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Sphingosine and Its Analog, the Immunosuppressant 2-Amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol, Interact with the CB_1 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 Δ^9-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 CB_1 but not CB_2 cannabinoid receptors. FTY720 and sphingosine inhibited the binding of the CB_1-selective antagonist [^{3}H]*N*-(piperidinyl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide ([^{3}H]SR141716A) and the cannabinoid agonist [^{3}H](−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol ([^{3}H]CP55,940) in a concentration-dependent manner in both CB_1-expressing cell lines and mouse cerebellum. However, these compounds did not significantly alter [^{3}H]CP55,940 binding to CB_2 receptors. In G-protein activation assays, FTY720 and sphingosine inhibited the maximal stimulation of guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding by the cannabinoid agonist *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)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 CB_1 receptors and attenuated WIN55,212-2-stimulated internalization of a fluorescence-tagged CB_1 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 CB_1 antagonists. These results suggest that the CB_1 receptor could be a novel target of FTY720 and that sphingosine could be an endogenous CB_1 antagonist.

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Sphingosine-1-phosphate (S1P) and cannabinoid receptors belong to the lysolipid family of GPCRs (Toman and Spiegel, 2002). Subtypes for each receptor, $S1P_{1–5}$ and CB_1 and CB_2, 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-

ABBREVIATIONS: S1P, sphingosine-1-phosphate; BSA, bovine serum albumin; CNS, central nervous system; ERK, extracellular signal regulated kinase; p-, phosphorylated; GPCR, G-protein-coupled receptor; CHO, Chinese hamster ovary; Pen-Strep, penicillin/streptomycin; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; mCB_1-CHO, Chinese hamster ovary-K1 cells stably expressing the mouse CB_1 receptor; HEK, human embryonic kidney; hCB_1-HEK, human embryonic kidney-293 cells stably transfected with the human CB_1 receptor; GFP, green fluorescent protein; rCB_1-GFP, green fluorescent protein-tagged rat CB_1 receptor; GTPγS, guanosine-5′-*O*-(γ-thio)-triphosphate; WIN55,212-2, *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate; CP55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol; SR141716A, *N*-(piperidinyl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; FTY720, 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol.

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 $G_{i/o}$ family, for cannabinoid and $S1P_{1-5}$ receptors and of the $G_{12/13}$ or $G_{q/11}$ families for $S1P_{2-5}$ receptors (Spiegel and Milstien, 2003). The cannabinoid receptors have long been of interest because they mediate the effects of Δ^9-tetrahydrocannabinol, the active component of marijuana. CB_2 receptors are located primarily in the immune system, whereas CB_1 receptors are abundant in the CNS and are present in several peripheral organs (Howlett et al., 2002). CB_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 $S1P_2$. 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 $S1P_{1,3,4,5}$ 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). WIN55,2122-2, GTPγS, 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}S]GTPγS (1150–1300 Ci/mmol), D-erythro-[3-^{3}H]sphingosine (5 μCi/mmol), and [^{3}H]CP55,940 (158 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]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

