cannabinoid receptors that the presence of guanine nucleotides and sodium ions decreases the binding of agonists but not antagonists (Childers & Snyder, 1980; Rinaldi-Carmona *et al*., 1996). Therefore, displacement studies were conducted with SR 141716A, O-584 and O-1184 in identical conditions to the GTPγS binding experiments, or in the absence of guanine nucleotides and sodium ions. It was found that in the presence of sodium ions, GDP and GTPγS, the K_i of both O-584 and O-1184 was reduced by 7.26 fold and 4.84 fold respectively whereas the K_i of SR 141716A was unaffected. This supports the possibility that O-584 and O-1184 may indeed be agonists, and it is simply the very low efficacy of these compounds which results in the lack of stimulatory effect on [^{35}S]-GTPγS binding with the assay conditions used in this study.

However, the main aim of this study was to examine potential efficacy differences resulting from alterations of the Δ^8-THC side-chain, rather than to directly predict the exact behaviour of a compound in an alternative functional assay. For this reason, our assay conditions were designed to maximize efficacy differences between compounds, with lower efficacy agonists, such as THC, producing little or no stimulation of GTPγS binding and concurrently maximizing the stimulation of binding produced by higher efficacy compounds (Griffin *et al*., 1998).

In summary, the results contained in this study demonstrate several important points. Firstly, lengthening the aliphatic side-chain of the classical cannabinoid structure increases affinity for the CB_1 receptor. Secondly, the steric conformation of this side-chain, and specifically its rigidity and orientation in the region immediately adjoining the A ring of the Δ^8-THC molecule, greatly affects the efficacy of the molecule at the CB_1 receptor. The results also demonstrate how this assay may be of particular value in examining efficacy differences between receptor ligands. In light of the paucity of potent, selective cannabinoid ligands of varying efficacies, this may prove to be important in the development of such ligands by providing a means with which to evaluate the structural mechanisms behind drug efficacy.

This work was supported in part by National Institute on Drug Abuse Grants DA-09978, DA-05274, DA-09789 and the Council for Tobacco Research Grant CTR-4482.

References

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248 – 254.

BURKEY, T.H., QUOCK, R.M., CONSROE, P., ROESKE, W.R. & YAMAMURA, H.I. (1997). Δ^9-tetrahydrocannabinol is a partial agonist of cannabinoid receptors in mouse brain. *Eur. J. Pharmacol.,* **323,** R3 – R4.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) on an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099 – 3108.

CHILDERS, S.R. & SNYDER, S.H. (1980). Differential regulation by guanine nucleotides of opiate agonist and antagonist receptor interactions. *J. Neurochem.,* **34,** 583 – 593.

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.

GRIFFIN, G., ATKINSON, P.J., SHOWALTER, V.M., MARTIN, B.R. & ABOOD, M.E. (1998). Evaluation of cannabinoid receptor agonists and antagonists using the [^{35}S]GTPγS binding assay in rat cerebellar membranes. *J. Pharmacol. Exp. Ther.,* **285,** 553 – 560.

HIRST, R.A., ALMOND, S.L. & LAMBERT, D.G. (1996). Characterisation of the rat cerebella CB_1 receptor using SR141716A, a central cannabinoid receptor antagonist. *Neurosci. Lett.,* **220,** 101 – 104.

KENAKIN, T. (1996). The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol. Rev.,* **48,** 413 – 459.

MARTIN, B.R., THOMAS, B.F. & RAZDAN, R.K. (1995). Structural requirements for cannabinoid receptor probes. In *Cannabinoid Receptors* ed. R.G. Pertwee, pp 35 – 86, London: Academic Press.

PERTWEE, R.G. (1997). Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol. Ther.,* **74,** 129 – 180.

PERTWEE, R.G., FERNANDO, S.R., GRIFFIN, G., RYAN, W., RAZDAN, R.K., COMPTON, D.R. & MARTIN, B.R. (1996). Agonist-antagonist characterization of 6-cyano-2′-yne-Δ^8-tetrahydrocannabinol in two isolated tissue preparations. *Eur. J. Pharm.,* **315,** 195 – 201.

PERTWEE, R.G. & GRIFFIN, G. (1995). A preliminary investigation of the mechanisms underlying cannabinoid tolerance in the mouse vas deferens. *Eur. J. Pharm.,* **272,** 67 – 72.

PETITET, F., JEANTAUD, B., CAPET, M. & DOBLE, A. (1997). Interaction of brain cannabinoid receptors with guanine nucleotide binding protein: A radioligand binding study. *Biochem. Pharmacol.,* **54,** 1267 – 1270.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERRAR, P., SOUBRIE, P., BRELIERE, J.C. & FUR, G.L. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.,* **350,** 240 – 244.

RINALDI-CARMONA, M., PIALOT, F., CONGY, C., REDON, E., BARTH, F., BACHY, A., BRELIERE, J.-C., SOUBRIE, P. & LE FUR, G. (1996). Characterisation and distribution of binding sites for [^{3}H]SR141716A, a selective brain (CB1) cannabinoid receptor antagonist, in rodent brain. *Life Sci.,* **56,** 2033 – 2040.

SCHILD, H.O. (1949). pAx and competitive drug antagonism. *Br. J. Pharmacol.,* **4,** 227 – 280.

SELLEY, D.E., SIM, L.J., XIAO, R., LIU, Q. & CHILDERS, S.E. (1997). μ-opioid receptor-stimulated guanosine-5′-O-(γ-thio)-triphosphate binding in rat Thalamus and cultured cell lines: Signal transduction mechanisms underlying agonist efficacy. *Mol. Pharm.,* **51,** 87 – 96.

SELLEY, D.E., STARK, S., SIM, L.J. & CHILDERS, S.R. (1996). Cannabinoid receptor stimulation of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding in rat brain membranes. *Life Sci.,* **59,** 659 – 668.

SHOWALTER, V.M., COMPTON, D.R., MARTIN, B.R. & ABOOD, M.E. (1996). Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): Identification of cannabinoid receptor subtype selective ligands. *J. Pharmacol. Exp. Ther.,* **278,** 989 – 999.

SIM, L.J., HAMPSON, R.E., DEADWYLER, S.A. & CHILDERS, S.R. (1996). Effects of chronic treatment with Δ9-tetrahydrocannabinol on cannabinoid stimulated [^{35}S]GTPγS autoradiography in rat brain. *J. Neurosci.,* **16,** 8057 – 8066.

SIM, L.J., SELLEY, D.E. & CHILDERS, S.R. (1995). In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[γ-[^{35}S] thio]-triphosphate binding. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7242 – 7246.

WEILAND, T. & JAKOBS, K.H. (1994). Measurement of receptor-stimulated guanosine-5′-O-(γ-thio)triphosphate binding by G-proteins. *Methods Enzymol.,* **237,** 3 – 13.

(Received September 21, 1998
Revised January 8, 1999
Accepted January 13, 1999)