

Cannabinoid Agonist Signal Transduction in Rat Brain: Comparison of Cannabinoid Agonists in Receptor Binding, G-Protein Activation, and Adenylyl Cyclase Inhibition¹

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ABSTRACT

To investigate differences in agonist affinity, potency, and efficacy across rat brain regions, five representative cannabinoid compounds were investigated in membranes from three different rat brain regions for their ability to maximally stimulate [³⁵S]guanosine-5'-O-(3-thio)triphosphate (GTPγS) binding and bind to cannabinoid receptors (measured by inhibition of [³H]antagonist binding) under identical assay conditions. In all three brain regions, the rank order of potency for the stimulation of [³⁵S]GTPγS binding and the inhibition of [³H]SR141716A binding for these compounds were identical, with CP55940 ≈ levonantradol > WIN55212-2 ≥ Δ⁹-tetrahydrocannabinol (Δ⁹-THC) > methanandamide. The rank order of efficacy was not

related to potency, and relative maximal agonist effects varied across regions. Receptor binding fit to a three-site model for most agonists, stimulation of [³⁵S]GTPγS binding fit to a two-site model for all agonists, and high-affinity receptor binding did not appear to produce any stimulation of [³⁵S]GTPγS binding. WIN55212-2, methanandamide, and Δ⁹-THC also were assayed for the inhibition of adenylyl cyclase in cerebellar membranes. The rank orders of potency and efficacy were similar to those for [³⁵S]GTPγS binding, but the efficacies and potencies of methanandamide and Δ⁹-THC compared with WIN55212-2 were higher for adenylyl cyclase inhibition, implying receptor/G-protein reserve.

Cannabinoids include a family of compounds derived from *Cannabis sativa*, the most biologically active of which is Δ⁹-tetrahydrocannabinol (Δ⁹-THC) (Gaoni and Mechoulam, 1964). In addition, a number of compounds have been developed as specific receptor ligands, including agonists and antagonists (Compton et al., 1993; Rinaldi-Carmona et al., 1994). CB₁ receptors, and splice variant CB_{1A} (Shire et al., 1995), represent the principle cannabinoid receptor type so far found in rat brain (Matsuda et al., 1990), and mediate the central nervous system actions of cannabinoid compounds (Compton et al., 1993). Their actions are transduced via the activation of G-proteins (Howlett et al., 1986) that results in the inhibition (Howlett, 1984) or stimulation of adenylyl cyclase (Glass and Felder, 1997; Maneuf and Brotchie, 1997), inhibition of Ca²⁺ conductance (Mackie and Hille, 1992; Mackie et al., 1995), stimulation of K⁺ conductance (Mackie et al., 1995), and stimulation of the mitogen-activated protein kinase pathway (Bouaboula et al., 1995). Cannabinoid receptors couple to at least six different G_α-subunits in brain membranes (Prather et al., 2000).

Receptor activation of G-proteins can be measured by agonist-stimulated binding of the hydrolysis-resistant GTP analog [³⁵S]guanosine-5'-O-(3-thio)triphosphate (GTPγS) to G-protein α-subunits in membranes (Hilf et al., 1989; Selley et al., 1996) or brain sections (Sim et al., 1995). This technique is sensitive to differences in agonist efficacy and potency for G-protein activation (Lorenzen et al., 1996; Selley et al., 1997, 1998). Previous results with agonist-stimulated [³⁵S]GTPγS binding have demonstrated that Δ⁹-THC is a weak partial agonist (Sim et al., 1996), and anandamide is an intermediate efficacy partial agonist (Burkey et al., 1997) compared with WIN55212-2 or CP55940 in rodent brain membranes. Other studies have reported partial agonist activity of Δ⁹-THC and anandamide for inhibition of adenylyl cyclase (Howlett et al., 1986; Childers et al., 1994) and CP55940 for inhibition of Ca²⁺ currents (Shen et al., 1996). Previous work from our laboratory not only confirmed these differences in agonist efficacy (Breivogel et al., 1998) but also demonstrated that cannabinoid receptor activity varies across different regions of rat brain because receptors in each region exhibit different catalytic amplification factors, defined as the number G-proteins activated per agonist-occupied receptor (Breivogel et al., 1997).

Cannabinoid compounds inhibit adenylyl cyclase activity

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ABBREVIATIONS: Δ⁹-THC, tetrahydrocannabinol; GTPγS, guanosine-5'-O-(3-thio)triphosphate.

in cell lines (Howlett, 1984; Slipetz et al., 1995) and in brain membranes (Bidaut-Russell et al., 1990; Pacheco et al., 1991; Childers et al., 1994). In general terms, the pharmacology of cannabinoid-inhibited adenylyl cyclase matches that of cannabinoid receptor binding (Pacheco et al., 1991), including competitive antagonism by SR141716A (Rinaldi-Carmona et al., 1994). However, inhibition of adenylyl cyclase in membranes from different regions of rat brain was only detectable in cerebellum and striatum (Pacheco et al., 1991; Childers et al., 1994).

The present study compares the efficacies and potencies of several commonly used cannabinoid compounds for the stimulation of [35 S]GTP γ S binding and displacement of [3 H]SR141716A binding in rat brain membranes from three brain regions under identical assay conditions. Inhibition of adenylyl cyclase by several of these agonists also is determined in rat cerebellar membranes to compare the efficacies and potencies of agonists for G-protein activation with those for a downstream effector system. Although the efficacies of these compounds have previously been determined in rat cerebellar membranes, the relationship of agonist receptor occupancy to G-protein activation was not determined, and it is not known whether these efficacy differences are maintained in membranes from different regions of rat brain. To test the hypothesis that efficacy is related to receptor density, two regions were chosen that contain either similar (hippocampus) or different (hypothalamus) levels of cannabinoid receptors and cannabinoid-activated G-proteins compared with cerebellum. Furthermore, direct comparison of receptor binding and G-protein activation under identical conditions allows determination of the receptor states that are involved in agonist activity. Finally, determination of efficacy for adenylyl cyclase inhibition by these agonists will test the hypothesis that cannabinoid receptors in cerebellum exhibit greater receptor reserve at this effector than at G-proteins.