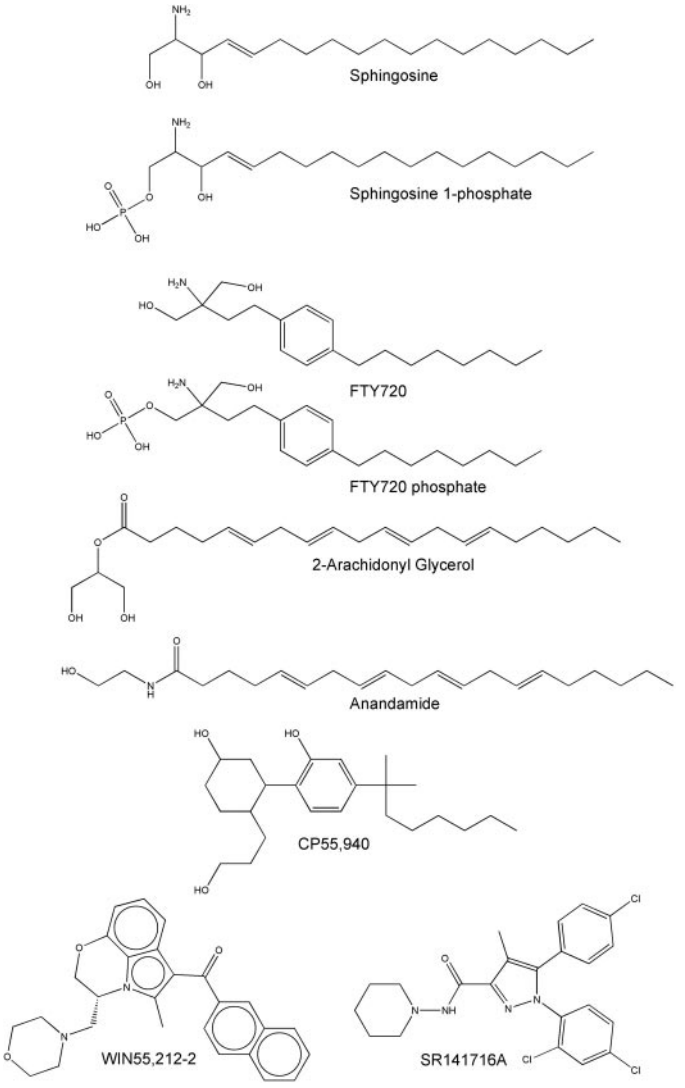

Fig. 1. Chemical structures of several sphingoid and cannabinoid compounds.

fluorescent protein (GFP)-fused rat CB_1 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 CB_1 or CB_2 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 CB_1 receptor (mCB_1-CHO) or the human CB_2 receptor (hCB_2-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 CB_1 receptor (hCB_1-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 $MgCl_2$, 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 $MgCl_2$, 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 [^{3}H]SR141716A, or in buffer B without NaCl with 1 nM [^{3}H]CP55,940 and varying concentrations of competing ligands or ethanol vehicle in a 0.5 ml total volume. Nonspecific binding was assessed in the presence of 5 μM unlabeled SR141716A in [^{3}H]SR141716A assays or 5 μM WIN55,212-2 in [^{3}H]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 ^{3}H after extraction of the filters in scintillation fluid.

Agonist-Stimulated [^{35}S]GTPγS Binding

Membranes were recovered in buffer B as described above and preincubated for 10 min at 30°C with adenosine deaminase (4 mU/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 [^{35}S]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 ^{35}S after extraction of the filters in scintillation fluid.

Sphingosine Kinase Assay

[^{3}H]Sphingosine phosphorylation by membrane preparations from mCB_1-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 [^{3}H]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 CB_1 receptor fused with GFP (CB_1-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 [^{35}S]GTPγS binding, basal binding is defined as specific [^{35}S]GTPγS binding in the absence of drug. Net-stimulated [^{35}S]GTPγS binding is defined as [^{35}S]GTPγS binding in the presence of drug minus basal. The percentage stimulation is expressed as (net stimulated [^{35}S]GTPγS binding/basal) × 100%. E_{max} or EC_{50} values were calculated from nonlinear regression analysis by iterative fitting of the concentration-effect curves to the Langmuir equation (E_{max}/[EC_{50} + 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[antagonist concentration/(DR − 1)], where DR is the agonist EC_{50} value with antagonist present/agonist EC_{50} value in the absence of antagonist. Competitor IC_{50} values and Hill coefficients were calculated according to the Hill equation $B/B_{total} = [C]^{nH}/([C]^{nH} + IC_{50}{}^{nH})$, where B is the specific binding of the radioligand, B_{total} is the specific binding of the radioligand in the absence of competitor, [C] is the concentration of the competitor, and n_H is the Hill coefficient. Competitor pK_i values were determined using the Cheng-Prussoff equation, pK_i = −log[IC_{50}/(1 + [*L]/K_D)], where [*L] is concentration of radiolabeled ligand, and K_D is the K_D 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 [^{3}H]SR141716A to the CB_1 Receptor. To determine whether FTY720 or sphingosine interact with the CB_1

