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Inverse Agonist Properties of *N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl (SR141716A) and 1-(2-Chlorophenyl)-4-cyano-5-(4-methoxyphenyl)-1*H*-pyrazole-3-carboxylic Acid Phenylamide (CP-272871) for the CB_1 Cannabinoid Receptor

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ABSTRACT. Two subtypes of cannabinoid receptors are currently recognized, CB_1, found in brain and neuronal cells, and CB_2, found in spleen and immune cells. We have characterized 1-(2-chlorophenyl)-4-cyano-5-(4-methoxyphenyl)-1*H*-pyrazole-3-carboxylic acid phenylamide (CP-272871) as a novel aryl pyrazole antagonist for the CB_1 receptor. CP-272871 competed for binding of the cannabinoid agonist ^{3}H-labeled (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol ([^{3}H]CP-55940) at the CB_1 receptor in rat brain membranes with a K_d value 20-fold greater than that of N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl (SR141716A). CP-272871 also competed for binding with the aminoalkylindole agonist ^{3}H-labeled (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]1,4-benzoxazin-6-yl](1-naphthyl)methanone ([^{3}H]WIN-55212–2), as well as the aryl pyrazole antagonist [^{3}H]SR141716A. Inverse agonist as well as antagonist properties were observed for both SR141716A and CP-272871 in signal transduction assays in biological preparations in which the CB_1 receptor is endogenously expressed. SR141716A augmented secretin-stimulated cyclic AMP (cAMP) accumulation in intact N18TG2 neuroblastoma cells, and this response was reversed by the agonist desacetyllevonantradol. CP-272871 antagonized desacetyllevonantradol-mediated inhibition of adenylyl cyclase in N18TG2 membranes, and increased adenylyl cyclase activity in the absence of agonist. SR141716A and CP-272871 antagonized desacetyllevonantradol-stimulated ^{35}S-labeled guanosine-5′-*O*-(γ-thio)-triphosphate ([^{35}S]GTPγS) binding to brain membrane G-proteins, and decreased basal [^{35}S]GTPγS binding to G-proteins. K^+ enhanced CP-272871 and SR141716A inverse agonist activity compared with Na^+ or $NMDG^+$ in the assay. These results demonstrated that the aryl pyrazoles SR141716A and CP-272871 behave as antagonists and as inverse agonists in G-protein-mediated signal transduction in preparations of endogenously expressed CB_1 receptors. BIOCHEM PHARMACOL **60**;9:1315–1323, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. [^{35}S]GTPγS binding; adenylyl cyclase; desacetyllevonantradol; CP-55940; inverse agonist; brain; neuroblastoma; tonsils; antagonist; cannabinoid receptor

Cannabimimetic compounds are a class of drugs that associate with the CB_1 cannabinoid receptor in the brain, as does Δ^9-THC,‡ the active agent of marijuana. There are two known subtypes of cannabinoid receptors: CB_1, found in brain and neuronal cells [1], and CB_2, found in spleen and immune cells [2]. The CB_1 receptor mediates responses such as analgesia, catalepsy, decreased body temperature, and a decrease in locomotor activity in rodents (for review articles, see Refs. 3 and 4), whereas the physiological responses mediated by the CB_2 receptor have yet to be determined. Agonists for the CB_1 and CB_2 receptors include cannabinoid ligands that resemble Δ^9-THC, including DALN and (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol (CP-55940). A second class of cannabinoid receptor agonists are the

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‡ *Abbreviations*: cAMP, cyclic AMP; CHO, Chinese hamster ovary; DALN, desacetyllevonantradol; DTT, dithiothreitol; FAF-BSA, fatty acid free-bovine serum albumin; GTPγS, guanosine-5′-*O*-(γ-thio)-triphosphate; NMDG, *N*-methyl-D-gluconate; and Δ^9-THC, Δ^9-tetrahydrocannabinol.

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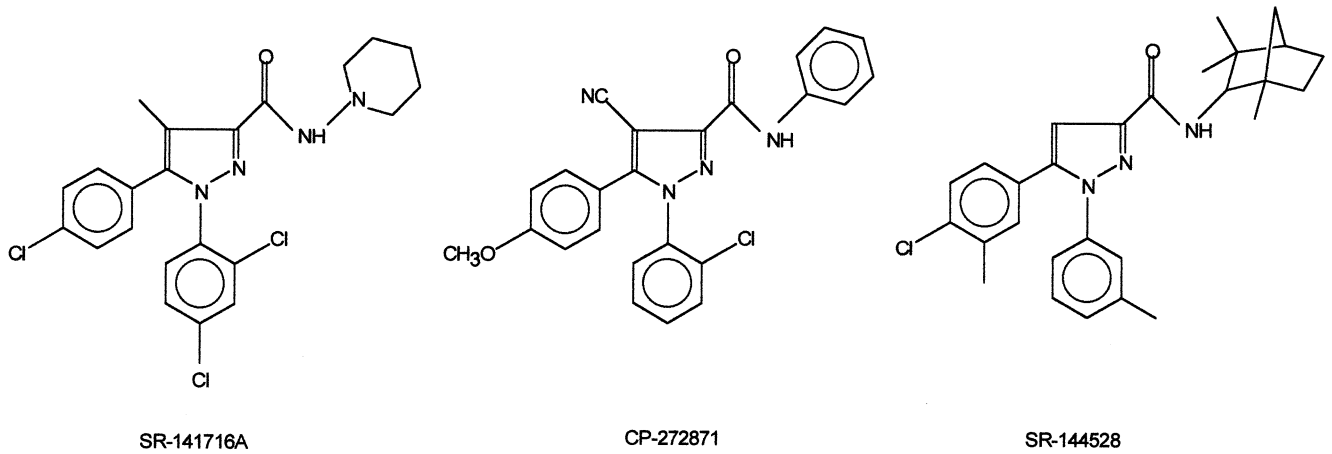

FIG. 1. Structures of SR141716A, CP-272871, and SR144528.

aminoalkylindoles, including (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]1,4-benzoxazin-6-yl](1-naphthyl)methanone (WIN-55212–2).

