Sphingosine and Its Analog, the Immunosuppressant 2-Amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol, Interact with the CB₁ Cannabinoid Receptor

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ABSTRACT

Sphingosine-1-phosphate (S1P) and cannabinoid receptors are G-protein-coupled receptors that mediate the effects of S1P and endocannabinoids, respectively. Cannabinoid receptors also mediate the effects of Δ⁹-tetrahydrocannabinol, the primary psychoactive ingredient in marijuana, whereas S1P receptors contribute to the immunosuppressant effects of 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol (FTY720). FTY720 is a sphingosine analog that can prevent renal graft rejections and suppress a variety of autoimmune disorders in animal models and clinical trials. We now report that both FTY720 and sphingosine interact with CB₁ receptors, but not CB₂ cannabinoid receptors. FTY720 and sphingosine inhibited the binding of the CB₁-selective antagonist [³H]N-(piperidinyl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide ([³H]SR141716A) and the cannabinoid agonist [³H](—)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-[3-hydroxypropyl]cyclohexanol ([³H]CP55,940) in a concentration-dependent manner in both CB₁-expressing cell lines and mouse cerebellum. However, these compounds did not significantly alter [³H]CP55,940 binding to CB₂ receptors. In G-protein activation assays, FTY720 and sphingosine inhibited the maximal stimulation of guanosine 5’-O-(3-[³²P]thio)triphosphate binding by the cannabinoid agonist R-(+)-[2,3-dihydro-5-methyl-3-[[morpholino]methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN55,212-2) in a concentration-dependent manner, and this antagonist effect was not mimicked by S1P. FTY720 and sphingosine also inhibited activation of extracellular signal-regulated kinases 1 and 2 and Akt by WIN55,212-2 in intact Chinese hamster ovary (CHO) cells expressing CB₁ receptors and attenuated WIN55,212-2-stimulated internalization of a fluorescence-tagged CB₁ receptor in CHO cells. Moreover, both FTY720 and sphingosine produced rightward shifts in the concentration-effect curves of cannabinoid agonists for G-protein activation, indicating that they act as competitive CB₁ antagonists. These results suggest that the CB₁ receptor could be a novel target of FTY720 and that sphingosine could be an endogenous CB₁ antagonist.

Sphingosine-1-phosphate (S1P) and cannabinoid receptors belong to the lysolipid family of GPCRs (Toman and Spiegel, 2002). Subtypes for each receptor, S1P₁–5 and CB₁ and CB₂, have been identified and exhibit unique tissue distributions and functional profiles. There are a number of similarities between S1P and cannabinoid receptors with a notably significant sequence homology of approximately 35% (Toman and Spiegel, 2002). Subtypes for each receptor, S1P₁–5 and CB₁ and CB₂, have been identified and exhibit unique tissue distributions and functional profiles. There are a number of similarities between S1P and cannabinoid receptors with a notably significant sequence homology of approximately 35% (Toman and Spiegel, 2002). S1P and cannabinoid receptors are acti-
vated by the endogenous lipid mediators S1P, and anandamide and 2-arachidonylglycerol, respectively (Howlett et al., 2002; Toman and Spiegel, 2002). Agonist binding activates G-proteins, primarily of the G\textsubscript{i/o} family, for cannabinoid and S1P\textsubscript{1–5} receptors and of the G\textsubscript{12/13} or G\textsubscript{q/11} families for S1P\textsubscript{2–5} receptors (Spiegel and Milstien, 2003). The cannabinoid receptors have long been of interest because they mediate the effects of \textDelta^9\text{-tetrahydrocannabinol}, the active component of marijuana. CB\textsubscript{2} receptors are located primarily in the immune system, whereas CB\textsubscript{1} receptors are abundant in the CNS and are present in several peripheral organs (Howlett et al., 2002). CB\textsubscript{1} and S1P receptors stimulate G-protein activity in the CNS and are codistributed in many regions (Sim et al., 1995; Waeber and Chiu, 1999). Interest in the S1P receptors has increased because of the therapeutic potential of the immunomodulatory drug FTY720, which targets all of these receptors, except S1P\textsubscript{2}. Because of the similarities between S1P and cannabinoid receptors, it is possible that some ligands might bind to both receptors.