Experimental Procedures

Materials. Male Sprague-Dawley rats were purchased from Zivic Miller Laboratories, Inc. (Zelienople, PA). [35 S]GTP γ S (1250 Ci/mmol), [α - 32 P]ATP (800 Ci/mmol), and [3 H]cAMP (25 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [3 H]SR141716A (53–55 Ci/mmol) was obtained from Amersham Life Sciences (Arlington Heights, IL). CP55940 and levonantradol were obtained from Pfizer, Inc. (Groton, CT). Δ^9 -THC was provided by National Institute on Drug Abuse/Research Triangle Institute (Research Triangle Park, NC). WIN55212-2, anandamide, and *R*-(+)-methanandamide were purchased from Research Biochemicals International (Natick, MA). SR141716A was a generous gift from Dr. Francis Barth at Sanofi Recherche (Montpellier, France). GDP and GTP γ S were purchased from Boehringer Mannheim (New York, NY). All other reagent grade chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Agonist-Stimulated [35 S]GTP γ S Binding and [3 H]SR141716A Competition Assays. Cerebellum, hippocampus, and hypothalamus were dissected from fresh rat brains on ice and pooled. Each region was homogenized with a Tisumizer (Tekmar, Cincinnati, OH) in cold membrane buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, pH 7.7) and centrifuged at 48,000g for 10 min at 4°C. Pellets were resuspended in membrane buffer, and then centrifuged again at 48,000g for 10 min at 4°C. Pellets from the second centrifugation were homogenized in membrane buffer and stored at -80°C until use. Frozen membranes were thawed and diluted in membrane buffer, homogenized,

and preincubated for 10 min at 30°C in 0.004 U/ml adenosine deaminase (240 U/mg of protein; Sigma Chemical Co.) to remove endogenous adenosine, and then assayed for protein content before addition to assay tubes. Assays were conducted at 30°C for 2 h in membrane buffer, including 8 to 10 μ g (cerebellum and hippocampus) or 10 to 20 μ g (hypothalamus) of membrane protein with 0.1% (w/v) BSA, 50 μ M GDP, 0.5 nM SR141716A (3 H-labeled for competition assays), and 0.05 nM GTP γ S (35 S-labeled in stimulation assays) in a final volume of 1 ml. Both assays were performed simultaneously by incubating membranes with various concentrations of each ligand. Each rack also included determination of [35 S]GTP γ S binding with 3 μ M levonantradol to be able to normalize the amount of stimulation by each agonist to that obtained with a maximally effective concentration of levonantradol. Nonspecific binding was determined in the absence of agonists and the presence of 30 μ M unlabeled GTP γ S (35 S]GTP γ S assays) or 10 μ M unlabeled SR141716A (3 H]SR141716A assays). Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold Tris buffer, pH 7.4. For [3 H]SR141716A binding, filters were presoaked for approximately 2 h in Tris buffer containing 0.5% (w/v) BSA, and cold Tris rinse buffer contained 0.05% (w/v) BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for 35 S or 45% efficiency for 3 H after overnight extraction of the filters in 4 ml of ScintiSafe Econo 1 scintillation fluid (Fisher Scientific). Typical [35 S]GTP γ S binding results included 500 to 700 dpm for nonspecific binding, 200 to 450 dpm [35 S]GTP γ S bound/mg of protein basal binding (depending on the region), and 850 to 1100 dpm/mg bound in the presence of maximally effective concentrations of levonantradol.

Agonist Inhibition of Adenylyl Cyclase. Assays were performed according to the method of Salomon (1979) with some modifications. Fresh cerebella were dissected on ice and homogenized in membrane buffer with a ground glass homogenizer. Membrane suspensions were centrifuged at 48,000g for 10 min at 4°C, and then pellets were resuspended and homogenized in membrane buffer. Membranes were assayed for protein content before addition to assay tubes. Membranes (~15 μ g) were incubated for 10 min at 30°C in membrane buffer in the presence of various concentrations of each agonist with 50 μ M cAMP, 50 μ M GTP, and 50 μ M ATP plus 1.5 μ Ci [α - 32 P]ATP with 10 mM theophylline, 5 mM phosphocreatine, 20 U/ml creatine phosphokinase (250 U/mg of protein; Sigma Chemical Co.), and 0.1% (w/v) BSA in a final volume of 0.1 ml. Reactions were terminated by boiling for 3 min and addition of stopping solution (2% sodium lauryl sulfate, 45 mM ATP, and 1.3 mM cAMP in Tris buffer, pH 7.5). [3 H]cAMP standard (50 μ l; ~15,000 cpm) and 1 ml of deionized water were added to each tube before addition of samples to Dowex columns and processing according to a previously published method (Salomon, 1979). Recovery of 32 P and 3 H were determined by liquid scintillation spectrophotometry at 99% efficiency for 32 P and 45% efficiency for 3 H in 3.5 ml of 0.1 M imidazole buffer, pH 7.3, and 18 ml of scintillation fluid. The solvents used to dissolve the cannabinoid compounds (dimethyl sulfoxide for WIN55212-2 and 95% ethanol for all others) had no effect on cAMP formation at the highest concentration of each vehicle present in the assay (0.1%).

Data Analysis. Net agonist-stimulated [35 S]GTP γ S binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist) and were normalized to the values obtained for a maximally effective concentration of levonantradol (3 μ M) measured in the same assay rack. The amount of [32 P]cAMP formed was determined by normalizing 32 P cpm to the fraction of total 3 H cpm recovered from the columns. Data analyses, including agonist concentration-effect curves and displacement curves, were conducted by iterative nonlinear regression by using JMP for Macintosh (SAS, Cary, NC) or Prism for Windows (GraphPad Software, San Diego, CA) to obtain EC₅₀, E_{max}, IC₅₀, I_{max}, and *n*_H (Hill slope) values. Determination of which model best fit the data was made by an *F* test with Prism to compare two models simultaneously. [35 S]GTP γ S binding and [3 H]SR141716A displacement data were found to fit

better to two- or three-component models than to a one-component model with variable Hill slope. Percentage of total [³H]SR141716A binding data was fit to two- or three-component models with specific binding constrained to between 0 and 100%. *K_i* and *K_s* values were estimated from IC₅₀ and EC₅₀ values, respectively, by the Cheng-Prusoff equation and antagonist *K_B* values were determined by the equation *K_B* = [Ant]/(CR - 1), where [Ant] is the concentration of antagonist and CR is the ratio of the agonist EC₅₀ values in the presence and absence of antagonist (Pratt and Taylor, 1990). Differences among agonists and regions were determined by ANOVA followed by the Tukey-Kramer test for multiple comparisons at *P* < .05. Unless otherwise indicated, all data presented are mean ± S.E. of at least three experiments performed in duplicate.