The CB_1 receptor is coupled to G_i-proteins, which inhibit the activity of adenylyl cyclase [5]. Association of agonists with the CB_1 receptor results in GDP release and subsequent binding of GTP, resulting in the dissociation of the α subunit from the $\beta\gamma$ dimer. The GTP-bound α subunit of G_i interacts with adenylyl cyclase, resulting in its inhibition. Hence, cannabimimetic compounds decrease cellular cAMP and shift protein kinase A target proteins to the dephosphorylated state [6]. The CB_1 receptor has also been shown to decrease Ca^{2+} currents in neurons, to activate mitogen-activated protein kinase and focal adhesion kinase, and to induce immediate early gene expression (reviewed in Ref. 4).

A high-affinity antagonist of the aryl pyrazole class, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl (SR141716A; Fig. 1), binds to the CB_1 receptor with a K_d of 1–2 nM and exhibits a 1000-fold greater affinity for the CB_1 receptor than for the CB_2 receptor [7]. *N*-[(1*S*)-Endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide (SR144528; Fig. 1) is an aryl pyrazole analog that shows selective antagonism of the CB_2 receptor [8]. Previous studies in other laboratories have described constitutive activity of the CB_1 cannabinoid receptor as indicated by the ability of SR141716A to behave as an inverse agonist in some signal transduction pathways [9, 10] (for reviews of inverse agonists, see Refs. 11–13). These studies have utilized host cells transfected with exogenous CB_1 receptors. The argument can be posed that overexpression of G-protein-coupled receptors in cells that do not normally express these receptors may introduce artifacts of stoichiometric disequilibrium between receptors and G-proteins that may give the appearance of constitutive activity of the receptor [13]. The present investigation examines a novel aryl pyrazole, 1-(2-chlorophenyl)-4-cyano-5-(4-methoxyphenyl)-1*H*-pyrazole-3-carboxylic acid phenylamide (CP-272871; Fig. 1), as a cannabinoid receptor antagonist. We showed that SR141716A and CP-272871 behave as inverse agonists in biological preparations endogenously expressing CB_1 cannabinoid receptors.

MATERIALS AND METHODS

Materials

Reagent grade chemicals obtained from the Sigma Chemical Co. were used for these studies. DALN, CP-272871, and SR141716A for these studies were gifts of Pfizer, Inc. CHO cells expressing the human CB_1 receptor (CHO-HCR) and control untransfected CHO cells were provided by Dr. M. Parmentier at the Université Libre de Bruxelles [14].

The P2 membranes from rat brains were prepared using the method of Devane *et al.* [15], and the human tonsil membranes were prepared employing the same method, except that the tissue was disrupted with a Polytron instead of a Dounce homogenizer. The protein concentrations were determined using the Bradford assay [16].

Radioligand Receptor Binding

Radioligand binding assays were performed in Regisil-treated (Regis Technologies) 96-well assay plates (Corning). Each well (200 μL final volume) contained radioligand plus TME buffer (20 mM Tris–Cl, pH 7.4, 3 mM $MgCl_2$, and 1 mM Tris–EDTA), the unlabeled ligand or vehicle, and P2 rat brain or human tonsil membrane protein (0.015 mg) suspended in TME buffer. Radioligand in ethanol was dried under N_2 gas, resuspended in 100 μL of a 50 mg/mL solution of FAF-BSA, and subsequently diluted 800-fold with TME buffer. The final concentration of [^{3}H]CP-55940 in the assay was 350 pM (the K_d of [^{3}H]CP-55940 that had been determined by Scatchard analysis for these assay conditions). [^{3}H]WIN-55212–2 and [^{3}H]SR141716A binding assays were performed at 3.6 and 4.0 nM, respectively, which is twice the K_d values reported for these ligands (WIN-55212–2 K_d = 1.9 nM for the CB_1 cannabinoid receptor in cerebellar membranes [17];

SR141716A K_d = 1.8 nM [7]). The ligands to be studied were dissolved in a solution containing 0.1 mg/mL of FAF-BSA in TME buffer. Nonspecific binding was determined using 0.1 μM DALN as the displacing ligand.

The 96-well plates were incubated at 30° for 1 hr with gentle shaking. After the incubation, 50 μL of a 50 mg/mL solution of BSA was added, and the solution was washed through a Tomtec Harvester onto a glass fiber "B" filter. The filter was dried in a microwave oven for 3 min, and Wallac Scintillation Fluid (Wallac) was added to each filter square (25 μL on both sides of the filter). The radioactivity on the filters was counted using a Wallac 1205 Betaplate (Wallac).

cAMP Studies

The adenylyl cyclase assay in N18TG2 membranes was performed according to the protocol of Howlett [18]. All drugs were added to the reaction mixture, and the reactions were initiated by the addition of N18TG2 membranes.

The cAMP accumulation in intact N18TG2 cells was determined as previously described [5], and cAMP was quantitated using the filtration method for the cAMP–protein kinase binding assay [19]. Briefly, N18TG2 or CHO-HCR cells were dislodged from the culture flask using PBS containing 0.65 mM EDTA, and preincubated for 30 min at 37° in Gey's balanced salt solution (buffered with 10 mM Na HEPES to pH 7.4) plus a phosphodiesterase inhibitor, 4-[(3-butoxy-4-methoxyphenyl)methyl]2-imidazolidinone (Ro20–1724) (0.1 mM). Secretin (30 nM) or forskolin (10 μM) plus the indicated concentrations of SR141716A were added, and cAMP accumulation was continued for 4 min at 37° before stopping the incubation.

[^{35}S] GTPγS Binding Assay

A reaction mixture containing 0.375 nM [^{35}S]GTPγS, 1 mM DTT, 10 μM GDP, and 100 mM NaCl (or KCl or $NMDG^+Cl$ as specified) in TME, the indicated concentrations of ligands, and rat brain P2 membranes (0.05 mg protein/mL) were added to each well of a Regisil-treated 96-well opaque polypropylene plate. The final volume was 100 μL. Test compounds were diluted in a 0.1 mg/mL solution of FAF-BSA in TME buffer (pH 7.4). The reaction mixture was incubated at 30° for 1 hr and filtered through a glass fiber "B" filter using the Tomtec Harvester, and radioactivity was counted using a Wallac 1205 Betaplate.

