0026-895X/04/6605-1275–1284$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:1275–1284, 2004

Vol. 66, No. 5
604/1178284
Printed in U.S.A.

Long-Term Administration of Δ^9-Tetrahydrocannabinol Desensitizes CB_1-, Adenosine A_1-, and $GABA_B$-Mediated Inhibition of Adenylyl Cyclase in Mouse Cerebellum

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Received March 18, 2004; accepted July 28, 2004

ABSTRACT

Cannabinoid CB_1 receptors in the cerebellum mediate the inhibitory effects of Δ^9-tetrahydrocannabinol (THC) on motor coordination. Intracellular effects of CB_1 receptors include inhibition of adenylyl cyclase via activation of $G_{i/o}$ proteins. There is evidence for the convergence of other neuronal receptors, such as adenosine A_1 and $GABA_B$, with the cannabinoid system on this signaling pathway to influence motor function. Previous studies have shown that brain CB_1 receptors are desensitized and down-regulated by long-term THC treatment, but few studies have examined the effects of long-term THC treatment on downstream effector activity in brain. Therefore, these studies examined the relationship between CB_1, adenosine A_1, and $GABA_B$ receptors in cerebella of mice undergoing prolonged treatment with vehicle or THC at the level of G protein activation and adenylyl cyclase inhibition. In control cerebella, CB_1 receptors produced less than additive inhibition of adenylyl cyclase with $GABA_B$ and A_1 receptors, indicating that these receptors are localized on overlapping populations of cells. Long-term THC treatment produced CB_1 receptor down-regulation and desensitization of both cannabinoid agonist-stimulated G protein activation and inhibition of forskolin-stimulated adenylyl cyclase. However, G protein activation by $GABA_B$ or A_1 receptors was unaffected. It is noteworthy that heterologous attenuation of $GABA_B$ and A_1 receptor-mediated inhibition of adenylyl cyclase was observed, even though absolute levels of basal and forskolin- or G_s-stimulated activity were unchanged. These results indicate that long-term THC administration produces a disruption of inhibitory receptor control of cerebellar adenylyl cyclase and suggest a potential mechanism of cross-tolerance to the motor incoordinating effects of cannabinoid, $GABA_B$, and A_1 agonists.

Δ^9-Tetrahydrocannabinol (THC), the psychoactive ingredient in marijuana, produces its central nervous system effects via activation of cannabinoid CB_1 receptors (Zimmer et al., 1999). CB_1 receptors activate pertussis toxin-sensitive G proteins of the $G\alpha_{i/o}$ subfamily, which produce intracellular effects including inhibition of adenylyl cyclase activity and modulation of calcium and potassium channel conductance (Howlett et al., 2002). CB_1 receptors are widely distributed in brain, with the greatest density in basal ganglia, hippocampus, and cerebellum (Herkenham et al., 1991). Behavioral effects of CB_1 receptor activation include antinociception, hypothermia, memory impairment, and modulation of motor function (Howlett et al., 2002). Cannabinoid-induced motor disturbances include ataxia and incoordination, which are mediated by CB_1 receptors in the cerebellum (Dar, 2000; Patel and Hillard, 2001).

Previous studies have demonstrated that both CB_1 (Pacheco et al., 1993) and adenosine A_1 receptors (Wojcik et al., 1985) in the cerebellum exhibit convergent regulation of adenylyl cyclase with $GABA_B$ receptors. This convergence is thought to occur mainly at parallel fiber-Purkinje cell synapses, because 1) mutant mice deficient in granule cells, the axon of which is the parallel fiber, are deficient in cerebellar CB_1, $GABA_B$, and A_1 receptors and receptor-mediated activity (Goodman et al., 1983; Pacheco et al., 1993) and 2) electrophysiological studies found that all three receptors pre-

These studies were supported by United States Public Health Service grants DA05274, DA14277, and DA03672 from the National Institute on Drug Abuse.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.000604.

ABBREVIATIONS: THC, Δ^9-tetrahydrocannabinol; GTPγS, guanosine-5′-*O*-(γ-thio)-triphosphate; BSA, bovine serum albumin; PIA, (−)-N^6-(2-phenylisopropyl)-adenosine; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-OH]enkephalin; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; RGS, regulators of G protein signaling.

synaptically inhibit the release of glutamate from parallel fibers (Takahashi et al., 1995; Takahashi and Linden, 2000). Moreover, because granule cells are the most abundant neuronal cell type in cerebellum, a major portion of the adenylyl cyclase signal in cerebellar preparations probably results from granule cells. The functional significance of convergent signaling has been demonstrated in studies showing interactions between A_1 and CB_1 or $GABA_B$ agonists on cerebellar-mediated motor incoordination (Dar, 1996, 2000).

Long-term administration of cannabinoid agonists produces tolerance to cannabinoid-mediated behaviors (Fan et al., 1994) and dependence, which is characterized by antagonist-precipitated withdrawal (Tsou et al., 1995). In the central nervous system, cellular adaptations to long-term cannabinoid administration include decreased CB_1 receptor levels (down-regulation) and desensitization of CB_1 receptor-mediated G protein activation in most CB_1 receptor-containing brain regions, including cerebellum (Fan et al., 1996; Sim et al., 1996; Sim-Selley and Martin, 2002). The mechanism of this desensitization, or decrement in CB_1 receptor signaling, is currently unclear; however, it is thought to result from a combination of CB_1 receptor down-regulation and uncoupling of CB_1 receptors from G proteins. It is noteworthy that desensitization of CB_1 receptor-mediated inhibition of adenylyl cyclase has not yet been demonstrated in brain (Fan et al., 1996), despite the finding that desensitization of CB_1-mediated inhibition of adenylyl cyclase occurs after long-term cannabinoid treatment in cell culture models (Dill and Howlett, 1988; Rhee et al., 2000). Moreover, recent studies have shown that basal and forskolin- and Ca^{2+}-stimulated adenylyl cyclase activity increase in cerebellum during CB_1 antagonist-precipitated withdrawal (Hutcheson et al., 1998; Tzavara et al., 2000). Similar findings have been reported in cell culture models, which further demonstrated that this "superactivation" of forskolin-stimulated adenylyl cyclase activity by long-term treatment with cannabinoids depends on the specific adenylyl cyclase isoform expressed in the cell (Rhee et al., 2000). These findings suggest that the regulation of cerebellar adenylyl cyclase activity by long-term treatment with cannabinoids is partly a function of the predominant adenylyl cyclase types expressed in cerebellar granule cells. Another factor that might affect the regulation of cerebellar adenylyl cyclase activity by long-term treatment with cannabinoids is convergent regulation by multiple receptors, such as A_1 adenosine or $GABA_B$. Long-term administration of A_1 adenosine or $GABA_B$ agonists produces desensitization of their respective receptors (Vendite et al., 1998; Wetherington and Lambert, 2002a,b), and heterologous desensitization of A_1 receptor-mediated effector activity by $GABA_B$ agonists has also been observed in cultured neurons (Wetherington and Lambert, 2002a,b). In contrast, long-term THC treatment did not produce heterologous desensitization of $GABA_B$ or A_1 receptor-mediated G protein activation in the brain (Sim et al., 1996; Breivogel et al., 1999). Nonetheless, De-Santy and Dar (2001) reported that long-term treatment with either CB_1 or A_1 agonist produced cross-tolerance to agonists for both CB_1 and A_1 receptors in a motor incoordination paradigm, suggesting that cerebellar A_1 receptor signaling might be altered downstream of receptor-G protein coupling after long-term THC administration.