S1P can be produced by sphingosine kinase-mediated phosphorylation of sphingosine and inactivated by S1P phosphatases or degraded by S1P lyase (Spiegel and Milstien, 2003). FTY720 can also be phosphorylated by sphingosine kinase type 2 to produce the active compound phospho-FTY720, which can bind to S1P\textsubscript{1,3,4,5} Receptors (Paugh et al., 2003; Allende et al., 2004). Immunomodulation through FTY720 is believed to be mediated by the induction of lymphopenia in blood and the thoracic duct via sequestration of lymphocytes from circulation to secondary lymphoid organs, away from inflamed peripheral tissues and graft sites (Brinkmann and Lynch, 2002; Mandala et al., 2002). A phase 2 clinical trial of FTY720 indicates that it can prevent kidney transplantation rejection (Tedesco-Silva et al., 2005). FTY720 administration can also prevent the development of experimental autoimmune encephalitis, an animal model of multiple sclerosis (Fujino et al., 2003), and can reduce symptoms in a long-term model of experimental autoimmune encephalitis (Webb et al., 2004). Preclinical studies have reported that FTY720 can prevent the development of several autoimmune diseases, including type 1 diabetes (Maki et al., 2005), adjuvant-induced arthritis (Matsuura et al., 2000), myocarditis (Kitabayashi et al., 2000), uveoretinitis (Kurose et al., 2000), systemic lupus erythematosus (Okazaki et al., 2002), and colitis (Mizushima et al., 2004).

Because of the sequence similarities of S1P and cannabinoid receptors, the structural resemblance of FTY720 to cannabinoid receptor ligands (Fig. 1), and the potential effectiveness of FTY720 in treating inflammatory disorders of the CNS, we investigated the possibility that FTY720 might interact with cannabinoid receptors. Sphingosine was also investigated in these studies because its structure is similar to FTY720 (Fig. 1) and the possibility that this endogenous lipid, which is abundant in the CNS and distinct from the endocannabinoids, might affect cannabinoid receptor function.

**Materials and Methods**

**Materials**

Sphingosine was purchased from Avanti Polar Lipids (Birmingham, AL). S1P was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). FTY720 was purchased from Cayman Chemical Co. (Ann Arbor, MI). WIN55,212-2, GTP\gamma\text{S}, GDP, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). CP55,940 and SR141716A were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). All drugs were originally dissolved at a concentration of 5 to 10 mM in 95% ethanol and then diluted to the appropriate stock concentrations in the relevant buffer before assay. \[^{35}\text{S}\text{GTP}\gamma\text{S}\] (1150–1300 Ci/mmol), \[^{3}\text{H}\text{sphingosine (5 \mu Ci/mmol)}\] and \[^{3}\text{H}\text{CP55,940 (158 Ci/mmol)}\] were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). \[^{3}\text{H}\text{SR141716A (44.0 Ci/mmol)}\] was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). ICR mice (male, 24–30 g) were obtained from Harlan (Indianapolis, IN). Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-12 medium, penicillin/streptomycin (Pen-Strep), hygromycin-B, G418 (Geneticin), fetal bovine serum (FBS), and Lipofectamine reagent were purchased from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence reagent was purchased from Pierce Biotechnology (Rockford, IL). Anti-pAkt, anti-pERK1/2, anti-Akt, and anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other reagent-grade chemicals were purchased from Sigma Chemical Co. or Fisher Scientific (Pittsburgh, PA). The cDNA construct encoding the green protein p65.
fluorescent protein (GFP)-fused rat CB1 receptor was obtained from Dr. Zsolt Lenkei (The City of Paris Industrial Physics and Chemistry Higher Educational Institution, Paris, France). Cell lines stably expressing mouse or human CB1, or CB2, receptors were obtained from Dr. Mary E. Abood (California Pacific Medical Center, San Francisco, CA).

Cell Culture

Chinese hamster ovary (CHO) K1 cells (CRL-1573; American Type Culture Collection, Manassas, VA) and CHO-K1 cells stably transfected with Flag-tagged mouse CB1 receptor (mCB1-CHO) or the human CB1 receptor (hCB1-CHO) were cultured in media consisting of 50% high-glucose DMEM containing 2 mM l-glutamine and 50% Ham’s F-12 supplemented with 5% heat-inactivated FBS, 1% Pen-Strep, and 0.25 mg/ml hygromycin B. Human embryonic kidney (HEK)-293 cells stably transfected with the human CB1 receptor (hCB1-HEK) were cultured in DMEM containing 10% FBS, 1% Pen-Strep, and 0.25 mg/ml G418. HEK-293 cells transiently expressing sphingosine kinase 1 were cultured in DMEM containing 10% FBS and 1% Pen-Strep.

Membrane Preparation

Cultured Cell Lines. Cells were harvested by replacement of the media with phosphate-buffered saline containing 0.4% EDTA and gentle agitation. The cells were then subject to centrifugation at 500 g, resuspended in 20 volumes of ice-cold buffer A (50 mM Tris-HCl, 3 mM MgCl₂, and 1 mM EGTA, pH 7.4), and homogenized. All membrane pellets were resuspended in buffer A, centrifuged at 48,000 g, and resuspended in buffer B (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4) and stored in aliquots at −80°C.

Cerebellum. Mice were killed by decapitation, and cerebella were dissected on ice and placed in 20 volumes of cold buffer A. Cerebella were homogenized and centrifuged at 48,000 g at 4°C for 10 min, and membranes were prepared for storage as above.