Results

Stimulation of [³⁵S]GTPγS Binding and Inhibition of [³H]SR141716A Binding by Cannabinoid Agonists. Concentration-effect curves were generated for both the stimulation of [³⁵S]GTPγS binding and competition for [³H]SR141716A binding by the cannabinoid agonists WIN55212-2, levonantradol, CP55940, Δ⁹-THC, and methanandamide in rat cerebellar (Fig. 1), hippocampal, and hypothalamic membranes. Both assays were performed under

the same conditions, with 0.5 nM SR141716A in [³⁵S]GTPγS binding assays and 0.05 nM GTPγS in [³H]SR141716A binding assays. Both assays included 50 μM GDP and 100 mM NaCl, both of which favor agonist stimulation of [³⁵S]GTPγS binding and promote the low-affinity state of cannabinoid receptors for agonist binding. Agonist-stimulated [³⁵S]GTPγS binding in cerebellar membranes was blocked by the CB₁-selective antagonist SR141716A (data not shown), and the *K_B* value of SR141716A in shifting WIN55212-2 concentration-effect curves to the right was 0.24 ± 0.08 nM, similar to the previously reported *K_D* value for [³H]SR141716A obtained by Scatchard analysis in cerebellar membranes (0.19 ± 0.01 nM; Breivogel et al., 1997), and consistent with competitive antagonism of CB₁ receptors.

Levonantradol significantly increased [³⁵S]GTPγS binding in all three brain regions; net agonist-stimulated binding (pmol/mg) was approximately the same in the three regions, but percentage of stimulation by levonantradol over basal ranged from 140 to 620% (Table 1). These differences in percentage of stimulation by agonist were due to differences in basal [³⁵S]GTPγS binding levels across regions. CB₁ receptor binding, measured with 0.5 nM [³H]SR141716A, ranged from 1.5 to 3.9 pmol/mg (Table 1). Because the *K_D* of SR141716A in brain membranes is 0.24 nM and does not vary between regions (Breivogel et al., 1997), *B_{max}* values of [³H]SR141716A binding can be estimated (Table 1) between 2.3 and 5.9 pmol/mg. These values are similar to those previously reported in these tissues (Breivogel et al., 1997).

Concentration-effect curves of all five agonists in stimulating [³⁵S]GTPγS binding (A) and displacing [³H]SR141716A binding (B) in cerebellar membranes are shown in Fig. 1. The efficacies (*E_{max}*) of the agonists for stimulating [³⁵S]GTPγS binding to cerebellar membranes were similar to those previously determined (Breivogel et al., 1998). *E_{max}* values normalized to maximal stimulation produced by levonantradol are presented in the text and Table 2. In cerebellar membranes, WIN55212-2 (106%) was only slightly more efficacious than levonantradol, but this difference was not significant. However, levonantradol and WIN55212-2 were both more efficacious than the other agonists (*P* < .05), and CP55940 (74%) was more efficacious than methanandamide (65%), which was more efficacious than Δ⁹-THC (20%) (Fig. 1). In hippocampal and hypothalamic membranes (Table 2), WIN55212-2 was significantly the most efficacious agonist, stimulating 128 and 130% of the [³⁵S]GTPγS binding stimulated by levonantradol, respectively. CP55940 was signifi-

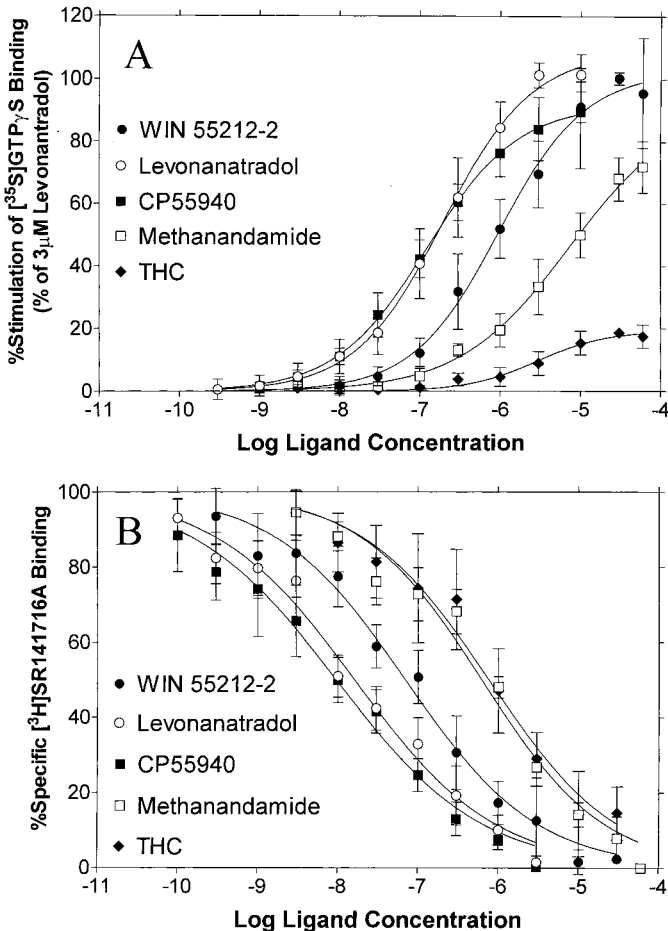


Fig. 1. Concentration-effect curves for cannabinoid stimulation of [³⁵S]GTPγS binding (A) and displacement of [³H]SR141716A binding (B) in rat cerebellar membranes. Assays were performed under identical conditions as described under *Experimental Procedures*. Net agonist-stimulated [³⁵S]GTPγS binding is shown as percentage of that obtained with 3 μM levonantradol. Curve fits are to a one-site model with variable Hill slope. Data are mean ± S.E. from four assays performed in duplicate.

TABLE 1
Binding parameters for cannabinoid-stimulated [³⁵S]GTPγS binding and [³H]SR141716A binding in brain membranes
Membranes from the indicated regions were analyzed for maximal stimulation of [³⁵S]GTPγS binding by 3 μM levonantradol and for [³H]SR141716A binding. Data for [³⁵S]GTPγS binding are presented as net levonantradol-stimulated binding (pmol/mg), and percentage of stimulation of [³⁵S]GTPγS binding over basal by levonantradol. Data for [³H]SR141716A binding are presented as specific binding determined with 0.5 nM [³H]SR141716A, and the estimated *B_{max}* of [³H]SR141716A binding assuming a *K_D* of 0.24 nM for SR141716A. Data are mean values ± S.E. from four separate experiments.

Region	Net Levonantradol-Stimulated [³⁵ S]GTPγS Binding		Specific [³ H]SR141716A Binding (<i>B_{max}</i>)	
	pmol/mg	% stimulation	pmol/mg	
Cerebellum	0.24 ± 0.02	620 ± 36	3.9 ± 0.5	(5.9)
Hippocampus	0.21 ± 0.01	210 ± 12	3.7 ± 0.2	(5.5)
Hypothalamus	0.25 ± 0.01	140 ± 15	1.5 ± 0.3	(2.3)

TABLE 2

Relative agonist efficacies for the stimulation of [35 S]GTP γ S binding and inhibition of adenylyl cyclase ([32 P]cAMP) determined using a one-site model

Agonist concentration-effect curves were generated for stimulation of [35 S]GTP γ S binding and inhibition of adenylyl cyclase ([32 P]cAMP) in brain membranes. $E_{\max}$ values for [35 S]GTP γ S binding and $I_{\max}$ values for adenylyl cyclase inhibition were normalized to the values obtained by maximally effective concentrations of levonantradol (3 μ M) or WIN55212-2 (1 μ M), respectively, before curve fitting. Normalized values for adenylyl cyclase inhibition are shown in parentheses. Data are mean values $\pm$ S.E. from four separate experiments.