The focus of the present investigation was to further examine the regulation of cerebellar adenylyl cyclase activity by CB_1 and other $G_{i/o}$-coupled receptors, with an emphasis on the response of this effector system to long-term administration of THC. These studies used a THC administration paradigm in mice, whereby the dose was escalated over a 15-day period to produce a maximal level of tolerance to the behavioral effects of THC and a high level of adaptation in CB_1 receptor signaling throughout the brain (Sim-Selley and Martin, 2002). This paradigm avoids overt behavioral or physiological toxicity, because each dose is administered for 3 days, allowing tolerance to develop before the dose is increased again. The specific goals of this study were to determine whether 1) CB_1, $GABA_B$, and A_1 receptors converge on overlapping populations of G proteins or adenylyl cyclase in cerebellum; 2) long-term THC treatment desensitizes CB_1 receptor-mediated inhibition of adenylyl cyclase in cerebellum; and 3) long-term THC treatment produces heterologous desensitization of A_1- or $GABA_B$-mediated inhibition of cerebellar adenylyl cyclase.

Materials and Methods

Materials. [^{35}S]GTPγS (1150–1300 Ci/mmol) and [α-^{32}P]ATP were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Male ICR mice (24–30 g) were obtained from Harlan (Indianapolis, IN). Econo-1 scintillation fluid was obtained from Fisher Scientific (Pittsburgh, PA). Ecolite scintillation fluid was obtained from MP Biomedicals (Irvine, CA). Bovine serum albumin (BSA), GTPγS, GDP, baclofen, PIA, and WIN 55,212-2 were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals (reagent grade) were obtained from Sigma-Aldrich or Fisher Scientific. CP 55,940 and THC were provided by the Drug Supply Program of the National Institute on Drug Abuse.

Long-Term THC Administration. THC was dissolved in a 1:1:18 solution of ethanol, Emulphor, and saline. Mice received subcutaneous injections of THC or vehicle twice daily (7:00 AM and 3:00 PM) for 15 days. THC was initially administered at a dose of 10 mg/kg, and the dose was doubled every 3 days to a final dose of 160 mg/kg. Animals were observed for overt signs of physiological or behavioral toxicity of THC or its vehicle throughout the 15-day treatment period, and none were detected. Twenty-four hours after the final injection, mice were sacrificed by decapitation, and cerebella were dissected on ice and stored individually at −80°C until assay.

Membrane Preparation. The entire cerebellum of each animal was homogenized in approximately 10 volumes of 50 mM Tris-HCl, 3 mM $MgCl_2$, and 1 mM EGTA, pH 7.4 (membrane buffer), with 20 strokes from a glass homogenizer at 4°C. The homogenate was centrifuged at 48,000*g* at 4°C for 10 min, resuspended in 30 ml of membrane buffer, centrifuged again at 48,000*g* at 4°C for 10 min, and finally resuspended in 50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4 (assay buffer). Membrane protein levels were determined by the method of Bradford using BSA as the standard.

[^{35}S]GTPγS Binding. Membranes (5–10 μg of protein) were preincubated for 10 min at 30°C with adenosine deaminase (3 mU/ml) in assay buffer. Membranes were then incubated for 2 h at 30°C in assay buffer containing 0.1 nM [^{35}S]GTPγS, 20 μM GDP, and adenosine deaminase (3 mU/ml) with and without the indicated concentrations of each agonist. Nonspecific binding was measured with 20 μM GTPγS. Under these conditions, bound [^{35}S]GTPγS was generally within 15% of the total amount of added [^{35}S]GTPγS in each sample. The incubation was terminated by filtration through GF/B glass fiber filters, followed by 3 washes with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction of the filters in Econo-1 scintillation fluid.

Adenylyl Cyclase Assay. Membranes (5–25 μg of protein) were preincubated with adenosine deaminase as described above and then incubated for 15 min at 30°C in the presence or absence of 1 μM forskolin, with or without various drugs at the indicated concentrations, in assay buffer containing 50 μM ATP, [α-^{32}P]ATP (1.5 μCi), 0.2 mM dithiothreitol, 0.01% BSA, 50 μM cAMP, 50 μM GTP, 0.2 mM papaverine, 5 mM phosphocreatine, and 20 U/ml creatine phosphokinase in a final volume of 100 μl. Under these conditions, total [α-^{32}P]cAMP recovered was generally less than 1% of the total amount of added [α-^{32}P]ATP in each sample. The reaction was terminated by boiling for 3 min. [^{32}P]cAMP was isolated by the dual column (Dowex and alumina) method of Salomon (1979). [^{3}H]cAMP (10,000 dpm) was added to each tube before column chromatography as an internal standard. Radioactivity was determined by liquid scintillation spectrophotometry (45% efficiency for ^{3}H) after 4.5 ml of the eluate was dissolved in 14.5 ml of Ecolite scintillation fluid.

Data Analysis. Unless otherwise indicated, data are reported as mean values ± S.E. of 3 to 5 separate experiments, each of which was performed in triplicate. Net-stimulated [^{35}S]GTPγS binding is reported as agonist-stimulated binding minus basal binding. Theoretical additive stimulation of [^{35}S]GTPγS binding by a combination of agonists is defined as the summation of net stimulation (picomoles per milligram) by the individual agonists; actual [^{35}S]GTPγS binding is defined as the net stimulation (picomoles per milligram) of the actual combined agonists. The percentage of additivity of [^{35}S]GTPγS binding is defined as actual stimulation by a combination of agonists/(theoretical additive stimulation − the actual stimulation of the single agonist producing the greatest stimulation alone) × 100%. Net forskolin-stimulated adenylyl cyclase activity is defined as forskolin-stimulated activity − basal activity (picomoles per milligram per minute). The percentage of inhibition of forskolin-stimulated adenylyl cyclase activity is defined as (net forskolin-stimulated activity in the absence of agonist − net forskolin-stimulated activity in the presence of agonist/net forskolin-stimulated activity in the absence of agonist) × 100%. Theoretically additive adenylyl cyclase inhibition is defined as the summation of the percentage of inhibition of net forskolin-stimulated activity by individual drug treatments; actual adenylyl cyclase inhibition is defined as the percentage of inhibition by the combination of agonists. The percentage of additivity of [^{32}P]cAMP production is defined as (actual inhibition of forskolin-stimulated [^{32}P]cAMP production/theoretically additive inhibition of forskolin-stimulated [^{32}P]cAMP production – the actual inhibition of the single agonist producing the greatest inhibition alone). All curve-fitting and statistical analyses were performed using JMP (SAS Institute, Cary, NC). Concentration-effect curves were analyzed by iterative nonlinear regression to obtain EC_{50} and E_{max} values. Statistical significance of the data were determined by analysis of variance, followed by the nonpaired two-tailed Student's t test, with the following exceptions. The nonpaired two-tailed Student's t test with Bonferroni adjustment was used to determine whether the percentage of additivity was significantly different from 100%. Because the magnitude of EC_{50} values varied among different agonists, significant differences between pairs of EC_{50} values (vehicle versus THC) were determined by direct comparison with the nonpaired two-tailed Student's t test.