Receptor Binding Assays

Membrane homogenates were thawed and homogenized in buffer B and then assayed for protein content (Bradford, 1976). Membranes (10–30 µg) were incubated for 90 min at 30°C in buffer B with 0.5% BSA, 0.6 nM [3H]SR141716A, or in buffer B without NaCl with 1 nM [3H]CP55,940 and varying concentrations of competing ligands or ethanol vehicle in a 0.5 ml total volume. Nonspecific binding was determined in the presence of 20 µM unlabeled SR141716A in [3H]SR141716A assays or 5 µM WIN55,212-2 in [3H]CP55,940 assays. Incubations were terminated by vacuum filtration through Whatman GF/B glass fiber filters (Whatman, Clifton, NJ), followed by three washes with ice-cold 50 mM Tris-HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation counting at 45% efficiency for [3H] after extraction of the filters in scintillation fluid.

Agonist-Stimulated [35S]GTPγS Binding

Membranes were recovered in buffer B as described above and preincubated for 10 min at 30°C with adenosine deaminase (4 µU/ml) to remove endogenous adenosine (cerebellum only). Samples containing 10 µg of membrane protein were incubated for 90 min at 30°C in buffer B containing 10 µM GDP, 0.1 nM [35S]GTPγS, 0.5% BSA, and the indicated concentrations of agonists and/or antagonists. Nonspecific binding was determined in the presence of 20 µM unlabeled GTPγS. Reactions were terminated by rapid vacuum filtration through GF/B glass fiber filters, and radioactivity was measured by liquid scintillation counting at 95% efficiency for [35S] after extraction of the filters in scintillation fluid.

Sphingosine Kinase Assay

[3H]Sphingosine phosphorylation by membrane preparations from mCB1-CHO cells, mouse cerebellum or cell lysates from HEK-293 cells transfected with human sphingosine kinase 1 was measured as described previously (Paugh et al., 2003), using 100 µM [3H]sphingosine in the presence and absence of 1 mM ATP.

Western Immunoblotting

Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and then transblotted to nitrocellulose, as described previously (Paugh et al., 2003). Blots were probed with anti-pAkt, anti-p-ERK1/2, anti-Akt, and anti-ERK2 (1:1000; Santa Cruz Biotechnology) followed by anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (1:10,000; Immunoresearch Laboratories). Immunocomplexes were visualized by enhanced chemiluminescence (Pierce). Images were captured using the Alpha Innotech (San Leandro, CA) FluorChem SP. Densitometric analysis was performed using Alpha Innotech AlphaEaseFC version 4.0.0 software. The ratio of phosphoprotein to total protein in the presence of WIN55,212-2 alone was set at 1, and all values were expressed relative to that value.

Immunofluorescence and Confocal Microscopy

CHO-K1 cells transfected with a construct containing the CB1 receptor fused with GFP (CB1-GFP) were seeded on glass coverslips. Cells were serum-starved for 3 h and then treated as described in figure legends. Cells were then washed with phosphate-buffered saline, fixed in 3% formaldehyde for 10 min at room temperature, and visualized by confocal fluorescence microscopy (LSM model 510; Carl Zeiss Microimaging, Inc., Thornwood, NJ) with a 60× oil-immersion objective lens. At least 20 cells were examined in each experiment.

Data Analysis

All binding assays were performed in triplicate and replicated at least three times. All binding data are reported as specific binding. For [35S]GTPγS binding, basal binding is defined as specific [35S]GTPγS binding in the absence of drug. Net-stimulated [35S]GTPγS binding is defined as [35S]GTPγS binding in the presence of drug minus basal. The percentage stimulation is expressed as (net stimulated [35S]GTPγS binding/basal) × 100%. Emax or EC50 values were calculated from nonlinear regression analysis by iterative fitting of the concentration-effect curves to the Langmuir equation: Emax/[EC50 + agonist concentration] × agonist concentration) using JMP (SAS for Macintosh; SAS Institute, Cary, NC). Apparent pA2 values of antagonists were determined by the following equation: −log[agonist concentration/(DR − 1)], where DR is the agonist EC50 value with antagonist present/agonist EC50 value in the absence of antagonist. Competitor IC50 values were calculated according to the Hill equation B/Boff = [C]/[C]1/EC50 + IC50, and pK values were calculated using the Cheng-Prusoff equation, pK = −log(IC50/[1 + [L]/Kp]), where [L] is concentration of radiolabeled ligand, and Kp is the Kd value of the radiolabeled ligand. Statistically significant differences were determined by the two-tailed Student’s t test with Bonferroni adjustment for multiple comparisons or by analysis of variance with Tukey-Kramer post hoc test, where indicated. All inferential statistical analyses were performed using JMP.

