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Evaluation of Cannabinoid Receptor Agonists and Antagonists Using the Guanosine-5′-O-(3-[^{35}S]thio)-triphosphate Binding Assay in Rat Cerebellar Membranes[1]

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ABSTRACT

Cannabinoid receptors are members of the superfamily of G protein-coupled receptors. Their activation has previously been shown to stimulate guanosine 5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding in a range of brain regions using both membrane preparations and autoradiography. This study evaluates the activities of structurally diverse cannabinoid receptor ligands in the GTPγS binding assay, comparing the relationship between receptor binding and activation and also examining efficacy differences between compounds. Using rat cerebellar membrane preparations, the effects of GDP concentration on GTPγS binding and the activities of a range of cannabinoid receptor ligands, including the CB_1 selective antagonist SR141716A, were investigated. GDP concentration was found to have differing effects on cannabinoid-stimulated [^{35}S]GTPγS binding depending on the nature of the agonist used. The stimulation produced by high efficacy compounds, such as CP 55,940 and WIN 55212–2, was increased by raising the GDP concentration, but that of a low efficacy agonist, (−)-Δ-tetrahydrocannabinol, was decreased. Of the cannabinoid compounds tested, a wide range of potencies (EC_{50}) and levels of maximal stimulation (E_{max}) were observed. These ranged from CP 55,244 (E_{max} of 165, 148–183%, and an EC_{50} of 0.47, 0.22–0.96, nM) through (−)-Δ-tetrahydrocannabinol, cannabinol and anandamide, which produced no concentration-dependent stimulation of [^{35}S]GTPγS binding under the same conditions. SR141716A competitively antagonized all the agonists against which it was tested, providing equilibrium dissociation constants (K_d values) in the sub-nanomolar range (0.06–0.40 nM), implicating a CB_1 receptor mediated response. These results provide a more detailed characterization of the cannabinoid-stimulated [^{35}S]GTPγS binding assay than has previously been reported.

Cannabinoid drugs are thought to produce their unique pharmacological profile of effects through activation of specific membrane receptors (Devane *et al*., 1988; Munro *et al*., 1993). These receptors, termed CB_1 and CB_2, couple to guanine nucleotide binding proteins (G proteins) as described for a large variety of other receptors (Matsuda *et al*., 1990; Munro *et al*., 1993; Kenakin, 1996). Cannabinoid receptors are found throughout both the central nervous system and the periphery (Herkenham *et al*., 1991; Munro *et al*., 1993). Within the central nervous system, cannabinoid receptors are found to be localized in many brain areas, with the regions of densest receptor localization including the cerebellum, hippocampus, cortex and the basal ganglia (Herkenham *et al*., 1991). This distribution may be related to the pharmacological effects of administered cannabinoid drugs (Pertwee, 1993) and is very similar irrespective of species (Herkenham *et al*., 1991; Jansen *et al*., 1992; Glass *et al*., 1997).

Several functional assays are presently used to characterize cannabinoid compounds including whole animal tests, such as inhibition of locomotor activity and the development of hypothermia; inhibition of electrically stimulated contractions of isolated smooth muscle preparations; and inhibition of forskolin-stimulated cAMP accumulation in tissues and cell lines. In addition, the effects of cannabinoids on ion channel conductance and on the activity of mitogen-activated protein kinase have been used (for review see: Martin *et al*., 1995; Bouaboula *et al*., 1995).

Recently, a method for measuring cannabinoid-stimulated

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ABBREVIATIONS: [^{35}S]GTPγS, guanosine-5′-O-(3-[^{35}S]thio)-triphosphate; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; THC, (−)-Δ^9-tetrahydrocannabinol; CP 55,940, −)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride; WIN55212–2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone; JWH-073, 1-(1-butyl)-3-(1-naphthoyl)-indole; JWH-030, 3-naphthoyl-N-pentylpyrrole; O-1064, 2,16,16-trimethyl-all-*cis*-5,8,11,14-docosatetraenoyl-2′-fluoroethanolamide; HU-210, (−)-11-OH-Δ^8-THC-dimethylheptyl; ANOVA, analysis of variance; CB_1, central cannabinoid receptor; CB_2, peripheral cannabinoid receptor.

[^{35}S]GTPγS binding in brain membranes has been described (Sim *et al*., 1995). This model measures the first step in functional activation of the receptor as a result of agonist binding, therefore allowing for the delineation of agonist/antagonist activity regardless of the second messenger system involved. Following activation of the receptor by an agonist, the affinity of the G protein *alpha* subunit increases with respect to GTP *vs.* GDP. As a consequence, GDP is displaced from the G protein and GTP or GTPγS binds. If a radioactive label, such as [^{35}S], is attached to the GTPγS molecule, then the formation of the G protein/[^{35}S]GTPγS complex may be directly measured using liquid scintillation spectrophotometry (Weiland and Jakobs, 1994).