Data Analysis

Data were analyzed and curves were generated and graphed using Inplot 4 (Graph Pad). Receptor binding curves were generated from data points (means ± SEM) from three or more independent experiments. The curves, K_i values, slopes, and confidence intervals were determined for CP-272871 and SR141716A by nonlinear regression analysis using the formula for a sigmoidal plot. The adenylyl cyclase data points are the means ± SEM of three experiments. The [^{35}S]GTPγS binding assay curves were generated from data points averaged (means ± SEM) from multiple independent experiments and analyzed by nonlinear regression for a sigmoid plot. The K_{inh} for competitive antagonism for both the adenylyl cyclase and [^{35}S]GTPγS binding assay was determined using the equation

$$K_{inh} = [IC_{50}]/\{1 + ([DALN]/(K_{act} \text{ of DALN})\}$$

where DALN is the stimulating agonist.

RESULTS

CP-272871 Binding to Cannabinoid Receptors

The ability of CP-272871 to bind the CB_1 receptors in rat brain membranes was established using the CB_1 agonists [^{3}H]CP-55940 and [^{3}H]WIN-55212–2. CP-272871 displaced [^{3}H]CP-55940 with a K_i = 57 nM and [^{3}H]WIN-55212–2 with a K_i = 92 nM (Fig. 2). Heterologous competition of [^{3}H]CP-55940 demonstrated that SR141716A has a K_i = 3.1 nM. CP-272871 displaced [^{3}H]SR141716A with a K_i of 39 nM using these experimental conditions. These heterologous competition data demonstrated that CP-272871 binds to sites on the CB_1 receptor occupied by these classes of radioligands.

To define receptor subtype selectivity, the ability of CP-272871 to bind to the CB_2 cannabinoid receptor was determined. Radioligand binding assays were performed on human tonsil membranes possessing CB_2 but not CB_1 receptors [2], and employed the nonselective cannabinoid receptor agonist [^{3}H]CP-55940 (Fig. 2D). Increasing concentrations of CP-272871 were able to displace [^{3}H]CP-55940 with a K_i of 114 nM. Hence, CP-272871 had a 2-fold selectivity for binding to the CB_1 receptor in preference to the CB_2 receptor.

Inverse Agonist Properties of Aryl Pyrazole Antagonists Toward cAMP Synthesis

In intact N18TG2 cells, the ability of SR141716A to behave as a competitive antagonist for the inhibition of cAMP accumulation by DALN was confounded by the observation that SR141716A increased secretin- or forskolin-stimulated cAMP accumulation. SR141716A had little effect on basal cAMP levels in the neuronal cells. However, in the presence of secretin at its maximally active concentration, SR141716A concentration-dependently augmented the hormone-stimulated response (Fig. 3A). In data averaged from three experiments, 10 and 100 nM SR141716A increased secretin-stimulated cAMP accumulation by 0.5- and 2-fold, respectively. Figure 3B shows that the augmentation by SR141716A of the secretin-stimulated cAMP accumulation was reversed by increasing concentrations of DALN. Similar results were observed when forskolin was used to activate adenylyl cyclase. Figure 4A