Results

Convergence of Signal Transduction between CB_1, $GABA_B$, and A_1 Adenosine Receptors

Convergence of G Protein Activation. Additivity experiments were performed to determine whether CB1 receptors converge on the same pools of G proteins that are also activated by $GABA_B$ and adenosine A_1 receptors in the cerebellum. Evidence for convergent regulation of signal transduction by these receptors was obtained by determining whether the signal transduction responses were additive or less than additive when agonists for the respective receptors were assessed together or separately in the reaction mixture. WIN 55,212-2 was used to active CB_1 receptors, whereas baclofen and PIA were used to activate $GABA_B$ and adenosine A_1 receptors, respectively. Maximally effective concentrations of each agonist were determined in concentration-effect curves for stimulation of [^{35}S]GTPγS binding (data not shown).

To determine whether cerebellar CB_1 receptors activate common populations of G proteins as $GABA_B$ and adenosine A_1 receptors, stimulation of [^{35}S]GTPγS binding by WIN 55,212-2 (10 μM) was measured alone and in combination with baclofen (300 μM) and/or PIA (5 μM). Results (Fig. 1A) showed that net stimulation by each individual drug ranged from 0.27 ± 0.03 pmol/mg (WIN 55,212-2) to 0.35 ± 0.04 pmol/mg (baclofen). In two-drug combinations, net-stimulated [^{35}S]GTPγS binding ranged from 0.51 ± 0.05 pmol/mg (WIN 55,212-2 + PIA) to 0.59 ± 0.07 pmol/mg (WIN 55,212-2 + baclofen). Net stimulation by all three drugs in combination was 0.72 ± 0.06 pmol/mg. Although net stimulation of G protein activation produced by more than one agonist in

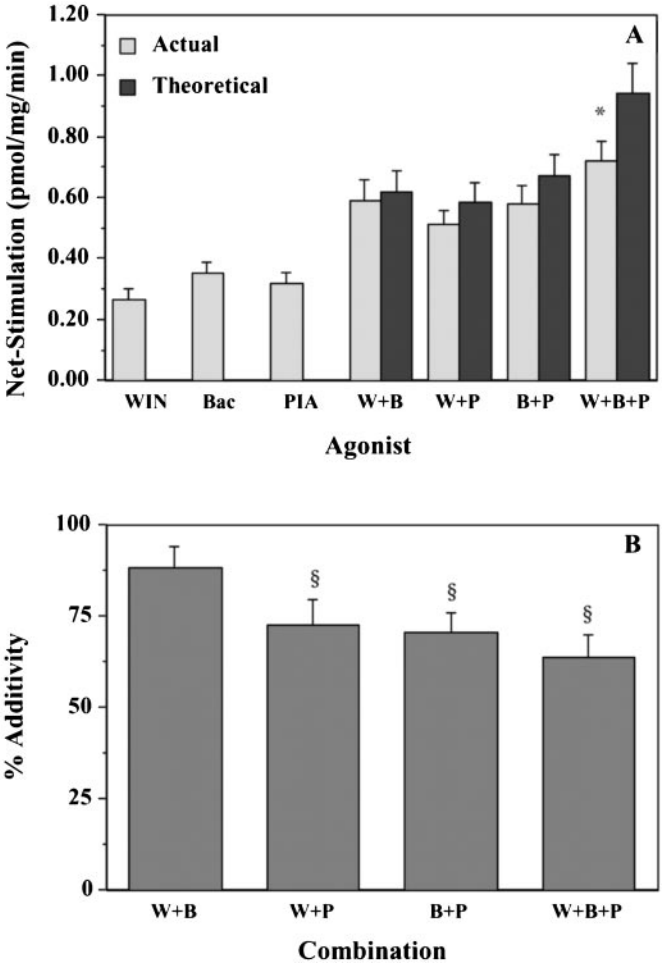

Fig. 1. Stimulation of [^{35}S]GTPγS binding by agonists of cerebellar CB_1, $GABA_B$, and A_1 receptors. Membranes were incubated with 20 μM GDP, 0.01 nM [^{35}S]GTPγS, and 10 μM WIN 55,212-2 (WIN, W); 300 μM baclofen (Bac, B); or 3 μM PIA (P) alone or in the indicated combinations as described under *Materials and Methods*. Data are mean values ± S.E.M. of (A) net-stimulated [^{35}S]GTPγS binding (picomoles per milligram) or (B) percentage of additivity. *, $p \leq 0.05$ different from theoretically additive stimulation; and §, $p \leq 0.05$ different from 100%.

combination was greater than with each agonist alone, this activity seemed to be slightly less than additive compared with the sum of individual agonist-stimulated activities, expressed as theoretical additive stimulation. However, because of normal interexperimental variability in the net-stimulated [^{35}S]GTPγS binding data, statistical analysis showed that only the WIN 55,212-2 + baclofen + PIA combination produced significantly less than theoretically additive stimulation.

To more accurately quantify whether each drug combination produced stimulation that was less than additive, the data were calculated as percentage of additivity (Fig. 2B). This calculation determined the relative additivity of each drug combination compared with the theoretical additive value within each experiment, defined as follows: (net stimulation by drug combination − net stimulation by the drug that produced the greatest stimulation alone)/(sum of net stimulation by each drug alone − net stimulation by the drug that produced the greatest stimulation alone) × 100%. According to this calculation, if there is complete overlap of activated G proteins between two receptors, the stimulation produced by any drug combination would equal the stimulation produced by the receptor that produced the greatest stimulation on its own, and this value would be defined as 0% additivity. On the other hand, if the stimulation produced by any receptor combination equals the sum of the net picomoles per milligram stimulated by the individual drugs, the percentage of additivity value would equal 100%. The results showed that all drug combinations exhibited partial additivity, ranging from approximately 64 to 88%. The WIN 55,212-2 + PIA, PIA + baclofen, and three-drug combination were all significantly less than additive, but the WIN 55,212-2 + baclofen combination was not statistically less than 100% additive. Thus, both CB_1 and $GABA_B$ receptors produced less than additive stimulation of G protein activation with A_1 receptors but not with each other. These results indicate that CB_1 and $GABA_B$ receptors partially converge with adenosine A_1 receptors on a common population of G proteins, which suggests at least partial colocalization of A_1 receptors on the same cells with CB_1 or $GABA_B$ receptors.

To confirm that these additivity results reflected convergent regulation of G proteins by receptors colocalized on the same cell membrane, we tested the hypothesis that receptors in membranes prepared from different cells could produce less than additive activation of G proteins when the membranes were suspended together in the same sample. For this purpose, membranes prepared from Chinese hamster ovary cells heterologously expressing μ-opioid receptors were homogenized together with membranes from mouse cerebellum, which expresses CB_1 but not μ-opioid receptors. Results showed that stimulation of G protein activation by WIN 55,212-2 and the full μ-agonist DAMGO were additive in this mixed membrane preparation. Net stimulation was 0.20 ± 0.02 pmol/mg by WIN 55,212-2, 0.29 ± 0.05 pmol/mg by DAMGO, and 0.47 ± 0.07 pmol/mg by WIN 55,212-2 + DAMGO. The percentage of additivity was 93 ± 6.4%, which was not significantly different from 100%. These results indicate that convergent activation of G proteins by receptors on different populations of cells does not occur to a significant extent, which supports the conclusion that less than additive activation of G proteins by different receptors within the same tissue is suggestive of colocalization on overlapping cell populations.