The aim of this study was to investigate the effects of a range of structurally diverse cannabinoid receptor ligands on [^{35}S]GTPγS binding. Further to this was an attempt to correlate a compound's ability to stimulate GTPγS binding with previously reported receptor affinity data and also with *in vivo* potency data.

Experimental Procedures

Materials. Male Sprague-Dawley rats (150–250 g) were obtained from Harlan (Dublin, VA). GDP and GTPγS were purchased from Boehringer Mannheim (Indianapolis, IN). [^{35}S]GTPγS (1000–1200 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Other reagent grade chemicals were purchased from Sigma (St. Louis, MO). THC and cannabinol were obtained from NIDA. SR141716A, CP 55,940 and CP 55,244 were generously provided by Pfizer (Groton, CT), and WIN 55212–2 was purchased from Research Biochemicals (Natick, MA). Anandamide, fluoromethanandamide and O-1064 were synthesized by Dr. Raj Razdan (Organix, Woburn, MA). HU-210 was generously provided by Prof. Raphael Mechoulam (Hebrew University, Jerusalem, Israel). Deoxy-HU-210, JWH-030 and JWH-073 were synthesized by Dr. John Huffman (Clemson University, Clemson, SC). All compounds were stored as 1 mg/ml solutions in ethanol.

Membrane preparation. Cerebella were dissected on ice from 3 fresh male Sprague-Dawley rat brains. The pooled tissue was suspended in centrifugation buffer (50 mM Tris HCl, 1 mM EGTA, 3 mM $MgCl_2$; pH 7.4) and homogenized using a Kontes Potter-Elvehjem glass-Teflon grinding system (Fisher Scientific, Sprinfield, NJ). The homogenate was centrifuged at 48,000 × *g* for 20 min at 4°C. The pellet was then resuspended in assay buffer (50 mM Tris HCl, 9 mM $MgCl_2$, 0.2 mM EGTA, 150 mM NaCl; pH 7.4), homogenized, and centrifuged as previously. The final P2 pellet was then resuspended in assay buffer, homogenized, and diluted to a concentration of ~ 2 μg/μl with assay buffer. The protein concentration was 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 containing .1% fatty acid free bovine serum albumin with GDP 1–100 μM, [^{35}S]GTPγS 0.05 nM and cannabinoid compounds/ethanol control in siliconized glass tubes. The total assay volume was 0.5 ml, which was incubated at 30°C for 30 min. An incubation time of 60 min was used in experiments with HU-210 as a time course experiment demonstrated this to be the optimal time for maximal stimulation of [^{35}S]GTPγS binding (data not shown). Experiments with anandamide also included 50 μM PMSF. The reaction was terminated by addition of 2 ml ice-cold wash buffer (50 mM Tris HCl, 5 mM $MgCl_2$; pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass-fiber 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 (RPI Corp., Mount Prospect, IL). Bound radioactivity was determined by liquid scintillation spectrophotometry after extraction in 5 ml BudgetSolve scintillation fluid. Non-specific binding was determined using 10 μM GTPγS. 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 levels (*i.e*., {[dpm (agonist) − dpm (no agonist)]/dpm (no agonist)}× 100).

Data analysis. Data are reported as mean ± S.E.M. of three to eight experiments, performed in triplicate. Nonlinear regression analysis of concentration-response data was performed using Prism 2.0 software for the Macintosh (GraphPAD Software, San Diego, CA) to calculate E_{max} and EC_{50} values. One-way ANOVA using Dunnett's *post-hoc* ($P < .05$) was used for statistical analysis. The equilibrium dissociation constant (K_d) for the interaction of the antagonist and the receptor has been calculated from the equation (dose ratio − 1) = [B] − K_d, where [B]is the concentration of the antagonist used in the experiment (Pertwee *et al*., 1995a).

Results

Effects of GDP on agonist-stimulated [^{35}S]GTPγS binding. Preliminary experiments were directed at optimizing the assay conditions for WIN 55212–2 stimulation of [^{35}S]GTPγS binding. To optimize this assay, the conditions should ideally overcome spontaneous agonist-independent guanine nucleotide exchange at the G protein, as has been demonstrated to occur with other G protein-coupled receptors (Kenakin, 1996), and also to maximize the ability of an agonist to induce GDP dissociation and [^{35}S]GTPγS association with the G protein following receptor activation (Weiland and Jakobs, 1994). The combination of these factors may be expected to result in both a decrease in basal and an increase in agonist-stimulated [^{35}S]GTPγS binding. Therefore, the influences of GDP, sodium and magnesium ions on WIN 55212–2-stimulated [^{35}S]GTPγS binding were investigated.