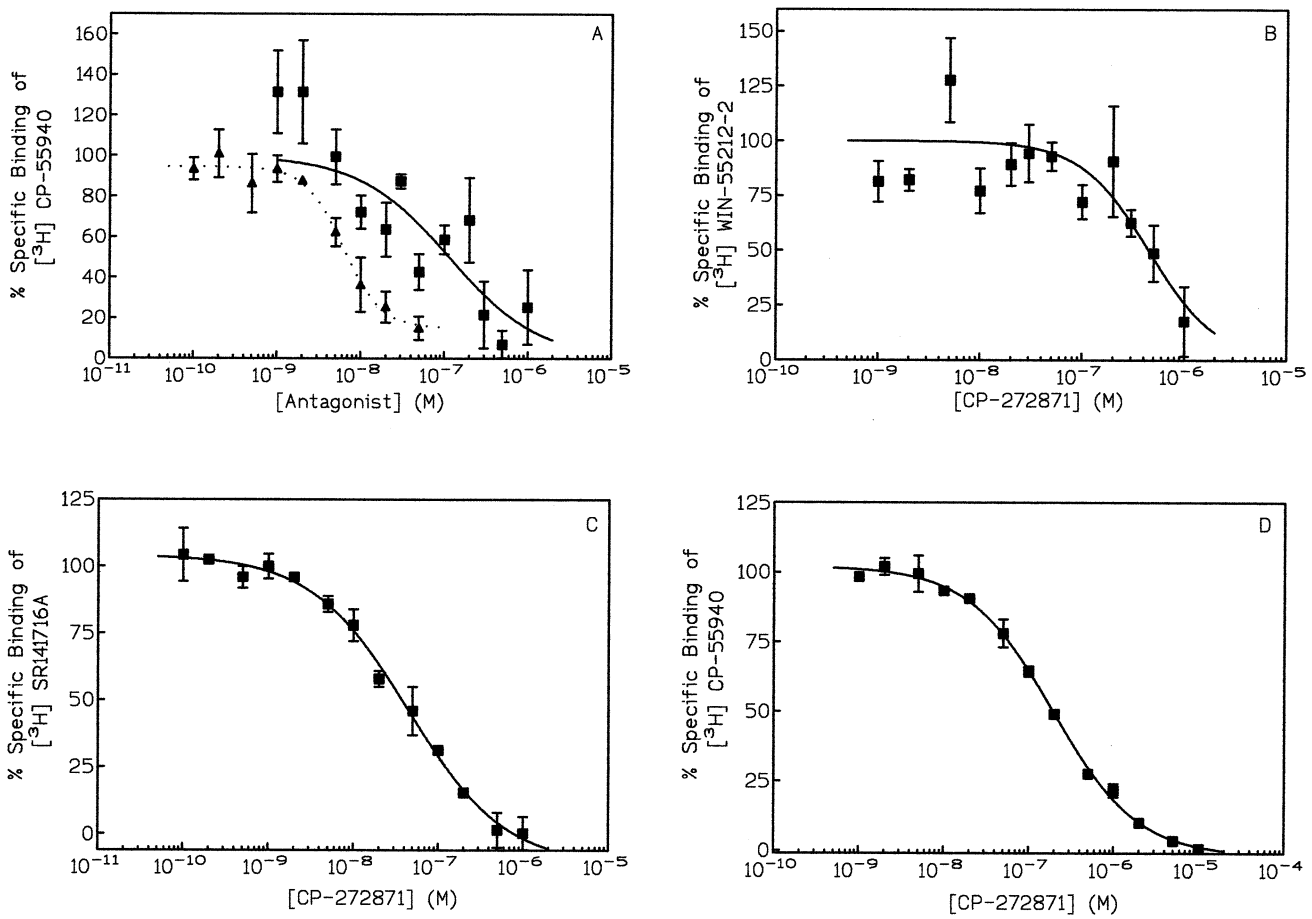

FIG. 2. Affinity of CP-272871 for CB_1 and CB_2 cannabinoid receptors. (A–C) CB_1 receptor radioligand binding: Heterologous competition with CP-272871. (A) [^{3}H]CP-55940 was the radioligand, and specific binding was defined as that which could be displaced by 100 nM DALN. The K_i for CP-272871 (■) was 57 nM (40–331 nM) and for SR141716A (▲) was 3.1 nM (2.1–4.4 nM) [mean (95% confidence interval) for N = 3 experiments]. (B) [^{3}H]WIN-55212–2 was the radioligand, and specific binding was defined as that which could be displaced by 100 nM DALN. The K_i for CP-272871 was 92 nM (48–174 nM) [mean (95% confidence interval) for N = 3 experiments]. (C) [^{3}H]SR141716A was the radioligand, and the K_i for CP-272871 was 39 nM (26–58 nM) [mean (95% confidence interval) for N = 3 experiments]. (D) Human tonsil membrane CB_2 receptor [^{3}H]CP-55940 binding: Heterologous competition with CP-272871. The K_i for CP-272871 was 114 nM (95–135 nM) [mean (95% confidence interval) for N = 3 experiments].

shows a concentration-dependent augmentation by SR141716A of forskolin-activated cAMP accumulation in N18TG2 cells. This curve was shifted to the right in the presence of 100 nM DALN, suggesting a competitive relationship between these two compounds. CHO-HCR cells expressing the human CB_1 receptor responded to the cannabinoid agonist 4-[4-(1,1-dimethylheptyl)-2-hydroxyphenyl]-6-hydroxymethyl-decahydro-napthalen-2-ol (CP-55244) with a 38% inhibition of cAMP accumulation at 10 nM. No inhibition was observed with the poorly active isomer CP-55243 at concentrations as great as 1 μM (data not shown), demonstrating the stable transfection of functional receptors. In CHO-HCR cells, SR141716A concentration-dependently increased forskolin-activated cAMP production, and this increase was reduced by DALN (Fig. 4B). SR141716A had little or no effect on basal cAMP accumulation in CHO-HCR cells (data not shown). These data are consistent with an ability of SR141716A to relieve constitutive inhibition of G_s-stimulated adenylyl cyclase activity due to CB_1 receptors endogenously expressed in N18TG2 cells or exogenously expressed in CHO-HCR host cells.

To demonstrate antagonist capabilities of CP-272871, its ability to block CB_1 receptor-mediated signal transduction was determined. CP-272871 antagonized CB_1 cannabinoid receptor-mediated adenylyl cyclase inhibition in N18TG2 membranes (Fig. 5). CP-272871 antagonized the response to the cannabinoid agonist DALN (1 μM) with a calculated K_{inh} = 69 nM. Hence, this compound has the ability to antagonize the effector response in the CB_1 cannabinoid receptor-mediated signal transduction pathway. CP-272871 (10 μM) administered alone produced a 20% increase in secretin-stimulated adenylyl cyclase activity. Thus, CP-272871 exhibited inverse agonist properties in the adenylyl cyclase assay in N18TG2 membranes possessing native CB_1 receptors.

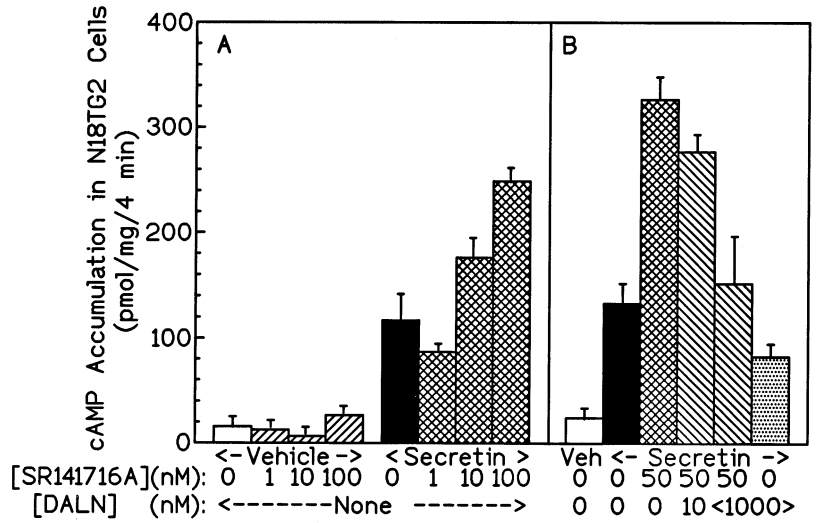

FIG. 3. Inverse agonist activity of SR141716A in hormone-stimulated cAMP accumulation in N18TG2 neuroblastoma cells. (A) Augmentation of the hormone-stimulated cAMP accumulation by SR141716A. N18TG2 cells were incubated with vehicle or secretin plus the indicated concentrations of SR141716A. The data bars are the means ± SEM of triplicate determinations, and this experiment is representative of three similar experiments. (B) Reversal by DALN of augmentation of cAMP accumulation by SR141716A. N18TG2 cells were tested with vehicle or secretin plus combinations of SR141716A and DALN at the indicated concentrations in the incubation. This experiment is representative of three experiments showing similar results.