Convergence of Adenylyl Cyclase Inhibition. To determine whether CB_1 receptors regulate the same population of adenylyl cyclase with $GABA_B$ or A_1 receptors in cerebellum, additivity studies of receptor-mediated inhibition of for-

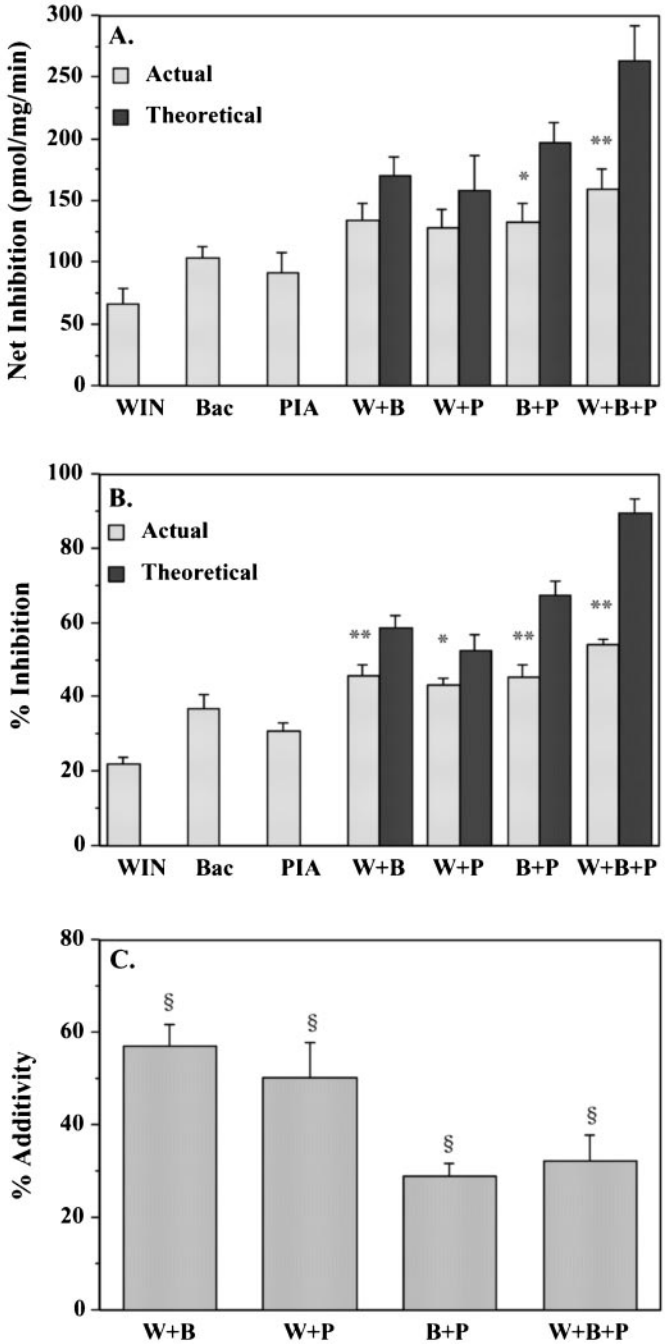

Fig. 2. Inhibition of adenylyl cyclase activity by agonists of cerebellar CB_1, $GABA_B$, and A_1 receptors. Membranes were incubated with 1.5 μCi [α-^{32}P]ATP and 10 μM WIN 55,212-2 (WIN, W), 300 μM baclofen (Bac, B), or 3 μM PIA (P) alone or in the indicated combinations as described under *Materials and Methods*. Data are mean values ± S.E.M. of (A) net-inhibited adenylyl cyclase activity (picomoles per milligram per minute), (B) percentage of inhibition of net forskolin-stimulated adenylyl cyclase activity, or (C) percentage additivity. *, $p \leq 0.05$; **, $p \leq 0.01$ different from theoretically additive stimulation; and §, $p \leq 0.05$ different from 100%.

skolin-stimulated adenylyl cyclase were performed. Maximally effective concentrations of WIN 55,212-2, baclofen, and PIA, as given above, were confirmed by concentration-effect curves for inhibition of adenylyl cyclase activity (data not shown). In the absence of inhibitory agonist, 1 μM forskolin stimulated cAMP synthesis to a rate of 459 ± 2.6 pmol/mg/min, which was a 2.82 ± 0.14-fold increase over basal activity. All drugs and drug combinations tested significantly inhibited forskolin-stimulated adenylyl cyclase activity but did not affect the basal activity of the enzyme (data not shown). Net inhibition of forskolin-stimulated activity by each drug alone ranged from 66 ± 12.4 pmol/mg/min (WIN 55,212-2) to 105 ± 7.4 pmol/mg/min (baclofen) (Fig. 2A), corresponding to approximately 22 to 30% inhibition of net forskolin-stimulated adenylyl cyclase activity (Fig. 2B).

Each drug combination seemed to produce inhibition of forskolin-stimulated adenylyl cyclase activity (picomoles per milligram per minute) that was less than the theoretical additive inhibition (Fig. 2A), but statistical analysis showed that only baclofen + PIA and the combination of all three drugs were significantly less than additive. However, because of normal interexperimental variability in the absolute rates of adenylyl cyclase activity, the percentage of inhibition of net forskolin-stimulated activity is a more precise measurement of the effect of each drug and drug combination. The percentage of inhibition of net forskolin-stimulated activity was found to be significantly less than additive for all drug combinations, based on the comparison to the sum of the percentage of inhibition by each drug alone (Fig. 2B). The percentage of additivity of each drug combination was then calculated for inhibition of adenylyl cyclase using the same relationship described above for G protein activation. The results showed that all drug combinations produced inhibition that was significantly less than 100% additive, with values ranging from 29 to 57% additivity (Fig. 2C). Thus, in cerebellum, CB_1 receptors produce less than additive inhibition of adenylyl cyclase with $GABA_B$ and A_1 adenosine receptors, which also produced less than additive inhibition with each other. These results suggest that there is a high degree of colocalization among these inhibitory receptors and that levels of adenylyl cyclase are a limiting factor relative to activated G proteins in this signal transduction pathway.