Sodium ions have been shown to modulate the affinity of the receptor for the G protein, reduce spontaneous coupling of opioid receptors and G proteins and to increase the inhibitory effect of GDP on basal [^{35}S]GTPγS binding (Kenakin, 1996; Weiland and Jakobs, 1994). High concentrations of sodium ions (100–150 mM) have also been demonstrated to increase the ability of WIN 55212–2 to stimulate [^{35}S]GTPγS binding in rat cerebellar membranes (Selley *et al*., 1996). Furthermore, magnesium ions have also been shown to influence agonist-stimulated[^{35}S]GTPγS binding to G proteins by increasing the affinity of the G protein for the receptor (Weiland and Jakobs, 1994). Therefore, the concentrations of these ions were varied to establish the optimal concentrations of 9 mM $MgCl_2$ and 150 mM NaCl.

Previously, the effect of GDP concentration on agonist-stimulated [^{35}S]GTPγS binding has been investigated (Selley *et al*., 1996). In the presence of excess GDP, the population of G proteins shifts toward an inactive state, thus ensuring both minimal basal binding and also maximal stimulation by agonists. To investigate the effect of GDP concentration on WIN 55212–2-stimulation of [^{35}S]GTPγS binding, membranes were incubated with various concentrations of GDP in the presence and absence of WIN 55212–2. Using the conditions of Sim *et al*. (1995), it was found that, at concentrations of 30 to 100 μM, GDP inhibited both basal binding and also that of 10 μM WIN 55212–2-stimulated binding in a concentration-dependent manner (fig. 1A). If the data are replotted as a

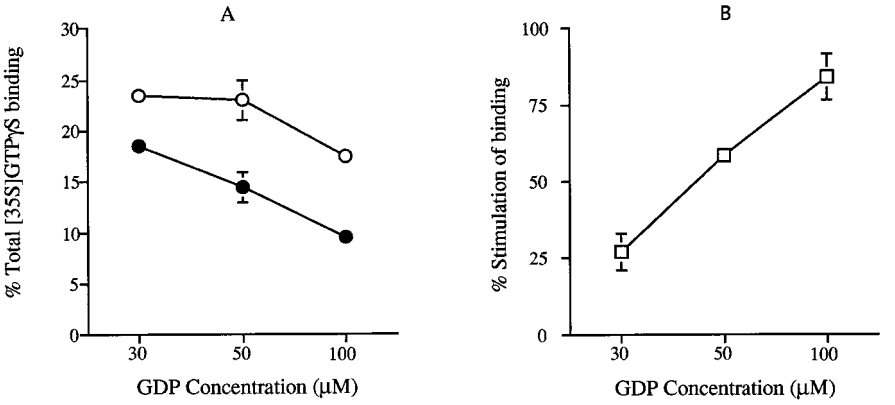

Fig. 1. Effect of GDP on basal and WIN 55212–2 (10 μM) stimulated [35S]GTPγS binding. A, Data represent percentage total specific [35S]GTPγS binding in the presence (○) and absence (●) of WIN 55212–2 (10 μM). B, Data represent percentage stimulation over basal binding induced by WIN 55212–2 (10 μM) (□). Results are presented as mean ± S.E. for $n = 4$ experiments.

percentage stimulation of binding over basal levels, it is found that increasing GDP concentrations increase the agonist-induced stimulation observed (fig. 1B). To maximize the stimulation produced by cannabinoid agonists, a GDP concentration of 100 μM was chosen.

The effect of GDP, at concentrations of 10 and 100 μM, on the concentration-response curves of three cannabinoid receptor ligands, WIN 55212–2, CP 55,940 and THC is shown in figure 2. The maximal stimulation (E_{max}) of each of these compounds under each condition is shown in table 1. WIN 55212–2 and CP 55,940 both produced a greater E_{max} with 100 μM GDP, but at this concentration, there was no concentration-dependent stimulation of [35S]GTPγS binding by THC. However, at 10 μM GDP, THC produced a significant concentration-dependent stimulation of [35S]GTPγS binding (one-way ANOVA, Dunnett's *post-hoc*, $P < .05$).

To summarize, to establish optimal conditions for maximal stimulation of binding, a systematic evaluation of WIN 55212–2 effects were undertaken. WIN 55212–2 was chosen as it has previously been shown to stimulate [35S]GTPγS binding in rat cerebellar membranes (Selley *et al*., 1996). The optimal conditions for WIN 55212–2-stimulated [35S]GTPγS binding were found to be: 10 μg protein per 0.5 ml assay volume; 9 mM $MgCl_2$; 150 mM NaCl; 100 μM GDP; 30 min incubation at 30°C. Under these conditions, WIN 55212–2, at a concentration of 10 μM, stimulated [35S]GTPγS binding by 156% (144–169%) over basal levels (table 2).