	$E_{\max}$ [35 S]GTP γ S %Levonantradol	$I_{\max}$ [32 P]cAMP %Inhibition (%WIN55212-2)
Cerebellum		
WIN55212-2	106 $\pm$ 2 ^a	21 $\pm$ 2 (100 $\pm$ 5) ^a
Levonantradol	100 $\pm$ 6 ^a	
CP55940	74 $\pm$ 2 ^b	
Methanandamide	65 $\pm$ 4 ^c	18 $\pm$ 3 (85 $\pm$ 5) ^a
Δ^9 -THC	20 $\pm$ 1 ^d	12 $\pm$ 1 (53 $\pm$ 7) ^b
Hippocampus		
WIN55212-2	128 $\pm$ 2 ^a	
Levonantradol	100 $\pm$ 2 ^b	
CP55940	87 $\pm$ 3 ^c	
Methanandamide	86 $\pm$ 2 ^c	
Δ^9 -THC	27 $\pm$ 1 ^d	
Hypothalamus		
WIN55212-2	130 $\pm$ 6 ^a	
Levonantradol	100 $\pm$ 4 ^b	
CP55940	82 $\pm$ 5 ^b	
Methanandamide	104 $\pm$ 8 ^b	
Δ^9 -THC Δ^9 -THC	12 $\pm$ 2 ^c	

^{a,b,c,d} Values labeled with different letters within a brain region are significantly different from one another ($P < .05$ by Tukey-Kramer test).

cantly less efficacious than levonantradol in hippocampus (87 $\pm$ 3%), but not in hypothalamus (82 $\pm$ 5%). Methanandamide was significantly different from levonantradol in hippocampus (86%), but not in hypothalamus (104%). In each region, Δ^9 -THC was the least efficacious agonist and was different from the other agonists, yielding 27 and 12% of the stimulation by levonantradol in hippocampus and hypothalamus, respectively. Thus, although normalized $E_{\max}$ values of each cannabinoid agonist across regions were similar, there were some significant differences. Agonist K_s values in stimulating [35 S]GTP γ S binding also were determined, and compared with K_i values for cannabinoid receptor binding determined under the same assay conditions. Agonist potencies for both assays followed the order CP55940 $\approx$ levonantradol > WIN55212-2 > Δ^9 -THC $\geq$ methanandamide. Figure 2 shows a comparison of the binding curves for one representative agonist, levonantradol, in all three brain regions. These curves show that although the potency of levonantradol for stimulating [35 S]GTP γ S binding appeared to vary across regions (most potent in hippocampus and least potent in cerebellum), the potency of levonantradol for displacing [3 H]SR141716A binding did not vary significantly across regions. This trend appeared for all agonists except Δ^9 -THC. The results of curve fitting parameters for fits to one-site models are shown in Table 3, and confirm this trend.

Comparison of the potencies of these agonists for displacing [3 H]SR141716A binding and stimulating [35 S]GTP γ S binding for each region also can be seen in Table 3. The rank order of potency of these agonists for receptor binding (CP55940 = levonantradol > WIN55212-2 > Δ^9 -THC > methanandamide) was approximately the same as for stimulation of [35 S]GTP γ S binding. However, EC_{50} values for Δ^9 -THC did not follow this order for [35 S]GTP γ S binding, possibly due to error resulting from the low efficacy of Δ^9 -

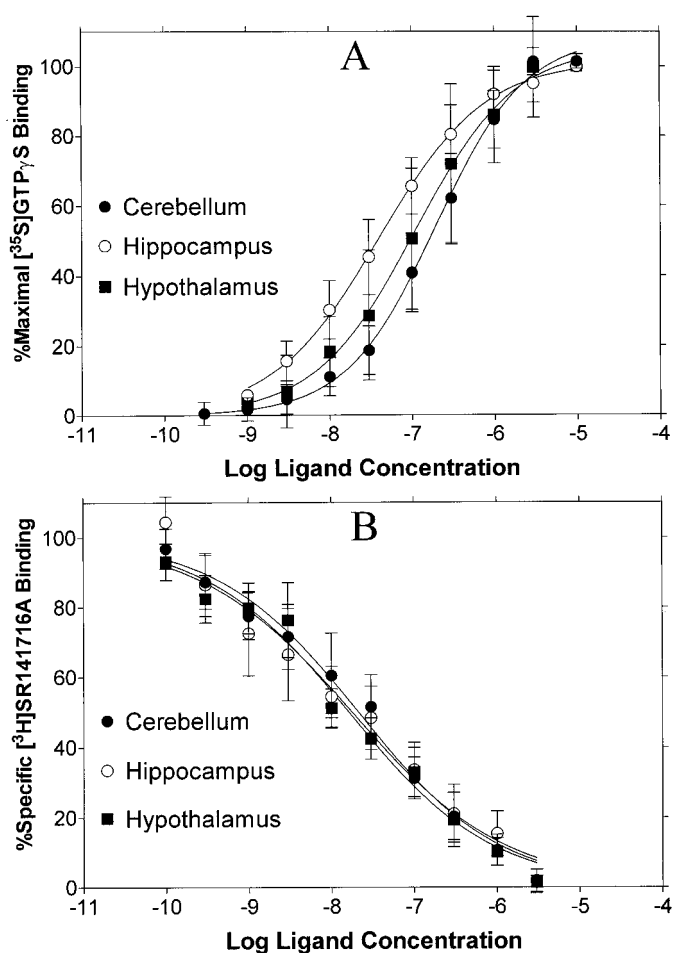


Fig. 2. Comparison of levonantradol-stimulated [35 S]GTP γ S binding (A) and inhibition of [3 H]SR141716A binding (B) in rat cerebellar, hippocampal, and hypothalamic membranes. Net agonist-stimulated [35 S]GTP γ S binding is shown as percentage of maximum stimulation obtained in each region. Curve fits are to a one-site model with variable Hill slope. Data are mean $\pm$ S.E. from four assays performed in duplicate.

THC in this assay. However, correlation of the K_i and K_s values for each agonist ($r = 0.90$ in cerebellum, $r = 0.92$ in hippocampus, and $r = 0.98$ in hypothalamus) were significant by ANOVA ($P < .05$). Moreover, each agonist was usually (Δ^9 -THC again being the exception) more potent in receptor binding than in stimulating [35 S]GTP γ S binding (Table 3). This leads to a problem in interpretation because it implies that greater than full receptor occupancy is required to achieve maximal effect, an issue that will be addressed by multicomponent binding analyses (see below).