Effects of Long-Term THC Administration on Signaling by CB_1, $GABA_B$, and A_1 Adenosine Receptors

Effects of Long-Term THC on G Protein Activation. Mice were injected twice daily with escalating doses of THC over a 15-day period to induce cannabinoid tolerance. The initial dose of THC was 10 mg/kg, and this dose was doubled every 3rd day up to a final dose of 160 mg/kg. This treatment paradigm has previously been shown to produce a high level of tolerance to THC-mediated antinociception, hypothermia, and hypolocomotion, as well as desensitization and down-regulation of CB_1 receptors throughout the brain, as measured by agonist-stimulated [^{35}S]GTPγS and [^{3}H]SR141716A autoradiography, respectively (Sim-Selley and Martin, 2002). To determine whether long-term administration of THC altered the ability of CB_1 receptors to activate G proteins in cerebellar membranes, concentration-effect curves of agonist-stimulated [^{35}S]GTPγS binding were conducted with the full CB_1 agonist WIN 55,212-2 and the high-efficacy partial agonist CP 55,940 (Breivogel et al., 1998). The results (Fig. 3) showed that long-term THC treatment produced an apparent decrease in CB_1 receptor-mediated G protein activation by both drugs; however, this decrease was greater in magnitude with CP 55,940 (Fig. 3B) than with WIN 55,212-2 (Fig. 3A). Nonlinear regression analysis of the concentration-effect curves supported this conclusion (Table 1). There was a decrease in the potency of WIN 55,212-2 to stimulate [^{35}S]GTPγS, as indicated by the 2-fold increase in the EC_{50} value. Although maximal stimulation by WIN 55,212-2 seemed to decrease by 20% in THC-treated relative to vehicle-treated mice, this was not statistically significant ($p = 0.17$). Nonetheless, maximal stimulation of [^{35}S]GTPγS binding by CP 55,940 in THC-treated mice was decreased by 44% relative to vehicle-treated mice, and there was a small (1.7-fold) increase in the EC_{50} value. Two lines of evidence suggest that residual THC in the tissue did not cause this apparent desensitization of G protein activation. First, basal [^{35}S]GTPγS binding in cerebellar membranes did not differ between long-term vehicle- and THC-treated mice (141 ± 14 versus 126 ± 15 fmol/mg, respectively). Second, there was no difference in the measured K_D value of cerebellar [^{3}H]SR141716A binding between long-term vehicle- and THC-treated mice (0.72 ± 0.01 versus 0.54 ± 0.18 nM,

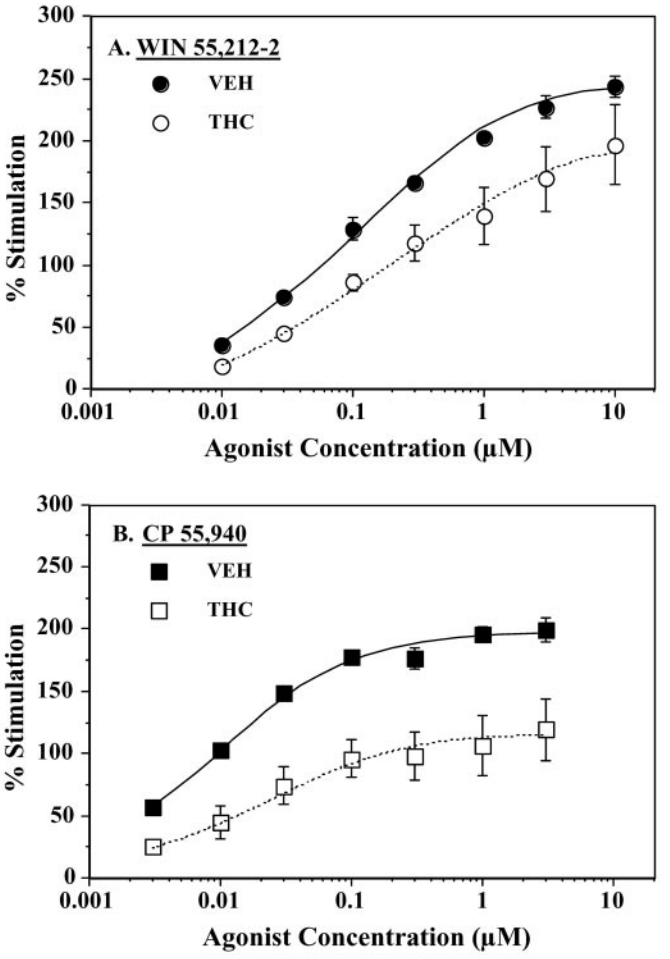

Fig. 3. Effect of long-term THC treatment on stimulation of cerebellar [^{35}S]GTPγS binding by cannabinoid agonists. Membranes from vehicle- (VEH) or THC-treated mice were incubated with (A) 20 μM GDP, 0.01 nM [^{35}S]GTPγS, and varying concentrations of WIN 55,212-2 or (B) CP 55,940 as described under *Materials and Methods*. Data are mean percent stimulation values ± S.E.M.

respectively). However, down-regulation of cerebellar CB1 receptor binding sites probably contributed toward the attenuated level of cannabinoid agonist-induced G protein activation, because the B_{max} value of [^{3}H]SR141716A binding was decreased by 55% after long-term THC treatment (4.18 ± 0.03 versus 1.79 ± 0.34 pmol/mg in vehicle- and THC-treated mice, respectively). Thus, long-term THC treatment down-regulated CB_1 receptor binding sites and desensitized cerebellar CB_1 receptor-mediated G protein activation by both the full agonist WIN 55,212-2 and the partial agonist CP 55,940, but the magnitude of this apparent desensitization was greater with CP 55,940.

To determine whether long-term administration of THC caused a heterologous desensitization of $GABA_B$ and A_1 receptor-mediated G protein activation in cerebellar membranes, concentration-effect curves for stimulation of [^{35}S]GTPγS binding were conducted with baclofen and PIA, respectively. In contrast to results obtained with CB_1 receptor-mediated G protein activation, there was no effect of long-term THC treatment on the potency or maximal stimulation produced by either baclofen or PIA (Fig. 4; Table 1). Therefore, long-term THC treatment produced homologous desensitization of CB_1 receptor-mediated G protein activation in cerebellum.

Effects of Long-term THC on Adenylyl Cyclase Inhibition. To determine the effects of long-term THC administration on CB_1 receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase activity, concentration-effect curves were generated with WIN 55,212-2, CP 55,940, and the low-efficacy partial agonist THC. Results showed little effect of long-term THC treatment on inhibition of cerebellar adenylyl cyclase by WIN 55,212-2 (Fig. 5A), but the inhibitory effects of CP 55,940 and THC were both attenuated (Fig. 5, B and C). Nonlinear regression analysis of the concentration-effect curves (Table 2) showed that, although there was no difference in maximal inhibition by WIN 55,212-2, there was a small but significant increase (1.9-fold) in the EC_{50} value of this full agonist in THC-treated mice. Moreover, long-term THC treatment decreased the maximal inhibition by CP 55,940 and THC by 21 and 42%, respectively, relative to the maximal inhibition produced by these drugs in vehicle-treated mice. Thus, long-term THC treatment desensitized CB_1 receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase in a manner inversely related to the efficacy of the agonist used to inhibit the enzyme.

To determine whether $GABA_B$ and adenosine A_1 receptor-mediated inhibition of cerebellar adenylyl cyclase was affected by long-term THC administration, concentration-effect curves were conducted with baclofen and PIA, respectively. Long-term THC treatment attenuated the inhibition of adenylyl cyclase by both baclofen and PIA (Fig. 6), in contrast to results obtained with these drugs in stimulating G protein activation. Nonlinear regression analysis confirmed that maximal inhibition of adenylyl cyclase by both PIA and baclofen was decreased by approximately 18%, and there was a trend toward an increase in the EC_{50} value of baclofen (1.8-fold, $p = 0.059$). Therefore, long-term THC treatment produced an apparent heterologous desensitization of CB_1, $GABA_B$, and A_1 receptor-mediated inhibition of cerebellar adenylyl cyclase.