Results

FTY720 and Sphingosine Inhibit Binding of the Antagonist [3H]SR141716A to the CB1 Receptor. To determine whether FTY720 or sphingosine interact with the CB1
FTY720 and Sphingosine Inhibit Binding of the Agonist [3H]CP55,940 to the CB1 Receptor. The finding that FTY720 and sphingosine inhibited [3H]SR141716A binding to mCB1-CHO cell membranes indicates that they compete for antagonist binding to CB1 receptors. To determine whether they also inhibit agonist binding, competition experiments were performed in this cell line with the cannabinoid agonist [3H]CP55,940 (Fig. 2B). As expected, WIN55,212-2 inhibited [3H]CP55,940 binding with a Ki value of 12.5 nM (pKi = 7.96) and a Hill coefficient that was not different from 1. FTY720 and sphingosine also inhibited [3H]CP55,940 binding, with Ki values of approximately 2 to 3 μM (pKi = 5.84 and 5.51) and Hill coefficients that were not different from 1. S1P did not significantly inhibit [3H]CP55,940 binding at concentrations up to 100 μM. These results show that FTY720 and sphingosine compete for agonist binding to CB1 receptors but with lower potency than for antagonist binding.

The experiments described above were conducted in cell lines expressing the mouse CB1 receptor. To determine whether FTY720 and sphingosine also inhibit cannabinoid binding to the human CB1 receptor, additional experiments were conducted with membranes prepared from HEK-293 cells expressing the human CB1 receptor (hCB1-HEK) (Fig. 3A). In this system, WIN55,212-2 inhibited [3H]CP55,940 binding with a Ki value of approximately 28 nM (pKi = 7.62; Table 1). FTY720 and sphingosine also inhibited the binding of [3H]CP55,940 to hCB1-HEK cell membranes with potencies (pKi = 5.06 and 5.23, respectively) similar to those observed in mCB1-CHO cell membranes and with Hill coefficients that were not different from 1 (Table 1). In contrast, S1P did not significantly inhibit [3H]CP55,940 binding to hCB1-HEK cell membranes at concentrations up to 100 μM. Thus, both FTY720 and sphingosine inhibited binding of the cannabinoid agonist to human CB1 receptors expressed in HEK-293 cells, with potencies similar to those observed with the mouse CB1 receptor.

To determine whether FTY720 and sphingosine inhibit [3H]CP55,940 binding to endogenous CB1 receptors in native brain tissue, competition binding experiments were performed in mouse cerebellar membranes (Fig. 3B). As expected, WIN55,212-2 inhibited [3H]CP55,940 binding with high potency (pKi = 8.36; Table 1) in this system. FTY720
and sphingosine inhibited [3H]CP55,940 binding to cerebellar membranes with \( K_i \) values in the low micromolar range (\( pK_i = 5.29 \) and 5.34, respectively; Table 1) and Hill coefficients of 1. In contrast, S1P exhibited a Hill coefficient that was significantly less than 1 (Table 1), and nonlinear regression analysis of the S1P data revealed that it produced incomplete inhibition (71 ± 5.9%). In contrast, when the inhibition produced by FTY720 and sphingosine was analyzed by nonlinear regression, these compounds produced maximal inhibition that was not different from 100%. Thus, FTY720 and sphingosine completely inhibited agonist binding to mouse CB1 receptors in cerebellum or in CHO cells but at higher concentrations than are required to inhibit antagonist binding. These results suggest that these ligands bind with greater affinity to an antagonist-prefering conformation of the CB1 receptor.

### TABLE 1

<table>
<thead>
<tr>
<th>Tissue, [3H]-Tagged Ligand, &amp; Competitor</th>
<th>( K_i )</th>
<th>( n_H )</th>
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<tr>
<td>mCB1-CHO [3H]SR141716A</td>
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<tr>
<td>WIN55,212-2</td>
<td>7.53 ± 0.23(^a)</td>
<td>0.80 ± 0.11</td>
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<tr>
<td>FTY720</td>
<td>6.17 ± 0.08(^b)</td>
<td>1.46 ± 0.13</td>
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<td>Sphingosine</td>
<td>6.46 ± 0.13(^b)</td>
<td>0.93 ± 0.04</td>
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<td>S1P</td>
<td>5.37 ± 0.16(^b)</td>
<td>0.52 ± 0.06(^*)</td>
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<tr>
<td>mCB1-CHO [3H]CP55,940</td>
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<tr>
<td>WIN55,212-2</td>
<td>7.96 ± 0.14(^b)</td>
<td>0.79 ± 0.22</td>
</tr>
<tr>
<td>FTY720</td>
<td>5.84 ± 0.22(^b)</td>
<td>0.73 ± 0.09</td>
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<tr>
<td>Sphingosine</td>
<td>5.51 ± 0.11(^b)</td>
<td>1.04 ± 0.08</td>
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<td>S1P</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>hCB1-HEK [3H]CP55,940</td>
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<tr>
<td>WIN55,212-2</td>
<td>7.62 ± 0.18(^a)</td>
<td>0.96 ± 0.20</td>
</tr>
<tr>
<td>FTY720</td>
<td>5.06 ± 0.14(^b)</td>
<td>1.21 ± 0.31</td>
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<td>Sphingosine</td>
<td>5.23 ± 0.05(^b)</td>
<td>1.36 ± 0.55</td>
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<td>N.D.</td>
<td>N.D.</td>
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<td>Cerebellum [3H]CP55,940</td>
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<td>WIN55,212-2</td>
<td>8.36 ± 0.21(^b)</td>
<td>0.79 ± 0.04</td>
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<tr>
<td>FTY720</td>
<td>5.29 ± 0.07(^b)</td>
<td>0.99 ± 0.14</td>
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<tr>
<td>Sphingosine</td>
<td>5.34 ± 0.16(^b)</td>
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<tr>
<td>S1P</td>
<td>4.82 ± 0.07(^b)</td>
<td>0.45 ± 0.09(^*)</td>
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</table>