Agonist Inhibition of Adenylyl Cyclase. Three agonists that exhibited a wide range of efficacies for stimulating the binding of [35 S]GTP γ S (WIN55212-2, methanandamide, and Δ^9 -THC) were used to assess the concentration-effect relationship for inhibition of adenylyl cyclase in rat cerebellar membranes. All three agonists produced significant (ANOVA, $P < .005$ for each agonist) concentration-dependent inhibition of adenylyl cyclase (Fig. 3). The effects of each agonist on adenylyl cyclase were completely blocked by 100 nM SR141716A, which had no effect on adenylyl cyclase by itself (data not shown). IC_{50} values for the three cannabinoid agonists in inhibiting adenylyl cyclase ranged from 32 to 155 nM, and they were significantly more potent than the corre-

TABLE 3

Agonist potencies for inhibition of [³H]SR141716A binding, stimulation of [³⁵S]GTPγS binding, and inhibition of adenylyl cyclase ([³²P]cAMP) determined using a one-site model

Agonist concentration-effect curves were generated under identical assay conditions for competition for [³H]SR141716A binding and stimulation of [³⁵S]GTPγS binding. Three representative compounds also were assayed in cerebellar membranes for inhibition of adenylyl cyclase ([³²P]cAMP). Curves were fit by iterative nonlinear regression to one-site models with variable Hill slopes to obtain IC₅₀ and EC₅₀ values. K_i and K_s values for binding were calculated as described under *Experimental Procedures*. Data shown are mean ± S.E. from four assays performed in duplicate.

	K _i [³ H]SR141716A	K _s [³⁵ S]GTPγS	IC ₅₀ [³² P]cAMP
	nM		
Cerebellum			
WIN55212-2	11 ± 3	250 ± 83	42 ± 14
Levonantradol	2.3 ± 0.3	60 ± 17	
CP55940	1.7 ± 0.5	35 ± 7	
Methanandamide	150 ± 10	1100 ± 260	155 ± 38
Δ ⁹ -THC	110 ± 52	1600 ± 480	32 ± 17
Hippocampus			
WIN55212-2	29 ± 5	130 ± 39	
Levonantradol	4.9 ± 1.0	11 ± 2	
CP55940	5.6 ± 1.4	8.5 ± 2.0	
Methanandamide	330 ± 46	630 ± 170	
Δ ⁹ -THC	170 ± 82	130 ± 48	
Hypothalamus			
WIN55212-2	14 ± 4	170 ± 64	
Levonantradol	2.9 ± 1.3	45 ± 27	
CP55940	2.0 ± 1.1	14 ± 6	
Methanandamide	240 ± 51	1000 ± 410	
Δ ⁹ -THC	37 ± 15	38 ± 12	

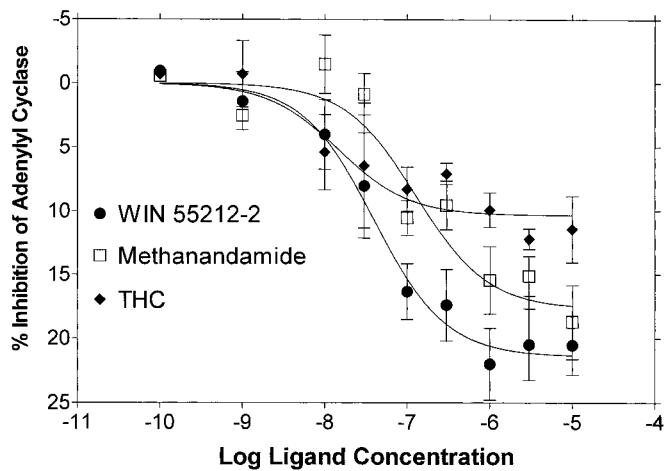


Fig. 3. Cannabinoid inhibition of adenylyl cyclase in rat cerebellar membranes. Assays were performed by incubating membranes with various concentrations of each agonist as described under *Experimental Procedures*. Data are percentage of inhibition of basal adenylyl cyclase activity presented as mean ± S.E. from four assays performed in duplicate.

sponding EC₅₀ values of each agonist in stimulating [³⁵S]GTPγS binding, but were similar to their K_i values in displacing [³H]SR141716A binding (Table 3). The cannabinoid agonists also exhibited different efficacies (I_{max} values) for inhibiting adenylyl cyclase (Table 2), with WIN55212-2 and methanandamide producing similar levels of inhibition, and Δ⁹-THC producing approximately 50% of the maximal inhibition produced by WIN55212-2 (*P* < .05, Tukey-Kramer test). The I_{max} values of methanandamide and Δ⁹-THC for adenylyl cyclase inhibition normalized to WIN55212-2 (85 ± 5 and 53 ± 4%, respectively) were significantly higher (*P* < .05, Mann-Whitney rank sum test) than those for [³⁵S]GTPγS binding normalized to WIN55212-2 (61 ± 4 and 19 ± 1%, respectively).

Multicomponent Analysis of Agonist-Stimulated [³⁵S]GTPγS Binding and Agonist-Inhibited [³H]SR141716A Binding Curves.

Inspection of the agonist concentration-effect curves for the stimulation of [³⁵S]GTPγS binding and inhibition of [³H]SR141716A binding revealed relatively shallow curves. In fact, all data fit better to a one-site model with variable slope (and all Hill slope values were less than one) than to a one-site model with Hill slope constrained to one. Moreover, comparison of the binding and stimulation curves in each region showed that each agonist appeared to occupy cannabinoid receptors at lower concentrations than those that stimulated [³⁵S]GTPγS binding (Fig. 4). In each region, approximately 10 to 30% of [³H]SR141716A binding was inhibited before any stimulation of [³⁵S]GTPγS binding occurred. For example, in hippocampus, 1 nM levonantradol displaced 25% of [³H]SR141716A binding, but it did not stimulate [³⁵S]GTPγS binding (Fig. 4). In contrast, complete receptor occupancy by each agonist, indicated by 100% inhibition of [³H]SR141716A binding, occurred at nearly the same concentration of agonist as maximal stimulation of [³⁵S]GTPγS binding (e.g., 3 μM levonantradol; Fig. 4).