This apparent heterologous desensitization could not be explained by changes in the levels of basal and forskolin- or $G\alpha_s$-stimulated adenylyl cyclase activity. No significant differences were observed between THC- and vehicle-treated mice in the absolute level of basal and GTPγS- or forskolin-stimulated adenylyl cyclase activity in cerebellar membranes (Fig. 7A). Moreover, the level of forskolin-stimulated adenylyl cyclase activity, calculated as a percentage of basal activity, was not different between THC- and vehicle-treated mice (301 ± 9.2 versus 308 ± 16%, respectively). Finally, concentration-dependent stimulation of cerebellar adenylyl cyclase activity by the β-adrenergic agonist isoproterenol was not different between THC- and vehicle-treated mice (Fig. 7B).

TABLE 1

E_{max} and EC_{50} values of agonist-stimulated [^{35}S]GTPγS binding in cerebellum

Data are mean values ± S.E.M. obtained from nonlinear regression analyses of the data shown in Figs. 3 and 4.

Drug	Treatment	E_{max}	EC_{50}
		% of stimulation	*nM*
WIN 55,212-2	Vehicle	230 ± 6.3	83 ± 15
	THC	187 ± 21	167 ± 21*
CP 55,940	Vehicle	193 ± 5.1	8.5 ± 0.8
	THC	130 ± 18*	15 ± 1.0**
PIA	Vehicle	255 ± 13	90 ± 16
	THC	249 ± 10	113 ± 20
Baclofen	Vehicle	233 ± 19	7615 ± 357
	THC	251 ± 18	8140 ± 1012

*, $p < 0.05$; **, $p < 0.01$ different from vehicle-treated mice.

Discussion

The present study is to our knowledge the first demonstration of long-term cannabinoid-induced desensitization of CB_1 receptor-mediated inhibition of adenylyl cyclase in brain. Previous studies demonstrated desensitization of adenylyl cyclase inhibition in CB_1 receptor-expressing cell lines (Dill and Howlett, 1988), but few have examined this question in brain. In one study (Fan et al., 1996), long-term administration of CP 55,940 produced no change in potency or maximal inhibition of cerebellar adenylyl cyclase by CP 55,940, despite a 50% decrease in the B_{max} value of [^{3}H]CP 55,940 binding. Potential explanations for these discrepant results include differences in treatment duration, dosing regimen, or the particular drug administered. In the present study, ex-

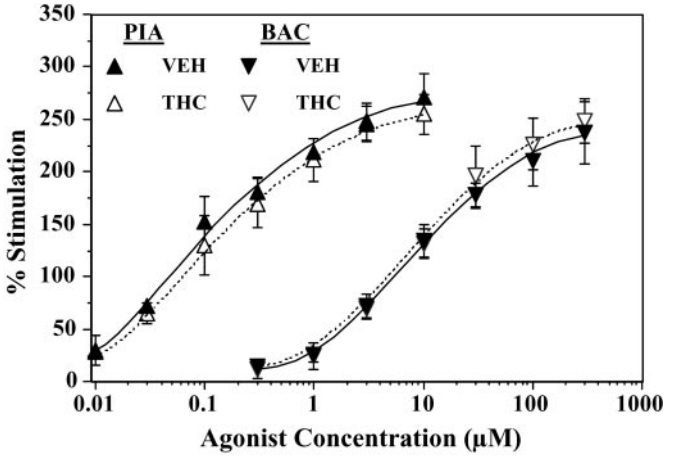

Fig. 4. Effect of long-term THC treatment on stimulation of cerebellar [^{35}S]GTPγS binding by A_1 or $GABA_B$ agonists. Membranes from vehicle- (VEH) or THC-treated mice were incubated with 20 μM GDP, 0.01 nM [^{35}S]GTPγS, and varying concentrations of PIA or baclofen as described under *Materials and Methods*. Data are mean percentage stimulation values ± S.E.M.

posure to escalating doses of THC over 15 days produced desensitization of CB_1 receptor-mediated G protein activation and inhibition of adenylyl cyclase, whereas the previous study examined a constant dose of CP 55,940 for 6.5 days. One potentially important factor is the difference in intrinsic efficacy between CP 55,940 and THC. We have previously found that long-term treatment with WIN 55,212-2 produced less desensitization than long-term treatment with THC in certain brain regions, including cerebellum (Sim-Selley and Martin, 2002). In general, previous studies found WIN 55,212-2 to be a full agonist for G protein activation relative to CP 55,940, and both exhibited greater efficacy than THC (Sim et al., 1996; Breivogel et al., 1998). Thus, there seems to be an inverse relationship between the efficacy of the treatment drug and CB_1 receptor desensitization in brain. The magnitude of desensitization also depended on the intrinsic efficacy of the cannabinoid used in the in vitro assay. Thus, greater desensitization was observed when testing CP 55,940 or THC compared with WIN 55,212-2. This relationship was previously shown for stimulation of G protein activity in cerebellum after long-term THC treatment (Breivogel et al., 2003), and the present results extend these findings to inhibition of adenylyl cyclase. In this case, CP 55,940 and THC both exhibited decreased maximal inhibition of adenylyl cyclase after long-term THC treatment, whereas WIN 55,212-2 only exhibited decreased potency.

This study also examined the interaction between CB_1, A_1, and $GABA_B$ receptors in cerebellum. In agreement with previous studies (Wojcik et al., 1985; Pacheco et al., 1993),