N.D., value could not be determined.  
\(^a\) \( p < 0.05 \) different from 1 by Bonferroni-adjusted Student’s \( t \) test.  
\(^b\) \( p < 0.05 \) different from FTY720 by Bonferroni-adjusted Student’s \( t \) test.  
\(^*\) Calculated \( K_i \) values for S1P are only apparent values because it produced incomplete inhibition of cannabinoid ligand binding.

**Fig. 3.** FTY720 and sphingosine inhibit [3H]CP55,940 binding to CB1 receptors. Membranes from hCB1-HEK cells (A) or mouse cerebellum (B) were incubated with 1 nM [3H]CP55,940 in the absence or presence of the indicated concentrations of WIN55,212-2, FTY720, sphingosine, or S1P. Data are expressed as mean ± S.E. of the percentage of maximal binding in the absence of unlabeled competitor (\( n = 3 \)).
membranes at concentrations as high as 10 μM (Fig. 5). Thus, FTY720 and sphingosine selectively inhibit the binding of cannabinoid ligands to CB₁ receptors. These results also make it unlikely that this inhibition is due to nonspecific perturbation of the membrane.

**FTY720 and Sphingosine Are CB₁ Receptor Antagonists.** Although the experiments described above indicate that FTY720 and sphingosine interact with the CB₁ receptor, they do not provide information as to whether these interactions are functionally relevant. The cannabinoid receptors, like S1P receptors, are coupled to the activation of G-proteins. G-protein activation upon ligand binding to GPCRs is due to the stimulation of GDP-GTP exchange of the G-protein α subunit. Binding of [³⁵S]GTPγS to the membranes is an effective method to determine the efficacy of GPCR ligands to activate G-proteins. To determine whether FTY720 or sphingosine stimulates G-protein activation via CB₁ receptors, membranes from CHO cells expressing the mouse CB₁ receptor or from untransfected CHO-K1 cells were incubated with varying concentrations of FTY720, sphingosine, S1P, or WIN55,212-2. As expected, the full CB₁ agonist WIN55,212-2 stimulated [³⁵S]GTPγS binding in a concentration-dependent manner in CHO cells expressing CB₁ receptors but not untransfected CHO cells (data not shown). FTY720 and sphingosine moderately stimulated [³⁵S]GTPγS binding but only at concentrations ≥10 μM (data not shown). However, a similar effect was also observed in CB₁-expressing and untransfected CHO-K1 cell membranes and was mimicked by S1P at concentrations ≥1 μM (data not shown). Thus, it seems likely that stimulation by sphingosine or FTY720 is due to activation of other GPCRs in these cells and not to the activation of CB₁ receptors.

The results described above, combined with the finding that FTY720 and sphingosine inhibited cannabinoid ligand binding to CB₁ receptors, suggest that these compounds might be CB₁ antagonists. To test this hypothesis, agonist-stimulated [³⁵S]GTPγS binding was measured in mCB₁-CHO cell membranes incubated with an EC₉₀ concentration of WIN55,512-2 and increasing concentrations of SR141716A, FTY720, or sphingosine (Fig. 6). FTY720 and sphingosine produced concentration-dependent inhibition of WIN55,212-2-stimulated [³⁵S]GTPγS binding with Kᵢ values of 1.0 and 1.3 μM (pKᵢ = 5.98 ± 0.05 and 5.92 ± 0.07, respectively). Although these are much lower potencies than those of the established CB₁ antagonist SR141716A, which had a Kᵢ value of 0.6 nM (pKᵢ = 9.3 ± 0.13), they are consistent with the apparent affinities of FTY720 and sphingosine in the CB₁ competition binding assays (Table 1). Moreover, their antagonist effects were not mimicked by S1P, which, when added to mCB₁-CHO cell membranes together with WIN55,212-2, produced a concentration-dependent stimulation of [³⁵S]GTPγS binding greater than that seen with WIN55,212-2 alone (Fig. 6). This stimulation by S1P was probably due to endogenous S1P receptors present in the CHO cells (Holdsworth et al., 2005), because the magnitude of stimulation by S1P in the presence of WIN55,212-2 was not significantly different from that produced by a maximally effective concentration of S1P alone in these cells (not stimulation = 32 ± 6 versus 24 ± 8 pmol/mg, respectively). Moreover, stimulation produced by 40 μM S1P alone in these cells was not significantly attenuated by SR141716A at concentrations up to 3 μM (data not shown), despite the finding that this concentration of SR141716A was greater than that required to completely block the stimulation produced by 10 μM WIN55,212-2 (Fig. 5). These results indicate that FTY720 and sphingosine, but not S1P, act as antagonists of the CB₁ receptor.