receptor, membranes from CHO-K1 cells stably expressing the mouse CB_1 receptor (mCB_1-CHO) were incubated with the CB_1 antagonist [^{3}H]SR141716A and increasing concentrations of WIN55,512-2, FTY720, sphingosine, or S1P (Fig. 2A). WIN55,512-2, an established cannabinoid agonist, produced a potent, concentration-dependent inhibition of [^{3}H]SR141716A binding with a K_i value of approximately 40 nM (pK_i = 7.53; Table 1) and a Hill coefficient that was not significantly different from 1. FTY720 and sphingosine also inhibited [^{3}H]SR141716A binding but with K_i values of 709 and 390 nM (pK_i = 6.17 and 6.46, respectively; Table 1). Both FTY720 and sphingosine inhibited [^{3}H]SR141716A binding with Hill coefficients that were not different from 1, similarly to WIN55,212-2. These results are in contrast to S1P, which produced the inhibition of [^{3}H]SR141716A binding with micromolar potency (apparent pK_i = 5.37) and a Hill coefficient that was significantly less than 1 (Table 1). The low Hill coefficient suggested that S1P inhibits [^{3}H]SR141716A binding with more than one affinity state (i.e., [^{3}H]SR141716A might bind to multiple binding sites with equal affinity, but S1P distinguishes between these sites). Therefore, the binding data were subjected to nonlinear regression analysis, which, unlike the Hill analysis, does not assume a maximal inhibition of 100%. This analysis revealed that maximal inhibition of [^{3}H]SR141716A binding by S1P was only 60 ± 7.6% compared with approximately 100% inhibition by FTY720 and sphingosine, indicating that the pK_i values of FTY720 and sphingosine were not different from the corresponding values obtained with the Hill analysis. In contrast, S1P seemed to be significantly more potent by nonlinear regression (pK_i = 6.23) than by Hill analysis. These data demonstrate that FTY720 and sphingosine, but not S1P, produce complete inhibition of [^{3}H]SR141716A binding to mCB_1-CHO cells in a manner indicating an interaction with a single binding site. Thus, these results suggest that FTY720 and sphingosine bind with moderate affinity to the cannabinoid ligand binding site of the CB_1 receptor. S1P, on the other hand, seems to inhibit a subset of [^{3}H]SR141716A binding sites by an unknown mechanism.

FTY720 and Sphingosine Inhibit Binding of the Agonist [^{3}H]CP55,940 to the CB_1 Receptor. The finding that FTY720 and sphingosine inhibited [^{3}H]SR141716A binding to mCB_1-CHO cell membranes indicates that they compete for antagonist binding to CB_1 receptors. To determine whether they also inhibit agonist binding, competition experiments were performed in this cell line with the cannabinoid agonist [^{3}H]CP55,940 (Fig. 2B). As expected, WIN55,212-2 inhibited [^{3}H]CP55,940 binding with a K_i value of 12.5 nM (pK_i = 7.96) and a Hill coefficient that was not different from 1 (Table 1). FTY720 and sphingosine also inhibited [^{3}H]CP55,940 binding, with K_i values of approximately 2 to 3 μM (pK_i = 5.84 and 5.51) and Hill coefficients that were not different from 1. S1P did not significantly inhibit [^{3}H]CP55,940 binding at concentrations up to 100 μM. These results show that FTY720 and sphingosine compete for agonist binding to CB_1 receptors but with lower potency than for antagonist binding.

The experiments described above were conducted in cell lines expressing the mouse CB_1 receptor. To determine whether FTY720 and sphingosine also inhibit cannabinoid binding to the human CB_1 receptor, additional experiments were conducted with membranes prepared from HEK-293 cells expressing the human CB_1 receptor (hCB_1-HEK) (Fig. 3A). In this system, WIN55,212-2 inhibited [^{3}H]CP55,940 binding with a K_i value of approximately 28 nM (pK_i = 7.62; Table 1). FTY720 and sphingosine also inhibited the binding of [^{3}H]CP55,940 to hCB_1-HEK cell membranes with potencies (pK_i = 5.06 and 5.23, respectively) similar to those observed in mCB_1-CHO cell membranes and with Hill coefficients that were not different from 1 (Table 1). In contrast, S1P did not significantly inhibit [^{3}H]CP55,940 binding to hCB_1-HEK cell membranes at concentrations up to 100 μM. Thus, both FTY720 and sphingosine inhibited binding of the cannabinoid agonist to human CB_1 receptors expressed in HEK-293 cells, with potencies similar to those observed with the mouse CB_1 receptor.