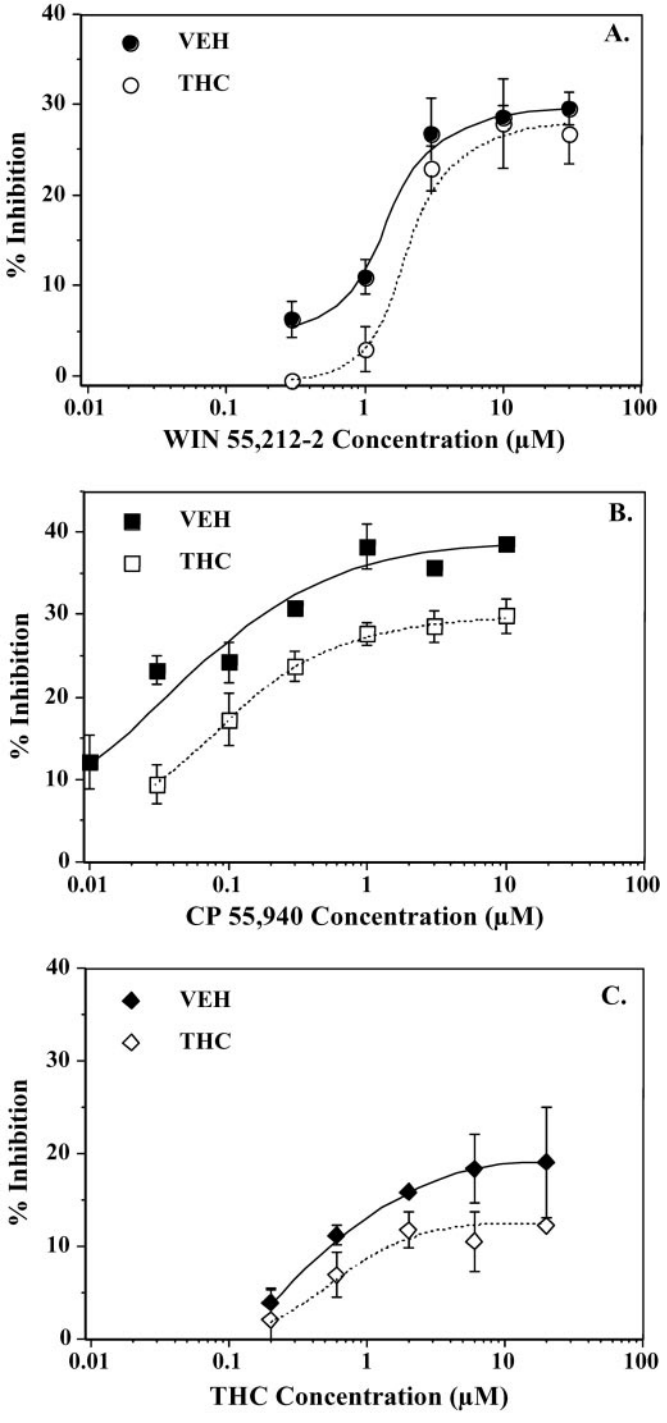

Fig. 5. Effect of long-term THC treatment on inhibition of cerebellar adenylyl cyclase activity by cannabinoid agonists. Membranes from vehicle- (VEH) or THC-treated mice were incubated with 1.5 μCi [α-^{32}P]ATP and varying concentrations of (A) WIN 55,212-2, (B) CP 55,940, or (C) THC as described under *Materials and Methods*. Data are mean percentage of inhibition of net forskolin-stimulated adenylyl cyclase values ± S.E.M.

TABLE 2

E_{max} and EC_{50} values of agonist inhibition of forskolin-stimulated adenylyl cyclase activity in cerebellum

Data are mean values ± S.E.M. obtained from nonlinear regression analyses of the data shown in Figs. 5 and 6.

Drug	Treatment	E_{max}	EC_{50}
		% of inhibition	*nM*
WIN 55,212-2	Vehicle	33 ± 2.0	1432 ± 386
	THC	32 ± 4.1	2705 ± 254*
CP 55,940	Vehicle	38 ± 1.6	44 ± 17
	THC	30 ± 1.4*	112 ± 43
THC	Vehicle	24 ± 3.3	654 ± 228
	THC	14 ± 1.4*	797 ± 290
PIA	Vehicle	48 ± 2.6	141 ± 19
	THC	39 ± 2.6*	147 ± 28
Baclofen	Vehicle	49 ± 2.6	2978 ± 818
	THC	40 ± 1.8*	5361 ± 613

*, $p < 0.05$; different from vehicle-treated mice.

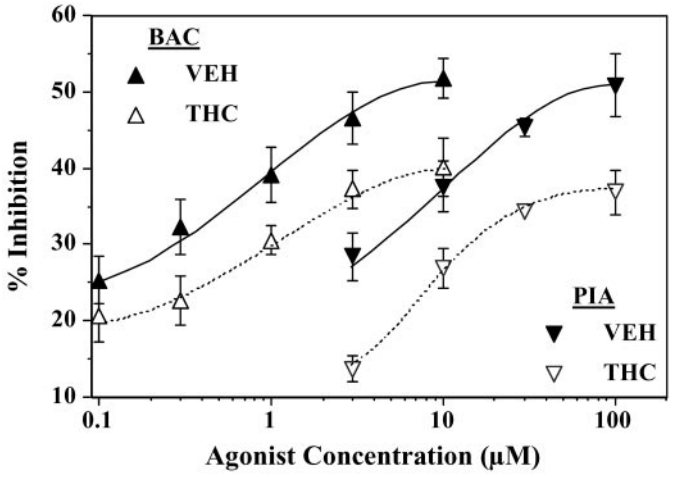

Fig. 6. Effect of long-term THC treatment on inhibition of cerebellar adenylyl cyclase activity by A_1 or $GABA_B$ agonists. Membranes from vehicle- (VEH) or THC-treated mice were incubated with 1.5 μCi [α-^{32}P]ATP and varying concentrations of PIA or baclofen as described under *Materials and Methods*. Data are mean percentage of inhibition of net forskolin-stimulated adenylyl cyclase values ± S.E.M.

$GABA_B$ receptors exhibited less than additive inhibition of adenylyl cyclase with either CB_1 or adenosine A_1 receptors. A novel finding of the present study is that CB_1 and A_1 receptors produced less than additive regulation of both G protein and adenylyl cyclase activity. The finding that activation of G proteins by a combination of receptors was closer to additive than was inhibition of adenylyl cyclase suggests that adenylyl cyclase is the limiting factor in this signal transduction pathway. A similar conclusion was reached in previous studies comparing G protein and adenylyl cyclase responses (Wojcik et al., 1985; Pacheco et al., 1993). The finding that cerebellar $GABA_B$ and CB_1 receptors produce additive activation of G proteins agrees with a previous report (Pacheco et al., 1993). However, the finding that G protein activation by $GABA_B$ and A_1 receptors was less than additive disagrees with a previous study that reported complete additivity (Wojcik et al., 1985). There are several potential explanations for this discrepancy, such as pretreatment of membranes with adenosine deaminase in the present study to inactivate endogenous adenosine and provide more accurate measurement of A_1 receptor activity, or the use of mice in this study versus rats in the earlier study. An additional possibility is that the previous study assessed G protein activation by measuring low K_m GTPase activity, whereas the present study measured [^{35}S]GTPγS binding. The former method may underestimate convergence, because each G protein may be activated by the receptor multiple times as a result of the Gα subunit reassociating with the G$\beta\gamma$ subunit after GTP hydrolysis and becoming available for subsequent activation. In the case of GTPγS, which is hydrolysis-resistant, activated receptors stimulate the accumulation of GTPγS-bound Gα subunits, which are no longer available for reactivation by receptors. Thus, agonist-stimulated [^{35}S]GTPγS binding might be more likely to reveal convergent activation of a finite population of G proteins by multiple receptors as a less than additive response.

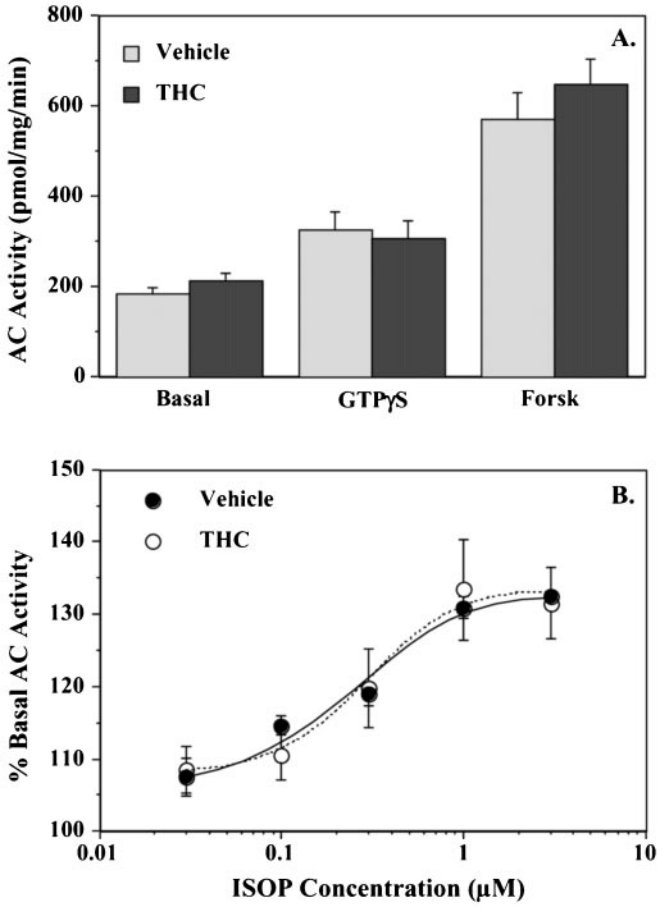

Fig. 7. Effect of long-term THC treatment on basal and stimulated cerebellar adenylyl cyclase activity. Membranes from vehicle- (VEH) or THC-treated mice were incubated with 1.5 μCi [α-^{32}P]ATP and (A) no drug, 10 μM GTPγS, or 1 μM forskolin, or (B) varying concentrations of isoproterenol (ISOP) as described under *Materials and Methods*. Data are mean values ± S.E.M. of (A) adenylyl cyclase activity (picomoles per milligram per minute) or (B) percentage of basal adenylyl cyclase activity.