Effects of various cannabinoid receptor ligands on [35S]GTPγS binding. To examine the effects of a number of structurally diverse cannabinoid ligands on[35S]GTPγS binding, membranes were incubated with varying concentrations of ligands from each of the four classes of cannabinoids; (1) classic cannabinoids (THC; HU-210; deoxy-HU-210; cannabinol), (2) nonclassic cannabinoids (CP 55,940; CP 55,244), (3) aminoalkylindoles (WIN 55212–2; JWH-030; JWH-073) and (4) eicosanoids (anandamide; fluoromethanandamide; O-1064). HU-210 and CP 55,244 were used as they have previously been shown, in several assay systems, to be high potency cannabinoid agonists (Howlett, 1995). Deoxy-HU-210 was used as this has a much higher affinity for CB_2 receptors than it does for CB_1 (Huffman *et al*., 1996). JWH-030 and JWH-073 were used as these indole analogues have been shown to have affinity for both CB_1 and CB_2 receptors and have not previously been tested in any functional assays (Showalter *et al*., 1996; B.R. Martin, unpublished results). Fluoromethanandamide and O-1064 were used as high affinity, metabolically stable anandamide analogues (Adams *et al*., 1995). The structures for each of these compounds are shown in figure 3. To keep conditions for the comparison of each compound identical, the GDP concentration was kept constant (100 μM). The results of these experiments are shown in table 2. CP 55,244 was the most potent of the compounds, and also produced the highest percentage stimulation of binding, with EC_{50} and E_{max} values of 0.47 (0.22–0.96) nM and 165% (148–183%), respectively. WIN 55212–2, deoxy-HU-210 and HU-210 all had E_{max} values that did not

TABLE 1
Effect of GDP concentration on the ability of three cannabinoid receptor agonists to stimulate [35S]GTPγS binding in rat cerebellar membranes

Agonist	GDP concentration	E_{max}
	μM	*% Stimulation of binding*
CP 55,940	10	61 (32–91)
	100	114 (97–131)
WIN 55212-2	10	89 (75–109)
	100	156 (144–169)
THC	10	51 (46–57)
	100	N/A

NA, not applicable; no significant concentration-dependent stimulation.
The numbers in parentheses represent 95% confidence limits.

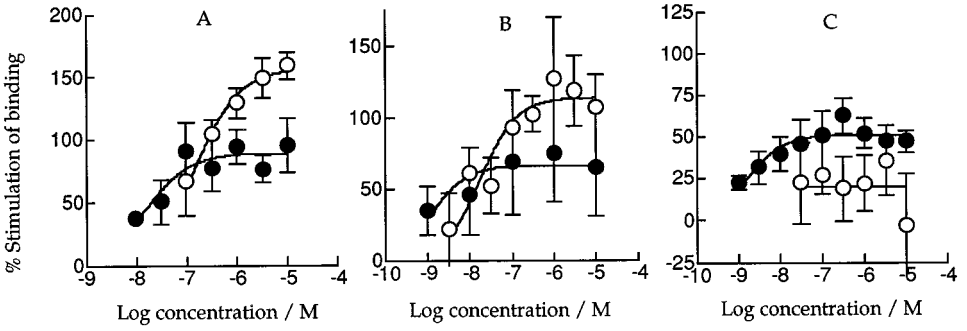

Fig. 2. Effect of 10 μM (●) and 100 μM (○) GDP on the concentration-response curves of WIN 55212–2 (A), CP 55,940 (B) and THC (C). Data represent percentage stimulation over basal binding. Results are presented as mean ± S.E. for $n = 4$ experiments.

TABLE 2
Ability of various cannabinoid receptor ligands to stimulate [^{35}S]GTPγS binding in rat cerebellar membranes at a GDP concentration of 100 μM

Compound	E_{max}	EC_{50}	CB1 K_i^i
	% Stimulation	*nM*	*nM*
CP 55,244	165 (148–183)	0.47 (0.22–0.96)	0.11[a]
WIN 55212-2	156 (144–169)	151.1 (101.6–227)	1.89[b]
Deoxy-HU-210	150 (95–205)	9.60 (0.91–95.6)	1.15[c]
HU-210	140 (117–152)	0.55 (0.25–1.20)	0.73[d]
CP 55,940	114 (97–131)	17.57 (7.03–43.94)	0.58[e]
Fluoromethanand-amide	97 (55–140)	25.37 (4.98–129.2)	5.70[e]
JWH-030	56 (37–76)	82.40 (14.0–484.1)	87[f]
JWH-073	29 (19–40)	104.8 (16.94–648.3)	8.90[g]
O-1064	60 (5–116)	246.2 (12.45–4869)	0.97[g]
Anandamide	NA	NA	89[h]
THC	NA	NA	40.70[e]
Cannabinol	NA	NA	308[e]

NA, not applicable; no significant concentration-dependent stimulation.
[a] Melvin *et al.* 1995.
[b] Kuster *et al.,* 1993.
[c] Huffman *et al.,* 1996.
[d] Compton *et al.,* 1993.
[e] Showalter *et al.,* 1996.
[f] Lainton *et al.,* 1995.
[g] B. R. Martin, unpublished results.
[h] Adams *et al.,* 1995.
[i] CB1, central cannabinoid receptor.
The numbers in parentheses represent 95% confidence limits.