Inverse Agonist Properties of Aryl Pyrazole Antagonists Toward G-Protein Activation

[^{35}S]GTPγS binding data were obtained to determine the ability of CP-272871 to antagonize CB_1 agonist-stimulated activation of G-proteins. CP-272871 antagonized DALN (1 μM)-stimulated [^{35}S]GTPγS binding with a calculated K_{inh} = 76 nM (Fig. 6A). The mechanism of antagonism for CP-272871 was determined using CB_1 agonist-stimulated [^{35}S]GTPγS binding curves generated in the presence of vehicle and CP-272871. DALN curves with CP-272871 showed a significant decrement in the maximum and minimum responses and a shift in their apparent K_{act} to the right compared with vehicle controls (Fig. 6B). Because basal activity as well as both the K_{act} and the maximum response of DALN-stimulated [^{35}S]GTPγS binding were affected by CP-272871, this compound exhibits complex antagonism with inverse agonist properties.

SR141716A shifted CB_1 agonist-stimulated curves to the right (Fig. 6C). Furthermore, SR141716A appeared to lower basal levels and decreased the maximum response; however, the changes in basal levels and maximum response were not significant. Hence, SR141716A did not seem to exhibit the same degree of complex antagonism as CP-272871 in the [^{35}S]GTPγS binding assay. Furthermore, SR141716A was less effective as an inverse agonist than CP-272871 under these assay conditions.

[^{35}S]GTPγS binding data obtained in studies of the μ-opioid receptor have demonstrated that different ionic conditions can affect both agonist and inverse agonist activity [20]. A theoretical discussion by Tian and Deth [21] describes the allosteric regulation of receptor–G-protein ternary complex by monovalent cations. In particular, increasing concentrations of Na^+ favor the uncoupled state of the receptor to the G-protein such that agonist activation appears to be augmented. Increasing concentrations of K^+ favor the precoupled state of the receptor and G-protein such that inverse agonist activity appears to be augmented. An ion such as $NMDG^+$ does not appear to enhance agonist or inverse agonist activity [21], and thus can be used to maintain ionic strength of the solution. We investigated the inverse agonist properties of CP-272871 and SR141716A in a rat brain membrane preparation possessing native CB_1 cannabinoid receptors. The inverse agonist properties of CP-272871 and SR141716A in the [^{35}S]GTPγS binding assay were characterized under different ionic conditions, employing Cl^- salts of either Na^+, K^+, or $NMDG^+$ (Fig. 7). Na^+ appeared to lower basal activity, thereby enhancing the agonist activity of DALN relative to basal (Fig. 7, panel A compared with panel C). K^+ increased basal activity and allowed the inverse agonist activity of CP-272871 and SR141716A to be revealed (Fig. 7, panel B compared with panel C). Na^+ and $NMDG^+$ failed to support inverse agonist activity (Fig. 7, A and C).

DISCUSSION

This study has introduced CP-272871 as a CB_1 cannabinoid receptor antagonist. CP-272871 exhibited a lower affinity for the CB_2 cannabinoid receptor, with relative selectivity for the CB_1 receptor of 2-fold. CP-272871 also exhibited competitive inhibitory properties against the cannabinoid agonist DALN in activation of G-proteins ([^{35}S]GTPγS binding) and the adenylyl cyclase signal transduction pathway.

The present study describes inverse agonist properties of the aryl pyrazole antagonists SR141716A and CP-272871

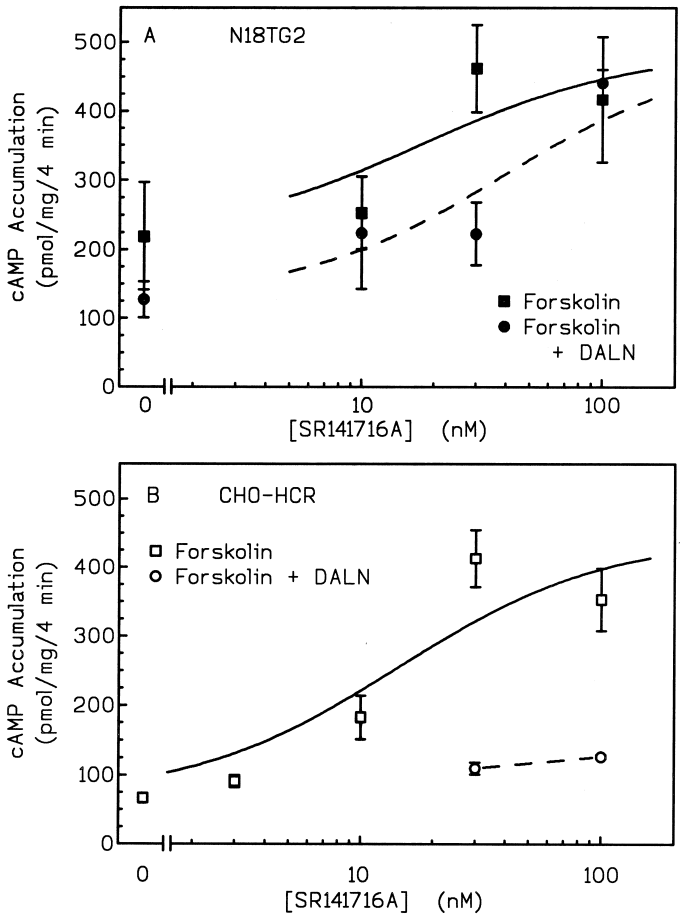

FIG. 4. Inverse agonist activity of SR141716A in forskolin-activated cAMP accumulation. (A) cAMP accumulation was tested in N18TG2 neuroblastoma cells with 10 μM forskolin (■) or forskolin plus 1 μM DALN (λ) at the indicated concentrations of SR141716A. (B) CHO-HCR cells were tested for cAMP accumulation with 10 μM forskolin (□) or forskolin plus 1 μM DALN (○) at the indicated concentrations of SR141716A. Data are the means ± SEM of triplicate determinations, and these experiments are representative of two similar experiments.