Convergent signaling by CB_1, A_1, and $GABA_B$ receptors in the cerebellum could have functional implications for the actions of psychoactive drugs. Studies by Dar and colleagues have documented a synergistic effect of A_1 receptor activation with cannabinoid-induced motor incoordination (Dar, 2000; DeSanty and Dar, 2001), whereby adenosine agonists and transport inhibitors synergistically enhanced the effects of cannabinoids in an A_1 antagonist-reversible manner. Another potential drug interaction could occur between cannabinoids and ethanol, because ethanol affects A_1, $GABA_B$, and CB_1 receptor systems (Colombo et al., 2000; Dunwiddie and Masino, 2001; Basavarajappa and Hungund, 2002) and has direct actions on cerebellar adenylyl cyclase via modulation of protein phosphorylation cascades (Tabakoff et al., 2001). In fact, behavioral studies have shown interactions between THC/ethanol and baclofen/ethanol in motor incoordination and modulation of these effects by A_1 agonists (Dar, 1996; Dar, 2000). Attenuation of these motor effects by pertussis toxin implicated interactions at the level of receptor-coupled G protein activity, and direct evidence for a role of adenylyl cyclase in adenosine-mediated modulation of ethanol-induced ataxia has been reported (Dar, 1997). Although the vehicle used in the present study contains ethanol, which might potentially enhance the heterologous effect of long-term THC treatment on $GABA_B$ and A_1 receptor signaling, the amount of ethanol administered was pharmacologically inactive and probably inadequate to produce any effect on signal transduction.

The finding that long-term THC administration produced heterologous attenuation of adenylyl cyclase inhibition by cerebellar $GABA_B$ and A_1 receptors has potential implications for in vivo cross-tolerance between agonists of these receptors. For example, studies have demonstrated cross-tolerance to cannabinoid-induced motor incoordination and its enhancement by A_1 agonists after long-term treatment with agonists of either receptor (DeSanty and Dar, 2001). The present results might provide a mechanism for this cross-tolerance and further suggest that long-term treatment with cannabinoid agonists might produce cross-tolerance to the synergistic enhancement of ethanol-induced motor incoordination by either A_1 or $GABA_B$ agonists.

There are several possible mechanisms by which heterologous attenuation of adenylyl cyclase inhibition might be produced by long-term THC treatment. Although long-term THC administration produces CB_1 receptor desensitization and down-regulation, this effect cannot explain heterologous desensitization of adenylyl cyclase inhibition by A_1 or $GABA_B$ receptors. Indeed, CB_1 receptor-stimulated G protein activity was homologously desensitized by long-term THC treatment in this and previous studies (Sim et al., 1996;

Breivogel et al., 1999). One potential explanation for attenuated effector regulation downstream of receptor-G protein coupling is an enhanced interaction of activated G proteins with regulators of G protein signaling (RGS), which accelerate G protein inactivation and modulate effector signaling (De Vries et al., 2000). At least two types of RGS proteins (RGS 7 and 10) are found in cerebellar granule cells (Gold et al., 1997), but their involvement in the regulation of cerebellar CB_1, A_1, or $GABA_B$ receptor signaling is unknown. Another potential mechanism of attenuated inhibition of adenylyl cyclase by $G_{i/o}$-coupled receptors is alteration in the expression of adenylyl cyclase isotypes that are differentially regulated by $G_{i/o}$ proteins. For example, types II and VII adenylyl cyclase are expressed in cerebellar granule cells (Hellevuo et al., 1996; Mons et al., 1998). These isotypes are not inhibited by $G\alpha_i$ but are stimulated by $G\beta\gamma$ in the presence of activated $G\alpha_s$ (Simonds, 1999), and studies in cell models have shown that CB_1 receptors stimulate these two isotypes (Rhee et al., 2000). The predominant G_i-inhibited adenylyl cyclase isotype in cerebellum is probably type I (Hellevuo et al., 1996; Storm et al., 1998). Thus, a decreased expression ratio of adenylyl cyclase type I to types II or VII could produce an apparent impairment of receptor-mediated inhibition of forskolin-stimulated activity. It is also possible that increased expression of type I adenylyl cyclase itself could produce an apparent heterologous loss of receptor-mediated inhibition, if the increase in this isotype overcame the capacity of inhibitory receptors to modulate its activity. Indeed, increased basal and forskolin- and Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity has been reported in cerebellum after antagonist-precipitated withdrawal after long-term cannabinoid agonist treatment (Hutcheson et al., 1998; Rubino et al., 2000; Tzavara et al., 2000). Because adenylyl cyclase I is the major Ca^{2+}-stimulated isotype in cerebellum (Storm et al., 1998), a significant portion of cannabinoid withdrawal-enhanced adenylyl cyclase activity could be caused by this isotype. Moreover, cannabinoid agonist-induced superactivation of adenylyl cyclase I has been demonstrated in cell culture models (Rhee et al., 2000). However, no significant increase in basal and forskolin- or G_s-stimulated adenylyl cyclase activity was observed in the present study. A likely explanation for this difference is that the manifestation of long-term cannabinoid-induced superactivation of adenylyl cyclase requires administration of the CB_1 antagonist SR141716A in both mice (Hutcheson et al., 1998) and cultured cells (Rhee et al., 2000). The removal of THC does not produce the same result as the administration of SR141716A, possibly because of the inverse agonist properties of this compound (Howlett et al., 2002). Moreover, because brains were obtained 24 h after the last THC injection in the present study, forskolin-stimulated adenylyl cyclase activity could have been elevated initially but then returned to baseline. Thus, it is unlikely that the apparent heterologous desensitization of CB_1-, A_1-, and $GABA_B$-mediated inhibition of adenylyl cyclase was caused by an elevation in adenylyl cyclase activity per se, although these two responses to long-term THC treatment might be related to a common underlying mechanism.

In conclusion, long-term THC administration in mice produced an apparent heterologous desensitization of CB_1, A_1, and $GABA_B$ receptor-mediated inhibition of cerebellar adenylyl cyclase. This adaptation occurred at a level of signal transduction that is downstream of the receptor-G protein interface and probably occurred in cells in which all three receptors converge on overlapping populations of adenylyl cyclase. These results provide a functional link between cerebellar endocannabinoid, adenosine, and GABA neurotransmitter systems that might explain the synergistic mediation and cross-tolerance in motor incoordination.

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