**FTY720 and Sphingosine Are Competitive Antagonists at CB₁ Receptors.** To determine whether FTY720 and sphingosine inhibit CB₁ receptor binding competitively or noncompetitively, varying concentrations of WIN55,212-2 were used at a fixed concentration (6 μM) of FTY720 and sphingosine. As shown in Fig. 7, FTY720 and sphingosine were competitive antagonists of CB₁ receptor-mediated G-protein activation because they produced a rightward shift in the WIN55,212-2 concentration-effect curve without decreasing the maximal stimulation. The apparent pA₂ values of

![Fig. 4. Lack of sphingosine phosphorylating activity in membrane preparations. Membrane fractions prepared from mCB₁-CHO cells and cerebellum were incubated with 100 μM [³H]sphingosine with or without 1 mM ATP, and the formation of [³H]S1P was determined. Cell lysate from HEK-293 cells transfected with SphK1 was used as a positive control. Data are mean picomoles per milligram per minute of [³H]S1P formed ± S.E. (n = 3).](image-url)

![Fig. 5. FTY720 and sphingosine do not inhibit [³H]CP55,940 binding to CB₂ receptors. Membranes from hCB₂-CHO cells were incubated with 1 nM [³H]CP55,940 in the absence and presence of the indicated concentrations of WIN55,212-2, FTY720, sphingosine, or S1P. Data are expressed as mean ± S.E. of the percentage of maximal binding in the absence of unlabeled competitor (n = 4).](image-url)
FTY720 and sphingosine to antagonize WIN55,212-2-stimulated G-protein activation were 5.72 ± 0.05 and 5.76 ± 0.1, respectively. Moreover, similar apparent pA2 values were obtained from CP55,940-stimulated [35S]GTPγS binding (data not shown), which were 5.43 ± 0.07 and 5.55 ± 0.09 for FTY720 and sphingosine, respectively (n = 4). These values are consistent with pKᵢ values for these compounds to inhibit CB₁ binding and CB₁-mediated G-protein activation, as described above. These results indicate that FTY720 and sphingosine act as competitive antagonists of CB₁ receptors.

**WIN55,212-2-Stimulated Activation of ERK1/2 and Akt Is Inhibited by FTY720 and Sphingosine.** To determine whether FTY720 and sphingosine antagonize CB₁ receptor signaling in intact cells, their ability to inhibit CB₁-mediated activation of downstream signaling was examined in mCB₁-CHO cells. In agreement with previous studies in CB₁-CHO cells (Bouaboula et al., 1995; Gomez del Pulgar et al., 2000), WIN55,212-2 markedly stimulated the phosphorylation of ERK1/2 and Akt, as determined with phosphospecific antibodies (Fig. 8). It is noteworthy that both FTY720 and sphingosine markedly inhibited WIN55,212-2-mediated phosphorylation of ERK1/2 and Akt. Neither FTY720 nor sphingosine alone significantly induced phosphorylation of ERK or Akt in mCB₁-CHO cells. These findings further support the hypothesis that FTY720 and sphingosine are functional CB₁ antagonists.

**CB₁ Internalization Induced by WIN55,212-2 Is Inhibited by FTY720 and Sphingosine.** It is established that activation of CB₁ receptors is followed by rapid internalization (Hsieh et al., 1999). To determine whether FTY720 and sphingosine inhibit agonist-stimulated CB₁ receptor internalization, CHO cells were transfected with a green fluorescent protein-tagged rat CB₁ receptor (rCB₁-GFP) that has been used previously to examine intracellular trafficking of the CB₁ receptor (Leterrier et al., 2004). Results showed that in the absence of agonist, rCB₁-GFP was expressed mainly on the plasma membrane of serum-starved cells (Fig. 9A), whereas GFP-vector was mainly cytosolic (data not shown). Treatment with WIN55,212-2 induced rapid internalization and significant redistribution of rCB₁-GFP into intracellular vesicles (Fig. 9B). It is noteworthy that although pretreatment with either sphingosine (Fig. 9C) or FTY720 (Fig. 9E) had no significant effect on localization of rCB₁-GFP, they inhibited WIN55,212-2-stimulated internalization of rCB₁-GFP (Fig. 9, D and F). These results provide further evidence of functional antagonism of CB₁ receptors by FTY720 and sphingosine.