in the lowering of basal [^{35}S]GTPγS binding in the presence of K^+ and the increase in adenylyl cyclase activity. This activity defines CB_1 receptors as constitutively active

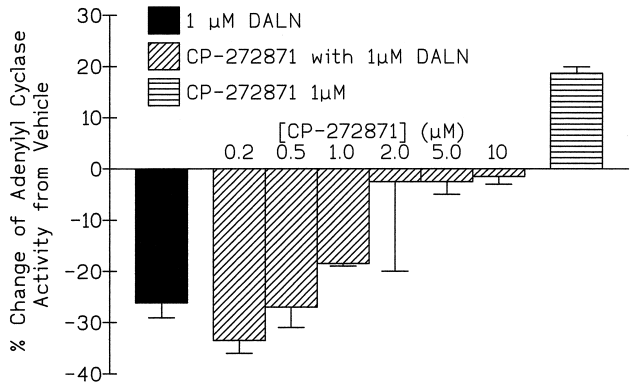

FIG. 5. Effect of CP-272871 on forskolin-stimulated (10 μM) adenylyl cyclase activity in N18TG2 membranes. DALN (1 μM) was used to inhibit the enzyme, and the effects of CP-272871 at the indicated concentrations on this inhibition were determined. For CP-272871, the calculated K_{inh} = 69 nM. Data are the means ± SEM of three independent experiments.

in a neuronal cell model in which CB_1 cannabinoid receptors are expressed endogenously. The inverse agonist properties of SR141716A have been characterized previously in a transfected cell system [9], in which SR141716A administered alone increased cAMP-mediated effects and decreased mitogen-activated protein kinase activity and immediate early gene expression. Another study has described inverse agonist activity of SR141716A on [^{35}S]GTPγS binding activity in CHO cells expressing CB_1 receptors [10]. Overexpression of receptors, which would increase the probability of precoupled receptor–G-protein complexes, is one explanation for constitutive activity of those receptors in transfected cell systems [22]. However, our studies showed that constitutive activity is demonstrable in neuronal cells which express CB_1 receptors endogenously and the G-proteins that transduce their responses. Inverse agonist activity would suggest that the cannabinoid receptor is precoupled to the G-protein in the absence of agonist. This is consistent with findings employing detergent-solubilized CB_1 cannabinoid receptor, in which G-proteins of the $G_{i/o}$