differ significantly from CP 55,244 and therefore may also be classified as full agonists. CP 55,940, fluoromethanandamide, O-1064, JWH-030 and JWH-073 all stimulated [^{35}S]GTPγS binding, but with a significantly lesser maximal effect, and may therefore be classed as partial agonists. The maximal percentage stimulations of these partial agonists ranged from 114% (97–131%) with CP 55,940 to 29% (19–40%) with JWH-073. THC, anandamide and cannabinol did not stimulate [^{35}S]GTPγS binding under these conditions. The ability of THC to antagonize CP 55,940-induced stimulation of [^{35}S]GTPγS was also tested. THC was found to produce a slight, but nonsignificant rightward shift of the concentration-response curve of CP 55,940 (results not shown). Following the observation that THC stimulated [^{35}S]GTPγS binding with a GDP concentration of 10 μM, but not at 100 μM, anandamide was also tested at this lower GDP concentration. However, it was found that anandamide did not stimulate [^{35}S]GTPγS binding at this GDP concentration. Furthermore, lowering the sodium and magnesium ion concentrations did not affect anandamide's ability to stimulate GTPγS binding. PMSF, at a concentration of 50 μM, was included in the assay buffer for experiments using anandamide and, at this concentration, was found not to effect [^{35}S]GTPγS binding. To examine the possibility that tissue metabolism accounted for the lack of stimulatory effect of anandamide on GTPγS binding, a radioligand displacement curve was constructed. It was found that in rat cerebellum, anandamide, in the presence of 50 μM PMSF, displaced 1 nM [^{3}H]CP 55,940 with a K_i = 145.4 nM (results not shown).

Effect of the selective CB_1 antagonist, SR141716A, on cannabinoid-stimulated [^{35}S]GTPγS binding. Since the discovery of the CB_1 selective antagonist in 1994 (Rinaldi-Carmona *et al*., 1994), this compound has been used in a wide variety of assays to assess the role of the CB_1 receptor in the production of a cannabimimetic effect (Rinaldi-Carmona *et al*., 1994; Pertwee *et al*., 1995a; Compton *et al*., 1996). To determine the role of the CB_1 receptor in the production of cannabinoid-stimulated [^{35}S]GTPγS binding, concentration-response curves of a number of the more potent and efficacious agonists used in this study were compared in the presence and absence of SR141716A.

The effect of three concentrations of SR141716A (1, 3 and 10 nM) on the log concentration-response curve of CP 55,940 is shown in figure 4. SR141716A produced concentration-dependent rightward shifts of the CP 55,940 concentration-response curve without affecting the E_{max} of the agonist (fig. 4). Construction of a Schild plot confirmed the competitive and reversible nature of the antagonism, as previously described for SR141716A using other assays (Rinaldi-Carmona *et al*., 1994; Pertwee *et al*., 1995a). The equilibrium dissociation constant, K_d, for SR141716A in the presence of CP 55,940 was found to be 0.14 (0.01–0.20) nM. SR141716A itself, at concentrations from 0.1 nM through 10 μM, had no effect on [^{35}S]GTPγS binding (results not shown). Table 3 shows K_d values calculated in the presence of a number of other cannabinoid agonists. The K_d values calculated from these experiments do not differ significantly between the various agonists suggesting the role of a single receptor, CB_1, in the production of the response to each of these agonists. Furthermore, the calculated K_d value correlates well with previously observed binding affinity (Rinaldi-Carmona *et al*., 1994; Showalter *et al*., 1996) and with K_d values observed in other functional assays (Pertwee *et al*., 1995a; Rinaldi-Carmona *et al*., 1994).

Discussion

Effects of GDP on agonist-stimulated [^{35}S]GTPγS binding. WIN 55212–2 was chosen for the initial optimization experiments as it has been used as a standard cannabinoid receptor agonist, acting as a potent full agonist, in many different assays (Howlett, 1995; Martin *et al*., 1995). It also displays a high affinity for both subtypes of cannabinoid receptor (Showalter *et al*., 1996) and has been demonstrated to stimulate [^{35}S]GTPγS binding in rat cerebellar membranes (Selley *et al*., 1996). Optimal WIN 55212–2-stimulated [^{35}S]GTPγS binding occurred with the conditions described in the results section and were used for all subsequent experiments. A significantly greater maximal percentage stimulation was observed at 100 μM GDP than at 10 μM. The E_{max} and the potency of WIN 55212–2 obtained under these conditions correspond with previous studies that used varying assay conditions with both rat cerebellar membranes and membrane preparations from other brain areas (Selley *et al*., 1996; Hosohata *et al*., 1997). When similar experiments were conducted using CP 55,940, an identical trend was observed. However, THC, at the higher GDP concentration (100 μM), produced no significant stimulation of binding whereas, at a GDP concentration of 10 μM, a clear concentration-dependent stimulation of [^{35}S]GTPγS binding was observed. This apparently contradictory set of results may be explained by considering the role of GDP in this assay. As previously discussed in the results section, GDP is included in the assay to promote G protein inactivation. High efficacy agonists, such as CP 55,940 and WIN 55212–2, are thought to be more efficient at overcoming this 'GDP block' than agonists of lower efficacy, such as THC (Selley *et al*.,