**Discussion**

The results of this study demonstrate that FTY720 and sphingosine are competitive antagonists of CB₁ but not CB₂ cannabinoid receptors. In contrast, S1P did not antagonize agonist-mediated CB₁ receptor activation, although it demonstrated partial inhibition of CB₁ ligand binding in a subset of tissues examined. The affinities of FTY720 and sphingosine for CB₁ receptors are clearly lower than those of the CB₁ agonist WIN55,212-2 or antagonist SR141716A, which are in the low nanomolar range, as measured in this and previous studies (Howlett et al., 2002). Likewise, the affinities of FTY720 and sphingosine for CB₁ receptors are also lower than the reported affinities of S1P or phospho-FTY720 for most S1P receptor types, which range from approximately 0.2 to 100 nM (Lee et al., 1998; Mandala et al., 2002; Rosen and Liao, 2003). However, they are similar to the affinities of FTY720 for most S1P receptor types, which are 0.3 and 2.6 μM for S1P₁ and S1P₅, respectively, and >5 μM for S1P₂₄ (Mandala et al., 2002). Moreover, ligands with affinities in the high nanomolar to low micromolar range can produce significant biological effects, as evidenced by the fact that some GPCR agonists bind within this concentration range to produce functional effects in intact cells (Toll, 1995). We have shown in the present study that 6 μM FTY720 or sphingosine produced ≥50% inhibition of ERK and Akt phosphorylation by a maximally effective WIN55,212-2 concentration in intact mCB₁-CHO cells.

These results could have implications for biological actions of FTY720 and cannabinoid compounds and provide a novel
putative mechanism for regulation of the endogenous cannabinoid system. The mechanism of FTY720-mediated immunomodulation is believed to be the induction of lymphocyte sequestration in lymph nodes (Mandala et al., 2002). There is evidence that this action is mediated by phosphorylated FTY720 acting as an agonist at S1P1 receptors (Brinkmann et al., 2002). In this regard, FTY720 is believed to be a prodrug whose conversion to phospho-FTY720 by sphingosine kinases is required for immunomodulation. It is tempting to speculate that in certain cases, FTY720 itself can bind to CB1 receptors in an antagonistic manner and block endocannabinoid-mediated signaling pathways that are otherwise important for normal immune cell functions. However, it is unclear whether this action contributes to immune regulation by FTY720. Although CB1 receptors are found in immune tissue, CB2 receptors seem to mediate the majority of cannabinoid effects on the immune system (Buckley et al., 2000). On the other hand, the administration of FTY720 could produce antagonism of CB1 receptors in other systems, notably the CNS. Cannabinoid agonists have been investigated as potential therapeutics for a number of conditions and are currently used to alleviate nausea during cancer chemotherapy and cachexia in patients with AIDS (Martin and Wiley, 2004). Moreover, CB1 antagonists can induce withdrawal in cannabinoid-dependent animals (Lichtman and Martin, 2002). It is possible that conditions exist that might be treated by both cannabinoids and FTY720, so the potential for this compound to antagonize cannabinoid-mediated effects should be noted.

The second major finding of this study is that sphingosine acted as an antagonist at CB1 receptors. This is the first evidence that an endogenous CB1 receptor antagonist exists, and this finding could reveal an important endogenous regulator of CB1 receptor function. Although CB1 receptors are expressed in a number of tissues, the predominant expression is in the CNS. Four of the five known S1P receptor subtypes (S1P1,2,3,5) are also expressed in the CNS (Toman and Spiegel, 2002), although few studies have attempted to ascertain the distribution or expression level of particular S1P receptor types. Agonist-stimulated [35S]GTPγS autoradiography using S1P has revealed S1P receptor-mediated G-protein activity throughout the brain (Waebner and Chiu, 1999). Of interest in the present study is the apparent overlap in the distribution of S1P and CB1 receptor-mediated activity in regions including the cerebellum, hippocampus, striatum, and cortex. Moreover, it is very well known that the brain is highly enriched in complex glycosphingolipids, suggesting that high levels of sphingosine should also be present in these regions as a precursor for S1P. S1P has been shown to regulate its own production from sphingosine through S1P receptor-driven increases in sphingosine kinase activity (Meyer zu Heringdorf et al., 2001). This could provide a mechanism whereby S1P receptors could regulate CB1 receptor function via the modulation of levels of the endogenous CB1 antagonist sphingosine. Moreover, CB1 receptor activation increases ceramide via both de novo synthesis and metabolism of sphingomyelin (Guzman et al., 2001), providing another potential mechanism for interaction between these systems. The CB1 receptor antagonist sphingosine is not produced by de novo synthesis but only from metabolism of complex sphingolipids to ceramide, which is then hydrolyzed by ceramidases to liberate sphingosine, or from dephosphorylation of S1P. Ceramide is known to be enriched in lipid rafts and might play a role in lipid raft fusion and consequent clustering of receptors and signaling proteins into complexes (Gulbins and Grassme, 2002). Furthermore, disruption of lipid rafts has been shown to affect CB1 signaling and trafficking in some cell types, suggesting that a portion of CB1 receptors is localized to lipid rafts (Keren and Sarne, 2003; Bari et al., 2005). Although ceramide generation and lipid raft interaction have not been demonstrated with CB1 receptors in neurons, they have been demonstrated in glial cells (Guzman et al., 2001; Bari et al., 2005). Therefore, it is conceivable that under conditions in which high levels of