To more accurately determine the potency and affinity of each ligand, a representative region (hippocampus) was as-

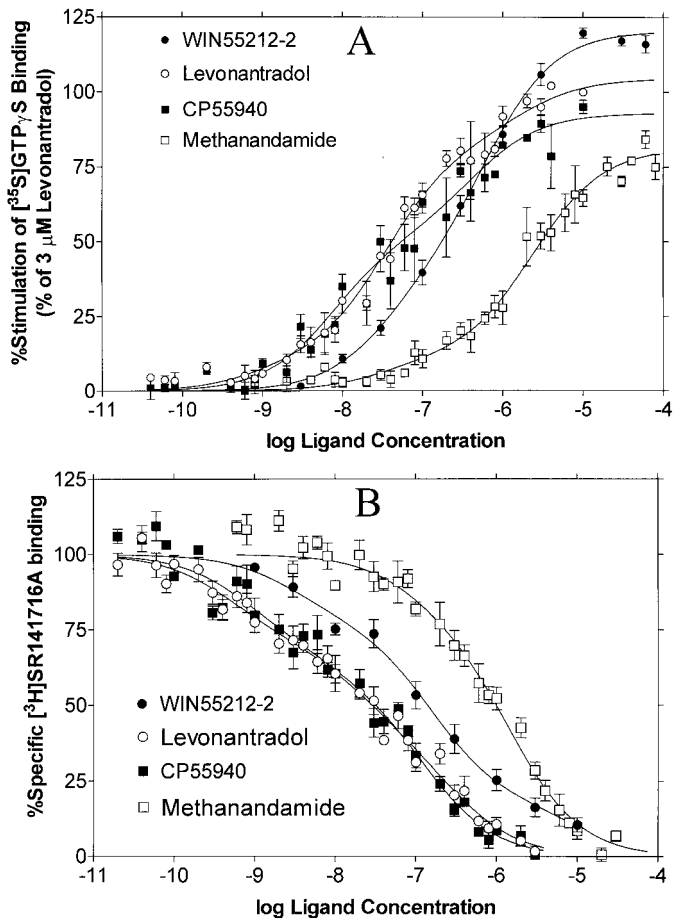


Fig. 4. Multicomponent analysis of cannabinoid stimulation of [³⁵S]GTPγS binding (A) and displacement of [³H]SR141716A binding (B) in hippocampal membranes. Data are mean ± S.E. from seven assays performed in duplicate. Net agonist-stimulated [³⁵S]GTPγS binding is shown as percentage of that obtained with 3 μM levonantradol. Curve fits are to two- and three-site models as appropriate.

sayed by using 34 concentrations of each agonist for displacement of [3 H]SR141716A binding and stimulation of [35 S]GTP γ S binding. Table 4 provides the analysis of displacement and stimulation curves for WIN55212-2, levonantradol, CP55940, and methanandamide. Data for Δ^9 -THC are not presented because the combination of low aqueous solubility and low affinity for this agonist made multicomponent analysis of the data unreliable. In all cases, for both [3 H]SR141716A and [35 S]GTP γ S binding, all agonists displayed Hill slope (n_H) values significantly less than one, with most Hill slope values approximately 0.5 (Table 4), suggesting the presence of multiple binding sites. Multicomponent analyses of agonist displacement and stimulation curves confirmed this suggestion. For [35 S]GTP γ S binding, agonist stimulation curves were best fit to a two-site model for all agonists, with high-affinity sites making up approximately 14 to 60% of the total number of sites. For receptor binding, the agonist displacement curves were best fit to a three-site model for all agonists except methanandamide, which fit best to a two-site model, consistent with its higher Hill slope (0.86) compared with those of other agonists (Table 4). For the three agonists producing a three-site fit, individual K_i values were at least 10-fold different from the other two K_i values for that agonist, with high-affinity sites ranging from 0.16 to 1 nM, intermediate-affinity sites ranging from 3 to 50 nM, and low-affinity sites ranging from 44 to 3200 nM. Although the potencies of levonantradol and CP55940 at each calculated site were very similar, each exhibited greater potency than WIN55212-2 at the corresponding site (Table 4).

The data in Table 4 allow a direct comparison between agonist potencies for displacement of [3 H]SR141716A binding compared with stimulation of [35 S]GTP γ S binding. However, because the [35 S]GTP γ S data were best fit with two-site models and [3 H]SR141716A data were fit to three-site models, it is not obvious how to determine which of the three receptor-binding sites best correlate with the two G-protein activation sites. The resolution of this problem is illustrated in Fig. 5, which shows the occupancy of receptors for levonantradol (Fig. 5A) and methanandamide (Fig. 5B). For levo-

nantradol, whose [3 H]SR141716A displacement curve was best fit to three sites, it is clear that occupancy of receptors occurred at lower concentrations of levonantradol than did stimulation of [35 S]GTP γ S binding. For example, 1 nM levonantradol produced 25% occupancy of receptor binding, but produced approximately 5% of maximal [35 S]GTP γ S stimulation. Therefore, for levonantradol, there is no high-affinity G-protein activation site that corresponds to the highest affinity receptor-binding site. For this reason, in Table 4, the "high"-affinity [35 S]GTP γ S site is compared directly with the intermediate receptor-binding site, and the low-affinity sites for both assays are compared with each other. The same situation existed for WIN55212-2 and CP55940: the low concentrations of agonist corresponding to the highest affinity receptor-binding site produced little or no stimulation of [35 S]GTP γ S binding (data not shown). In contrast, the situation was different for methanandamide (Fig. 5B), where receptor binding and [35 S]GTP γ S curves were essentially identical, and there was close to a 1:1 relationship between receptor occupancy by this agonist and stimulation of [35 S]GTP γ S binding. This agrees with the basic finding of multicomponent analysis of methanandamide curves that showed that a two-site model best fit the data for both assays (Table 4).

Now that the different affinity states for agonist displacement of [3 H]SR141716A binding and stimulation of [35 S]GTP γ S binding can be matched, K_i/K_s ratios can be calculated between receptor-binding and G-protein activation potencies (Table 4). With the exception of the low-affinity sites for WIN55212-2, which exhibited a K_i/K_s ratio of 16, the K_i/K_s ratios were all fairly similar for all agonists, and all close to one, indicating little if any receptor reserve for these agonists for the stimulation of [35 S]GTP γ S binding via cannabinoid receptors.

Discussion

For these studies, five agonists representing a wide range of efficacies and potencies for G-protein activation in rat

TABLE 4

Multicomponent analysis of agonist displacement of [3 H]SR141716A and stimulation of [35 S]GTP γ S binding in hippocampal membranes

Agonist concentration-effect curves were generated under identical assay conditions for displacement of [3 H]SR141716A binding and stimulation of [35 S]GTP γ S binding. Data were normalized to percentage of total specific (or agonist-stimulated) binding. Pooled data from seven separate experiments were fit to multicomponent equations by nonlinear regression, as described under *Experimental Procedures*. The "%" column refers to the percentage of total binding sites that are of high, intermediate, or low affinity as fit by nonlinear regression. IC_{50} and EC_{50} values are presented as mean log values $\pm$ S.E. of each fit, with corresponding K_i and K_s values in nM listed in parentheses. K_i/K_s ratios are shown for receptor binding and [35 S]GTP γ S-stimulating sites that are hypothesized to correspond to one another.