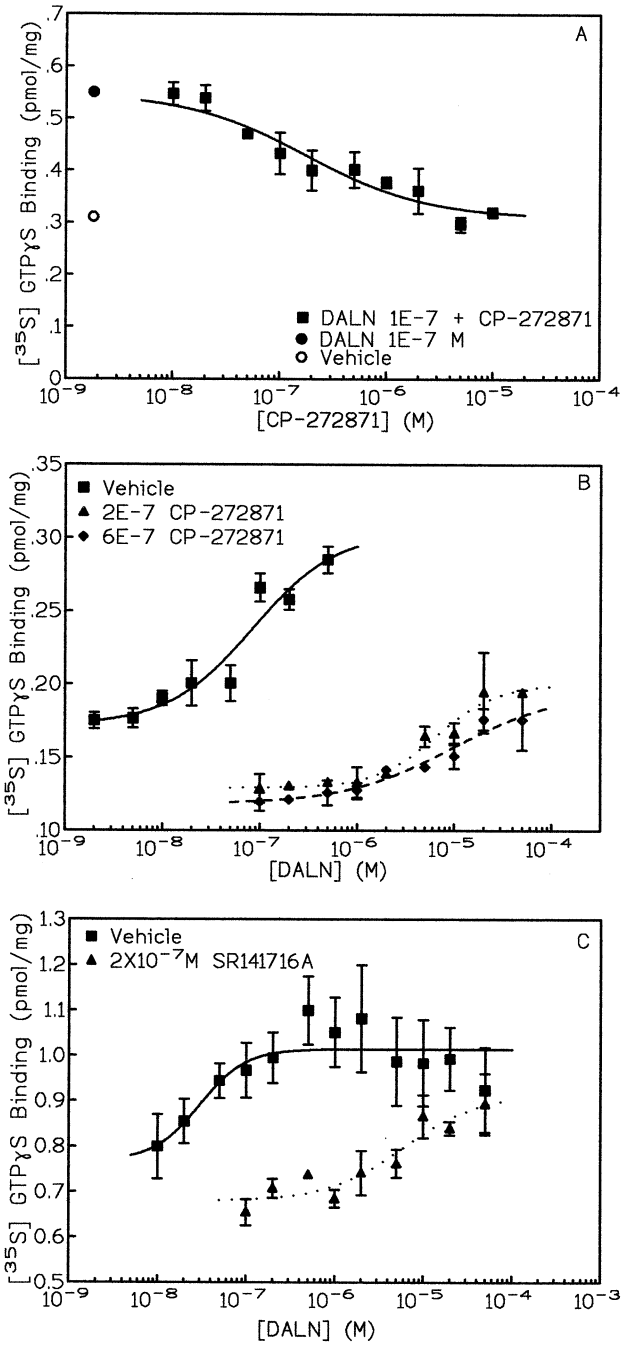

FIG. 6. Effect of DALN and CP-272871 on G-protein activation as determined by [^{35}S]GTPγS binding. (A) Antagonism of the response to DALN by CP-272871. Data are shown for vehicle (○), 100 nM DALN (●), and DALN plus CP-272871 at the indicated concentrations (■). The K_{inh} calculated for CP-272871 was 76 nM (56–614 nM) (95% confidence interval). Data are the means ± SEM of triplicate determinations, and these experiments are representative of two similar experiments. (B) DALN log concentration–response curves generated in the presence of vehicle (■), 200 nM CP-272871 (▲), and 600 nM CP-272871 (◆). For vehicle control, the K_{act} was 749 nM (134 nM–4.2 μM), the maximum response was 0.30 ± 0.04 pmol/mg, and the minimum response was 0.17 ± 0.01 pmol/mg. In the presence of 200 nM CP-272871, the K_{act} was 6.5 μM (3–14 μM), the maximum response was 0.20 ± 0.01 pmol/mg, and the minimum response was 0.13 ± 0.004 pmol/mg. In the presence of 600 nM CP-272871, the K_{act} was 10 μM (0.5–182 μM), the maximum response was 0.20 ± 0.03 pmol/mg, and the minimum response was 0.12 ± 0.01 pmol/mg. Data are the means ± SEM of triplicate determinations, and these experiments are representative of three similar experiments. (C) DALN log concentration–response curves generated in the presence of vehicle (■) and 200 nM SR141716A (▲). For vehicle control, the K_{act} was 30 nM (2.1–424 nM), the maximum response was 1.01 ± 0.02 pmol/mg, and the minimum response was 0.777 ± 0.16 pmol/mg. In the presence of 200 nM CP-272871, the $K_{act\ app}$ was 6.0 μM (1.9–19 μM), the maximum response was 0.916 ± 0.04 pmol/mg, and the minimum response was 0.679 pmol/mg. Data are the means ± SEM of three independent experiments.

family remain associated with the CB_1 receptor in the absence of cannabinoid agonists [23, *].

A less likely alternative explanation for the effects observed for CP-272871 and SR141716A is that these antagonists are blocking the response(s) to endogenous ligand(s) for the cannabinoid receptor [22]. Endogenous CB_1 cannabinoid receptor ligands that may be present in brain preparations include anandamide [24] or 2-arachidonylglycerol [25]. N18TG2 cells possess enzymes capable of the production of each of these endogenous ligands [26, 27]; however, N18TG2 cells are also capable of rapidly metabolizing these ligands [28, 29]. Addition of antagonists in the presence of these endogenous contaminants could reverse their effects, leading to a decrease in [^{35}S]GTPγS binding and an increase in adenylyl cyclase activity. The membrane preparations were washed twice in an effort to remove any potential endogenous ligands, although given the hydrophobic nature of these substances, it is not clear how effective these washes may be. More importantly, the effects of K^+ on the activity of these compounds suggest that they are not merely antagonizing endogenous substances. If these compounds were antagonizing endogenous substances, then the presence of K^+ would not enhance their activity when compared with vehicle controls. Thus, we believe that this alternative explanation is unlikely.

Both SR141716A and CP-272871 exhibit an ability to antagonize DALN-stimulated [^{35}S]GTPγS binding by increasing the apparent K_{act} value for agonist in the manner of a competitive antagonist and reducing the basal activity in the manner of an inverse agonist. However, CP-272871 but not SR141716A reduced the maximal activity in the manner of a noncompetitive antagonist, and this response was not surmountable by increasing concentrations of DALN. One possible explanation is that the concentration of agonist needed to restore maximal activity in the presence of CP-272871 is beyond the water solubility of the ligand, and the excess of lipophilic compound present in the assay may cause membrane fluidity effects leading to an artifactual decrease in [^{35}S]GTPγS binding. However, this is not a likely explanation because we have found that the

* Mukhopadhyay S and Howlett AC, Manuscript submitted for publication.

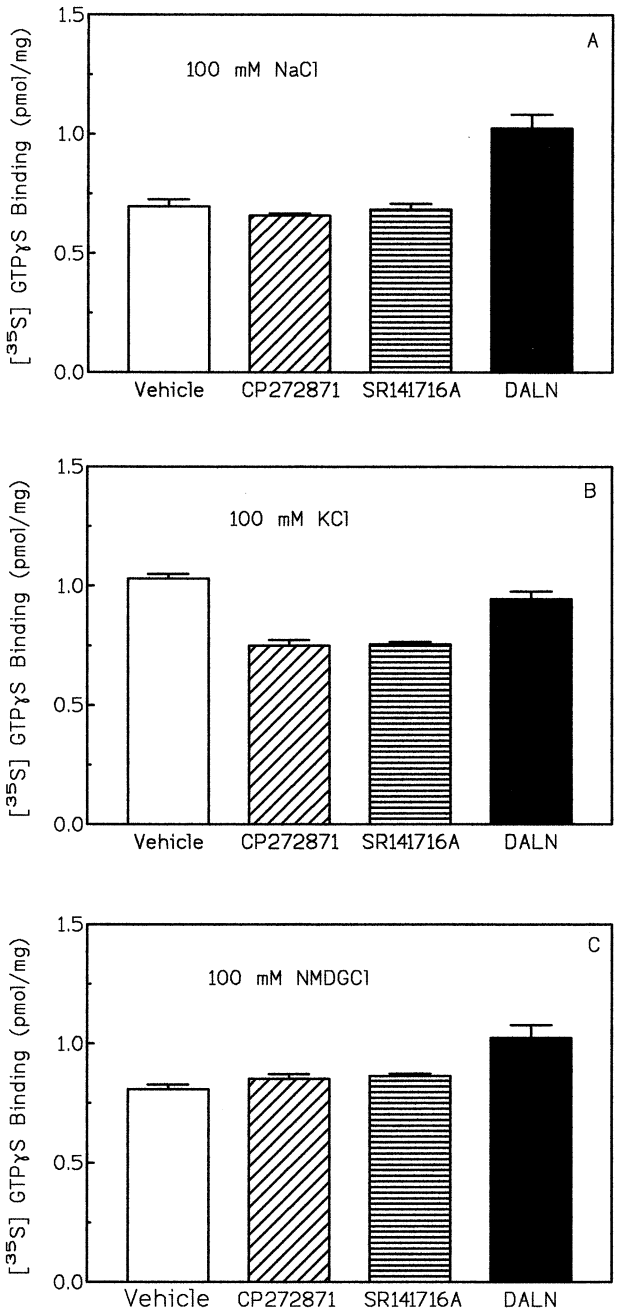

FIG. 7. Inverse agonist properties of CP-272871 (1 μM) and SR141716A (1 μM) and agonist properties of DALN (1 μM) in the presence of (A) 100 mM Na^+, (B) 100 mM K^+, and (C) 100 mM $NMDG^+$. Data are the means ± SEM of triplicate determinations, and these experiments are representative of three similar experiments.

same concentration of agonist was able to restore maximal [^{35}S]GTPγS binding in response to SR141716A. Another potential explanation is that CP-272871 is binding to the receptor irreversibly, causing a drop in the number of functional CB_1 receptors. However, preincubation of membranes with CP-272871 followed by washing did not preclude [^{3}H]CP-55940 radioligand binding appreciably (data not shown). This suggests that CP-272871 is not binding irreversibly to the receptor. Thus, at the present time, we are not able to explain the apparent noncompetitive component of the antagonism by CP-272871.