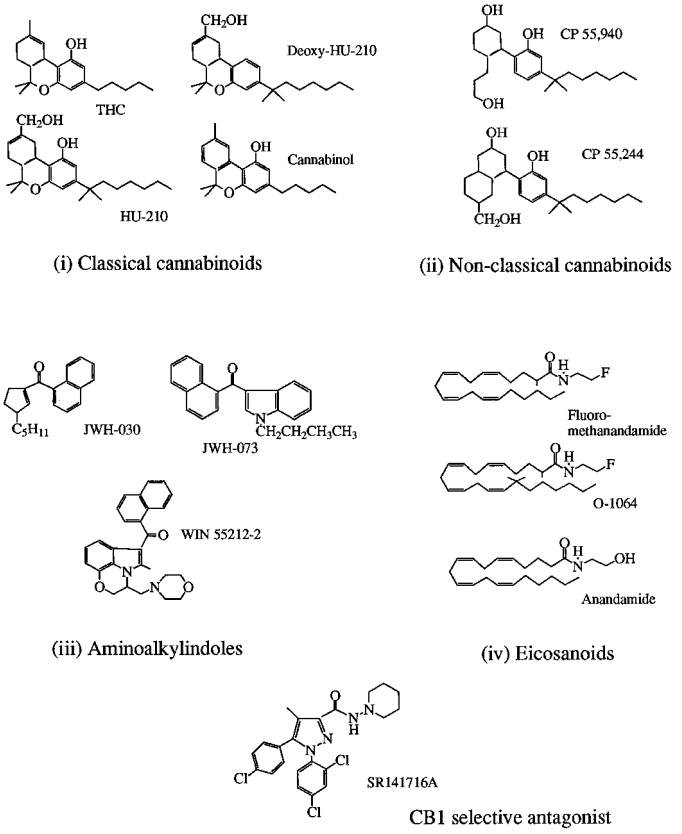

Fig. 3. Structures of compounds used in this study.

1996). This difference may be as a result of a better ability of high efficacy compounds to induce and/or stabilize changes in receptor conformation. Therefore, by reducing the "GDP block," THC may now be capable of stimulating [^{35}S]GTPγS binding. It has also been suggested that excess GDP may cause a decrease in the catalytic rate of G protein activation, to which high efficacy agonists may be less susceptible (Emmerson *et al*., 1996). The results presented here are consistent with these possibilities. This observation has previously been demonstrated in other studies (Selley *et al*., 1996; Breivogel *et al*., 1997a).

Effects of various cannabinoid receptor ligands on [^{35}S]GTPγS binding. The pharmacologies of the cannabinoid receptor ligands tested in this study, in the presence of 100 μM GDP, demonstrate a wide range of activities. The compounds ranged from the highly potent and efficacious through to compounds displaying little or no concentration-dependent stimulation of [^{35}S]GTPγS binding. Of the range of compounds tested; CP 55,244, HU-210, deoxy-HU-210 and WIN 55212–2 may be regarded as full agonists; CP 55,940, fluoromethanandamide, O-1064, JWH-030 and JWH-073 displayed varying degrees of partial agonism and THC, anandamide and cannabinol were not active (although THC was active at 10 μM GDP). This disparity of activities, and the finding that several compounds, known to act as full agonists in other functional assays, have little or no activity in this assay, requires further examination.

With many functional assays, high correlations have been demonstrated comparing pharmacological potency with receptor affinity. However, with the results of this study, this type of comparison yields inconclusive results, whether using potency (EC_{50}) or E_{max} levels for the comparison. Linear regression analysis of a comparison of EC_{50} values in the GTPγS assay with K_i values (CB_1) from affinity binding studies (see table 2) provides an r^2 value of .62. The K_i values used were obtained from a number of sources, each of which used slightly different experimental conditions and receptor preparations. This lack of a direct correlation between potency in the [^{35}S]GTPγS binding assay and receptor affinity may be related in part to the mixed effects of GDP. As discussed previously, the fact that a particular GDP concentration may be optimal for one compound does not necessarily mean that this applies to all compounds, as was found with THC and WIN 55212–2. In this study, each agonist was tested at a single GDP concentration, 100 μM, as this had been shown to be optimal for WIN 55212–2-stimulated [^{35}S]GTPγS binding and also to ensure consistency between experiments. However, it is likely, as was found to be the case with THC, that for some of these compounds, a lower concentration of GDP may be required for maximal binding. A further consideration is the differences in assay conditions used to produce receptor affinity data and those used in these experiments. It may be expected that high affinity agonist binding would be impaired by the high concentrations of sodium ions and guanine nucleotides used in our experiments. Furthermore, the affinity data used for the comparison is a combination of results from several studies, all of which use slightly different experimental conditions and membrane preparations. Additionally, the affinity data used is taken from displacement of cannabinoid receptor agonists, namely [^{3}H]CP 55,940 and [^{3}H]WIN55212–2. An alternative method would be to determine K_i values by conducting displacement assays under identical conditions as those for the GTPγS assay using [^{3}H]SR141716A as a radioligand. The