Agonist Site	$[^3\text{H}]$ SR141716A Binding			$[^{35}\text{S}]$ GTP γ S Binding			K_i/K_s Ratio
	n_H	%	Log IC_{50} (K_i) nM	n_H	%	Log EC_{50} (K_s) nM	
WIN55212-2	0.53			0.73			
High		22	-8.50 ± 0.51 (1.01)				
Intermediate		59	-6.81 ± 0.29 (50.8)		32	-7.46 ± 0.26 (11.4)	4.5
Low		19	-5.01 ± 0.62 (3200)		68	-6.20 ± 0.14 (207)	15.5
Levonantradol	0.50			0.66			
High		29	-9.31 ± 0.24 (0.16)				
Intermediate		29	-7.75 ± 0.62 (5.73)		60	-7.89 ± 0.12 (4.20)	1.4
Low		42	-6.57 ± 0.23 (87.3)		40	-6.49 ± 0.24 (105)	0.83
CP55940	0.56			0.56			
High		29	-9.11 ± 0.54 (0.25)				
Intermediate		14	-8.04 ± 2.52 (2.94)		55	-8.13 ± 0.13 (2.43)	1.3
Low		57	-6.87 ± 0.22 (44.0)		45	-6.38 ± 0.23 (137)	0.32
Methanandamide	0.86			0.67			
High		24	-6.87 ± 0.52 (44.3)		14	-7.46 ± 0.45 (11.2)	4.0
Low		76	-5.80 ± 0.18 (517)		86	-5.61 ± 0.09 (789)	0.66

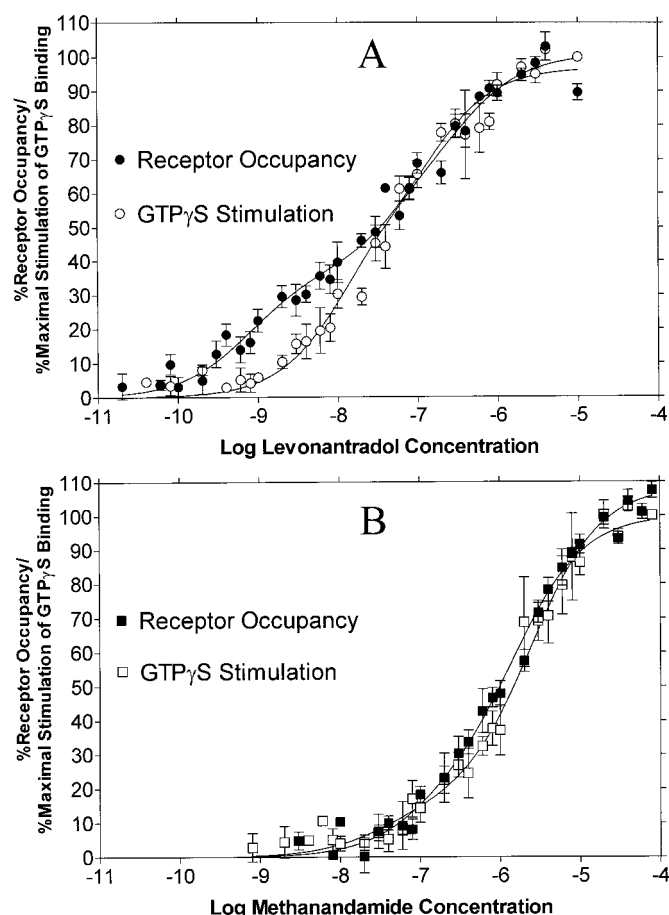


Fig. 5. Comparison of levonantradol (A) and methanandamide (B) stimulation of [35 S]GTP γ S binding and cannabinoid receptor occupancy. Data are mean $\pm$ S.E. from seven assays performed in duplicate. Net agonist-stimulated [35 S]GTP γ S binding is shown as percentage of maximum obtained with each agonist. Curve fits are to two- and three-site models as appropriate.

cerebellar membranes were chosen to determine whether these differences for G-protein activation persisted in other brain regions, and whether they also were manifested at the level of a downstream effector system, adenylyl cyclase. Hippocampus and hypothalamus were compared with cerebellum because hippocampus exhibited similar levels of cannabinoid receptors and cannabinoid-activated G-proteins, whereas hypothalamus exhibited lower levels of cannabinoid receptors and higher levels of cannabinoid-activated G-proteins (Breivogel et al., 1997). Methanandamide, a stable analog, was used instead of the endogenous cannabinoid anandamide because previous results demonstrated that these agonists acted essentially identically for both [35 S]GTP γ S binding (Breivogel et al., 1998) and receptor binding (Abadji et al., 1994) (provided that the membranes had been pre-treated with an esterase inhibitor).

It is clear that the responses measured in these studies were mediated by CB $_1$ receptors as assessed by using the CB $_1$ -selective antagonist SR141716A. Agonist-receptor binding was measured by inhibition of [3 H]SR141716A binding, stimulation of [35 S]GTP γ S binding was inhibited by SR141716A with a potency consistent with mediation by CB $_1$, and agonist inhibition of adenylyl cyclase was blocked potently by SR141716A. Furthermore, the relative affinities

for each agonist agreed with previously published relative affinities at CB $_1$ cannabinoid receptors (Rinaldi-Carmona et al., 1996).

Results of this study indicated that relative agonist potency is determined by agonist receptor affinity because agonist potencies for functional responses (activation of G-proteins and the inhibition of cAMP accumulation) correlated with agonist receptor-binding affinities. Although there were no apparent differences in agonist affinities across regions, most agonists (except Δ^9 -THC where efficacy was low) were most potent for [35 S]GTP γ S binding in hippocampus and least potent in cerebellum. This may be explained by regional differences in receptor reserve, with reserve being highest in hippocampus and lowest in cerebellum. However, agonist receptor affinities did not vary across regions, and the K_i/K_s ratios in hippocampus were all close to one, indicating little, if any, cannabinoid receptor reserve for [35 S]GTP γ S binding. This agrees with the observation that near complete receptor occupancy is required to obtain maximal stimulation of [35 S]GTP γ S binding (Fig. 4).