In summary, aryl pyrazoles CP-272871 and SR141716A competitively antagonize CB_1 cannabinoid agonist-mediated responses and act as inverse agonists at the CB_1 cannabinoid receptors in N18TG2 cells and in rat brain. The complex antagonism exhibited by CP-272871 suggests that this compound may be exhibiting some additional influence on [^{35}S]GTPγS binding activity that is manifested as noncompetitive antagonism. The therapeutic potential for a cannabinoid inverse agonist depends largely on whether constitutive activity exists endogenously. This report thus discloses constitutive activity of CB_1 receptors that are endogenously expressed along with their associated G-proteins and suggests that cannabinoid inverse agonists may be therapeutically useful by modulating activity of the CB_1 receptor system in the brain.

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References

1. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR and Rice KC, Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* **87:** 1932–1936, 1990.
2. Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G and Casellas P, Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* **232:** 54–61, 1995.
3. Howlett AC, Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol* **35:** 607–634, 1995.
4. Pertwee RG, Pharmacology of cannabinoid receptor ligands. *Curr Med Chem* **6:** 635–664, 1999.
5. Howlett AC, Qualy JM and Khachatrian LL, Involvement of G_i in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol Pharmacol* **29:** 307–313, 1986.
6. Childers SR and Deadwyler SA, Role of cyclic AMP in the actions of cannabinoid receptors. *Biochem Pharmacol* **52:** 819–827, 1996.
7. Rinaldi-Carmona M, Barth F, Héaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Néliat G, Caput D, Ferrara P, Soubrié P, Brelière JC and Le Fur G, SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350:** 240–244, 1994.
8. Rinaldi-Carmona M, Barth F, Millan J, Derocq J-M, Casellas P, Congy C, Oustric D, Sarran M, Bouaboula M, Calandra B, Portier M, Shire D, Breliere J-C and Le Fur GL, SR144528, the first potent and selective antagonist of the CB_2 cannabinoid receptor. *J Pharmacol Exp Ther* **284:** 644–650, 1998.
9. Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandras B, Pecceu F, Lupker J, Maffrand J-P, Le Fur G and Casellas P, A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:** 22330–22339, 1997.

10. Landsman RS, Burkey TH, Consroe P, Roeske WR and Yamamura HI, SR141716A is an inverse agonist at the human cannabinoid CB_1 receptor. *Eur J Pharmacol* **334:** R1–R2, 1997.
11. Schutz W and Freissmuth M, Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol Sci* **13:** 376–380, 1992.
12. Kenakin T, Pharmacological proteus? *Trends Pharmacol Sci* **16,** 256–258, 1995.
13. Milligan G, Bond RA and Lee M, Inverse agonism: Pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol Sci* **16:** 10–13, 1995.
14. Gerard CM, Mollereau C, Vassart G and Parmentier M, Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* **279:** 129–134, 1991.
15. Devane WA, Dysarz FA, Johnson MR, Melvin LS and Howlett AC, Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34:** 605–613, 1988.
16. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
17. Kuster JE, Stevenson JI, Ward SJ, D'Ambra TE and Haycock DA, Aminoalkylindole binding in rat cerebellum: Selective displacement by natural and synthetic cannabinoids. *J Pharmacol Exp Ther* **264:** 1352–1363, 1993.
18. Howlett AC, Cannabinoid inhibition of adenylate cyclase. Biochemistry of the response in neuroblastoma cell membranes. *Mol Pharmacol* **27:** 429–436, 1985.
19. Tao Y-P, Najafi L, Shipley S, Howlett A and Klein C, Effects of nitric oxide on adenylyl cyclase stimulation in N18TG2 neuroblastoma cells. *J Pharmacol Exp Ther* **286:** 298–304, 1998.
20. Costa T, Ogino Y, Munson PJ, Onaran HO and Rodbard D, Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: Thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol Pharmacol* **41:** 549–560, 1991.
21. Tian W-N and Deth RC, Precoupling of G_i/G_o-linked receptors and its allosteric regulation by monovalent cations. *Life Sci* **52:** 1899–1907, 1993.
22. Baxter GS and Tilford NS, Endogenous ligands and inverse agonism. *Trends Pharmacol Sci* **16:** 258–259, 1995.
23. Mukhopadhyay S, McIntosh HH, Houston DB and Howlett AC, The CB_1 cannabinoid receptor juxtamembrane C-terminal peptide confers activation to specific G proteins in brain. *Mol Pharmacol* **57:** 162–170, 2000.
24. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R, Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258:** 1946–1949, 1992.
25. Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A and Waku K, 2-Arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* **215:** 89–97, 1995.
26. Di Marzo V, De Petrocellis L, Sepe N and Buono A, Biosynthesis of anandamide and related acylethanolamides in mouse J774 macrophages and N18 neuroblastoma cells. *Biochem J* **316:** 977–984, 1996.
27. Di Marzo V, De Petrocellis L, Sugiura T and Waku K, Potential biosynthetic connections between the two cannabimimetic eicosanoids, anandamide and 2-arachidonoyl-glycerol, in mouse neuroblastoma cells. *Biochem Biophys Res Commun* **227:** 281–288, 1996.
28. Deutsch DG and Chin SA, Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol* **46:** 791–796, 1993.
29. Maurelli S, Bisogno T, De Petrocellis L, Di Luccia A, Marino G and Di Marzo V, Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. *FEBS Lett* **377:** 82–86, 1995.