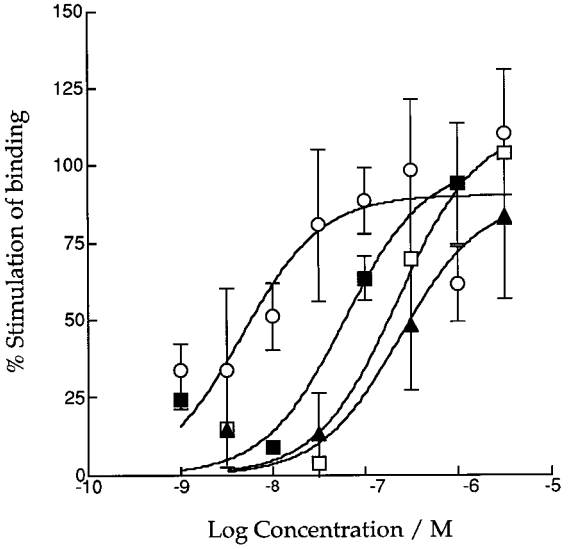

Fig. 4. Effect of SR141716A at concentrations of 1 nM (■), 3 nM (□) and 10 nM (▲) on the log concentration response curve of CP 55,940 (○). Data represent percentage stimulation over basal binding. Results are presented as mean ± S.E. for $n = 4$ experiments.

binding of [^{3}H]SR141716A is not altered by guanine nucleotides (Rinaldi-Carmona *et al*., 1996). However, Burkey *et al*. (1997) in a receent report in which identical radioligand and GTPγS binding conditions were used concluded that drug potency measurements were not the best measure of drug-mediated functional responses. Instead they propose relative efficacies (not E_{max}) may be more relevant). All of these factors may contribute to the observed differences in potency and affinity.

A similar comparison between the [^{35}S]GTPγS binding assay and other functional models also reveals a low correlation ($r^2 = .53$). One difference between this model and other functional models is the measured response itself. For example, inhibition of forskolin-stimulated cAMP accumulation, inhibition of neurotransmitter release and inhibition of smooth muscle contraction (for examples; Howlett and Fleming, 1984; Ishac *et al*., 1996; Pertwee and Griffin, 1995) all measure responses farther downstream of the initial receptor/G protein coupling, at the end of the signal transduction cascade. The effects of such measurements may be 2-fold. First, it is possible that in these models there is sufficient amplification of what may be a very low G protein signal to produce a full response. Second, the [^{35}S]GTPγS assay does not take into account any contributions of the G protein *beta gamma* subunits, which themselves have been shown to both directly modulate effectors and also to modify the activity of the *alpha* subunit (Kenakin, 1996). Both of these factors may contribute to differences between respective functional assays.

Furthermore, many assays measure the coupling of a particular type of G protein to the receptor (for example, inhibition of adenylate cyclase is thought to be largely mediated by G_i, Howlett, 1995, or inhibition of neuronal calcium channels by G_o, Hescheler *et al*., 1987). This assay, however, does not measure such a specific coupling. As GTPγS has affinities for all G proteins (Weiland and Jakobs., 1994), this assay simply measures the binding of [^{35}S]GTPγS to a G protein. It is therefore possible that some of the disparities between a

TABLE 3
Equilibrium dissociation constants (K_d values) of SR141716A calculated in the presence of five cannabinoid receptor agonists

Agonist used	K_d (95% limits)
	nM
WIN 55,212-2	0.33 (0.22–0.78)
CP 55,940	0.14 (0.01–0.20)
HU-210	0.40 (0.08–1.22)
Deoxy-HU210	0.06 (0.01–0.16)
Fluoro-methanandamide	0.33 (0.08–0.90)

compound's activity in the [^{35}S]GTPγS binding assay and other functional assays may reflect a more promiscuous receptor/G protein coupling, not only within subtypes of a particular G protein, but also with different G proteins. This has previously been demonstrated to occur with other G protein-coupled receptors, such as *alpha*-2 adrenoceptors (Eason *et al*., 1992) and *mu* and *delta* opioid receptors (Laugwitz *et al*., 1993; Prather *et al*., 1994).