Results (Tables 1 and 2) showed significant differences in relative agonist efficacy ($E_{\max}$) and potency across regions, but the reasons for these differences are not clear. The numbers of cannabinoid-activated G-proteins and cannabinoid receptors and the ratios between them (catalytic amplification factors) in these brain regions were previously reported by our laboratory. These factors were very similar for cerebellum and hippocampus, but hypothalamus exhibited higher amplification factors (Breivogel et al., 1997). Neither differences in receptor density nor amplification factor correlated with differences in relative agonist $E_{\max}$ or potency across these regions because relative agonist $E_{\max}$ values and potencies were most similar in hippocampus and hypothalamus. However, it is possible that the types of G-proteins activated by cannabinoid receptors in these regions vary. This is supported by the observation that cannabinoid-inhibited adenylyl cyclase is measurable in cerebellar but not in hippocampal or hypothalamic membranes, indicating that perhaps there is a higher ratio of adenylyl cyclase-inhibiting G-proteins (G $_{i\alpha}$ or certain G $_{\beta\gamma}$ complexes) to other types of G-proteins coupling to cannabinoid receptors in cerebellum (Pacheco et al., 1991; Childers et al., 1994). However, data obtained in another study by using a photoaffinity GTP analog, azidoanilido [32 P]GTP, found no evidence for a regional difference in activation of G-protein α -subunits that would account for this variation (Prather et al., 2000). Alternatively, it may be that these agonists acting at CB $_{1A}$ or at undiscovered cannabinoid receptor subtypes display different efficacies, and that the multiple receptor subtypes are present in different ratios across these three regions.

In cerebellar membranes, it is clear that the potencies of each agonist, and the efficacies of the partial agonists relative to WIN55212-2, are higher for inhibition of adenylyl cyclase than for stimulation of [35 S]GTP γ S binding. This is consistent with the existence of receptor/G-protein reserve for adenylyl cyclase inhibition, or greater receptor reserve for adenylyl cyclase inhibition than for G-protein activation. Receptor/effector reserve for the adenylyl cyclase would be predicted to produce greater apparent potency for the agonists because lower levels of receptor occupancy would be required to obtain the maximal effect. Because full agonists would activate excess G-proteins over what is necessary for maxi-

mal inhibition of adenylyl cyclase, partial agonists would have greater efficacy relative to a full agonist (e.g., WIN55212-2) for adenylyl cyclase inhibition than for G-protein activation.

This study clearly shows that cannabinoid agonists exhibit a wide range of efficacies for G-protein activation, translating into efficacy differences for at least one downstream effector system, adenylyl cyclase. Some of these differences were reported previously by other measurements. For example, both anandamide and CP55940 were partial agonists for inhibiting Ca^{2+} currents (Mackie et al., 1993; Shen et al., 1996). Chronic treatments with agonists of different efficacies have shown that more tolerance develops to agonists of greater efficacy (Elliott et al., 1997). Cultured N18TG2 cells exhibited greater desensitization of cannabinoid-inhibited cAMP after chronic desacetyllevonantradol than Δ^9 -THC treatment (Dill and Howlett, 1988), and mice treated chronically with CP55940 showed greater behavioral tolerance than those treated with Δ^9 -THC (Fan et al., 1994). Although it is difficult to demonstrate acute efficacy differences behaviorally (Fan et al., 1994), these chronic data suggest in vivo efficacy differences. Thus, it appears that these differences in efficacy at different effector systems are produced at the level of G-protein activation. Differences in efficacy may have profound implications for both drug abuse and for the use of cannabinoids as medicinal agents, particularly with long-term use.

[^{35}S]GTP γ S binding assays produced multicomponent concentration-effect curves for all agonists. Some of this heterogeneity may be due to the simultaneous presence of G-protein-coupled and -uncoupled cannabinoid receptors because both guanine nucleotides and sodium decrease high-affinity agonist binding to cannabinoid receptors by decreasing receptor/G-protein coupling (Devane et al., 1988; Pacheco et al., 1994). Alternatively, the two apparent potencies observed for the stimulation of [^{35}S]GTP γ S binding may have been due to coupling to different subtypes of G-protein α -subunits, as previously suggested (Prather et al., 2000).

The identity of the three apparent receptor-binding sites is not clear. The highest affinity sites probably represent precoupled cannabinoid receptors because these values were similar to those previously determined under high-affinity agonist binding conditions (Rinaldi-Carmona et al., 1996). Previous data indicating that coupling to different G_α -subtypes occurred with different potencies (Prather et al., 2000) suggest that the multiple agonist receptor-binding affinities observed for the agonists were due to this differential coupling. Coupling to different G-proteins has previously been proposed as the source of three receptor-binding affinities observed for muscarinic receptors in cardiac membranes (Green et al., 1997). Another possible explanation for the three apparent receptor-binding sites is that high-affinity receptor binding occurs when receptors are coupled to G-proteins that are not binding either GDP or GTP(γ S), and the remaining states arise from receptor coupling to different subtypes of G-proteins that have guanine nucleotide bound.

These data do not directly identify the receptor affinity state(s) responsible for G-protein activation, but they do provide evidence via a strong correlation. In this study, it appeared that highest affinity agonist-binding sites contributed to basal, and not to agonist-stimulated [^{35}S]GTP γ S binding. For example, a previous study suggested that the GDP affin-

ity on receptor precoupled G-proteins is approximately 30-fold lower than on uncoupled G-proteins, and is decreased only 8-fold further by a cannabinoid full agonist (Breivogel et al., 1998). Thus, receptor/G-protein coupling may promote some [^{35}S]GTP γ S binding even in the absence of agonist. A mathematical modeling study by Shea and Linderman (1997) supports the suggestion that precoupling of receptors and G-proteins may lead to complex binding and activation curves as were observed experimentally in the present study. Intermediate- and low-affinity receptor-binding sites appeared to correspond to the high- and low-affinity [^{35}S]GTP γ S-stimulating sites. This interpretation is somewhat complicated by findings with WIN55212-2 (Table 4), where the percentage of intermediate receptor sites (59%) more closely corresponded to the percentage of low-affinity [^{35}S]GTP γ S sites (68%). Nevertheless, for the other agonists, the percentage of low-affinity receptor sites corresponds well with the percentage of low-affinity [^{35}S]GTP γ S sites. The lack of correspondence between the percentages of intermediate receptor sites and high-affinity [^{35}S]GTP γ S sites can be explained by the existence of high-affinity receptor-binding sites that reduce the percentage of intermediate receptor sites. Previous findings that the ratio between cannabinoid receptor number and cannabinoid-activated G-proteins is not constant across brain regions (Breivogel et al., 1997) would predict that the ratio between these individual sites also might not be constant.

In contrast to the three-site model that fit receptor-binding data for WIN55212-2, levonantradol, and CP55940, methanandamide appeared to recognize only two-receptor-binding sites, indicating that this ligand binds to two of the sites recognized by the other ligands with equal or very similar affinity. Perhaps the inability of methanandamide to recognize a high-affinity receptor-binding site (higher than those stimulating [^{35}S]GTP γ S binding) is related to its lower efficacy for stimulating [^{35}S]GTP γ S binding. Regardless of the interpretation, these data show that cannabinoid receptors exhibit multiple affinities for at least some agonists, and that agonist occupancy of cannabinoid receptors often occurs at lower concentrations than are able to stimulate [^{35}S]GTP γ S binding to G-proteins.

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