![Fig. 8. FTY720 and sphingosine inhibit WIN55,212-2-induced ERK1/2 and Akt activation. mCB1CHO cells were serum-starved for 3 h and then incubated with vehicle (4 mg/ml BSA), FTY720 (6 μM), or sphingosine (6 μM) for 10 min. Cells were then treated without or with WIN55,212-2 (WIN, 5 μM) for 8 min. lyzed, and equal amounts of protein were analyzed by immunoblotting with pAkt and p-ERK1/2 antibodies. Blots were stripped and reprobed with Akt and ERK2 antibodies as loading controls. Blots were scanned, and bands were quantified by densitometry. Data are representative immunoblots (A) and mean O.D. values ± S.E. of p-Akt and p-ERK1/2 (B) as a proportion of the respective Akt and ERK loading controls (n = 3). *p < 0.05 different from WIN55,212-2 alone by Bonferroni-adjusted Student’s t-test.](image-url)
endocannabinoids are produced by neurons, they could activate CB1 receptors on adjacent glial cells to produce ceramide. Ceramide could then be metabolized to sphingosine, which acts as an endogenous CB1 antagonist. Whether sphingosine could be released from glial cells to affect neuronal CB1 receptors is unclear, but this possibility could be tested in future studies.

Unphosphorylated FTY720 could mimic the CB1 antagonist action of sphingosine. Although it is conceivable that locally generated sphingosine could reach micromolar levels in membrane microdomains, it is uncertain whether exogenously administered FTY720 would be expected to reach these concentrations in the unphosphorylated state. Nonetheless, some studies suggest that relevant FTY720 concentrations could be achieved in vivo. Administration of 0.5 mg/kg FTY720 to rodents via intravenous administration resulted in approximately equal levels of FTY720 and phospho-FTY720 in plasma (Mandala et al., 2002). Levels of 20 ng/ml (≈60 nM) FTY720 and phospho-FTY720 were achieved in this paradigm. Therefore, administration of 10 mg/kg FTY720 would be expected to result in a concentration of approximately 1 μM of FTY720 and phospho-FTY720. Similar results were reported in humans administered 5 mg/kg FTY720 for 7 days (Kovarik et al., 2004). These data showed that administration of FTY720 produced equal levels of FTY720 and phospho-FTY720 in the blood over the 24-h period monitored after drug administration. This same group reported that administration of 5 mg of FTY720 resulted in blood levels of approximately 20 ng/ml (≈60 nM) FTY720 and phospho-FTY720. However, these authors also noted that steady-state blood levels were not achieved during the study, indicating that higher levels are likely to be present in patient populations taking FTY720 for longer periods of time or in patients taking higher doses of the drug. Thus, it is possible that partial antagonism of CB1 receptors could result from clinical use of FTY720.

In summary, the present study demonstrates that sphingosine and its analog FTY720 are competitive antagonists of CB1, but not CB2 receptors. This property is not shared by S1P, indicating that phosphorylated sphingosine analogs do not significantly interact with CB1 receptors. Sphingosine, an endogenous metabolite of membrane sphingomyelin, could act as an endogenous antagonist of CB1 receptors. Because CB1 receptor activation in glia has been shown to stimulate sphingomyelin catalolism, it is possible that endocannabinoid-mediated CB1 receptor activation could stimulate the formation of sphingosine, which could then feed back and antagonize CB1 receptors. If so, then this report would be the first demonstration of endogenous cannabinoid antagonism. This function could be mimicked by FTY720 when administered as an immunomodulatory drug, with unknown consequences. Moreover, it is possible that other endogenous sphingolipid metabolites, such as ceramide or sphinganine (dihydro sphingosine), could also interact with cannabinoid receptors, perhaps with higher affinity than sphingosine. Thus, further investigation of this potentially important relationship between endocannabinoid and sphingolipid cellular mediators is needed.

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