Anandamide has been described as an endogenous ligand for the cannabinoid receptor (Devane *et al*., 1992), and therefore it is reasonable to expect that anandamide should also induce a stimulation of [^{35}S]GTPγS binding. However, this was not found to occur. THC stimulated [^{35}S]GTPγS binding at 10 μM but not at 100 μM GDP, and therefore anandamide was also tested at this lower GDP concentration. However, anandamide did not stimulate binding when tested at this concentration of GDP. It is possible that the potency of anandamide may be very low, as has been previously described (Selley *et al*., 1996), and therefore, in the concentration range used here, a concentration-dependent effect may not be seen (10 μM was the highest concentration used in any experiment due to both the high levels of ethanol used and also to avoid any nonspecific effects of these highly lipophilic compounds). Furthermore, the conditions used in these experiments were optimised for WIN 55212–2 stimulation of binding, and it is feasible that the potency of anandamide is simply too low to detect any stimulation of binding using this method. Although experiments were conducted with lower GDP concentrations, and also with lower sodium and magnesium ion concentrations, it is possible that further optimisation of conditions may improve the apparent potency of anandamide. Anandamide has also been shown to be susceptible to metabolism by several tissue and membrane preparations (Abadji *et al*., 1994; Pertwee *et al*., 1995b). The experiments with anandamide included the nonspecific amidase inhibitor, PMSF, in an attempt to prevent any metabolism of anandamide. However, the possibility that metabolism occurred in these experiments cannot be fully discounted. Anandamide, in the presence of 50 μM PMSF, displaced [^{3}H]CP 55,940 from cerebellar membranes with a K_i of 145 nM. This value is ~2- to 3-fold higher than previously reported values although the literature varies (Devane *et al*., 1992; Abadji *et al*., 1994; Showalter *et al*., 1996) and this may indicate that a small amount of metabolism is occurring, although the crudeness of the membrane preparation may also account for this apparent lower affinity. Metabolic inactivation is further supported by the observation that the high affinity, metabolically stable analogue of anandamide, fluoromethanandamide (Adams *et al*., 1995) did produce a significant concentration-dependent stimulation of

[35S]GTPγS binding. The pharmacology of anandamide within the GTPγS assay warrants further study.

Effect of the selective CB_1 antagonist, SR141716A, on cannabinoid-stimulated [35S]GTPγS binding. The results of this study demonstrate that in rat cerebellar membranes, cannabinoid-induced stimulation of [35S]GTPγS binding is mediated by specific cannabinoid receptors. The ability of SR141716A to antagonize cannabimimetic effects has been demonstrated in detail in many other assays. In isolated smooth muscle preparations, for example, SR141716A has been reported to antagonize electrically evoked contractions with K_d values ranging from ~1 through 10 nM (Rinaldi-Carmona *et al*., 1994; Pertwee *et al*., 1995a). Similarly, Selley *et al*. (1996) demonstrated that SR141716A, at a concentration of 200 nM, antagonized WIN 55212–2-induced stimulation of [35S]GTPγS binding in rat cerebellar membranes. Although the authors did not quote a K_d value, it was estimated to be no greater than 2 nM. The K_d values of SR141716A calculated in the presence of five cannabinoid receptor agonists correlate closely with each other, irrespective of the agonist used, suggesting that cannabinoid agonist-induced stimulation of [35S]GTPγS binding is mediated by a single receptor site, CB_1. The agreement of the K_d values found in this study with those previously reported using other experimental models is further evidence of the validity of this assay for the study of cannabinoid receptor antagonists.

In rat cerebellar membranes, SR141716A alone did not affect [35S]GTPγS binding, suggesting that it acts as a neutral antagonist, rather than as an inverse agonist, of the CB_1 receptor, under the conditions used in these experiments.

It has also been suggested that the cerebellum, as well as being very densely populated with CB_1 receptors, may also contain a number of CB_2 receptors (Skaper *et al*., 1996). The use of the CB_2-selective compound, deoxy-HU-210, was an attempt to investigate whether or not any of the stimulation of [35S]GTPγS binding produced by this compound was mediated by CB_2 receptors. If any of the observed stimulation was attributable to CB_2 receptors, then it may be expected that the CB_1 selective antagonist, SR141716A would not antagonize this component, therefore resulting in a higher estimate of the K_d value. The observation that the K_d value of SR141716A was not significantly different to that observed in the presence of the other, nonselective, agonists suggests that cannabinoid-induced stimulation of [35S]GTPγS binding was solely the result of CB_1 binding and activation. However, it is important to note that deoxy-HU-210, although CB_2 selective, still retains a high affinity for the CB_1 receptor, 1.15 nM (Showalter at al., 1996). The possible existence of CB_2 receptors in the cerebellum may be more conclusively tested using a CB_2 agonist with negligible CB_1 affinity or with the recently announced CB_2-selective antagonist, SR144528 (Barth *et al*., 1997).

In summary, the data presented in this report represent a further characterization of the cannabinoid-stimulated [35S]GTPγS binding assay in rat cerebellar membranes. The results demonstrate the importance of the assay conditions that are used, in particular that of GDP concentration, and the care which must be taken in the interpretation of data. We confirm the potential of the technique for the investigation of known cannabinoid receptor agonists and antagonists as well as its use for the delineation of novel cannabinoid receptor ligands.

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