To determine whether FTY720 and sphingosine inhibit [^{3}H]CP55,940 binding to endogenous CB_1 receptors in native brain tissue, competition binding experiments were performed in mouse cerebellar membranes (Fig. 3B). As expected, WIN55,212-2 inhibited [^{3}H]CP55,940 binding with high potency (pK_i = 8.36; Table 1) in this system. FTY720

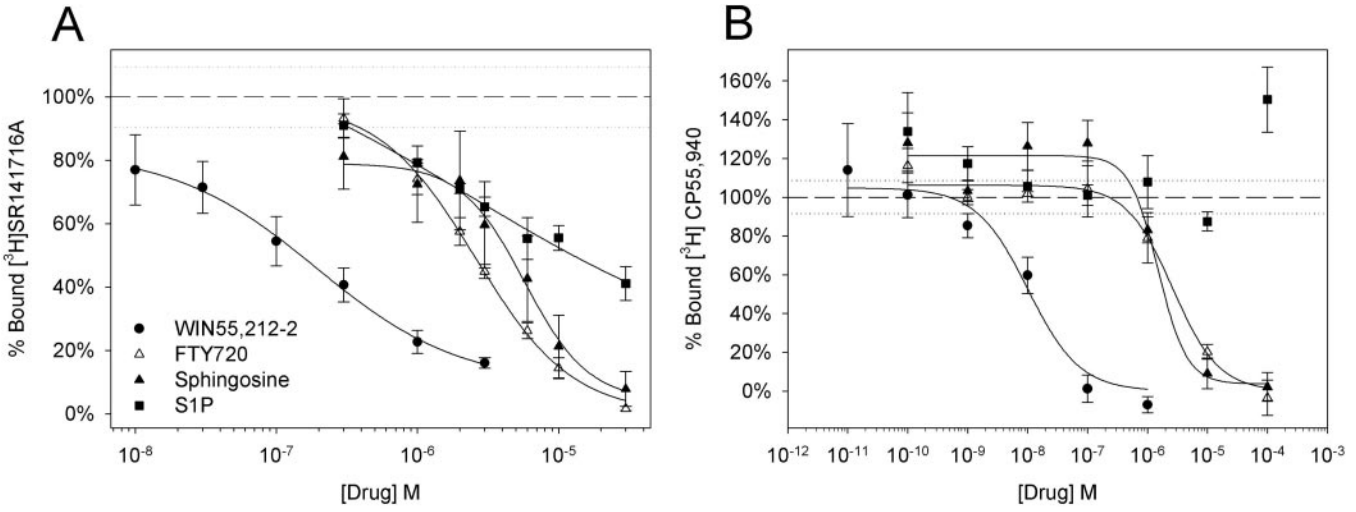

Fig. 2. FTY720 and sphingosine inhibit [^{3}H]SR141716A binding to CB_1 receptors. Membranes from mCB_1-CHO cells were incubated with 0.6 nM [^{3}H]SR141716A (A) or 1 nM [^{3}H]CP55,940 (B) in the absence or presence of the indicated concentrations of WIN55,212-2, FTY720, sphingosine, or S1P. Data are expressed as mean ± S.E. of the percentage of maximal binding in the absence of unlabeled competitor (n = 4).

and sphingosine inhibited [^{3}H]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 CB_1 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-preferring conformation of the CB_1 receptor.

TABLE 1

Competition binding values of WIN55,212-2, FTY720, sphingosine, and S1P for [^{3}H]cannabinoid ligand binding to CB_1 receptors

Membranes prepared from the indicated tissue were incubated with [^{3}H]SR141716A or [^{3}H]CP55,940 in the presence and absence of varying concentrations of WIN55,212-2, FTY720, sphingosine, or S1P. The competition binding data were fitted to the Hill equation to determine IC_{50} and n_H values. The resulting IC_{50} values were converted to pK_i values as described under *Data Analysis*. Values shown are mean ± S.E. of three to four experiments performed in triplicate. Pairs of pK_i values with different letter designations (*a*, *b*, *c*) within a particular cell type-radioligand competition group are $p < 0.05$ different from each other by Tukey-Kramer test, whereas those with the same letter designations are not significantly different.

Tissue, ^{3}H-Tagged Ligand, & Competitor	pK_i[§]	n_H
mCB_1-CHO		
[^{3}H]SR141716A		
WIN55,212-2	7.53 ± 0.23^a	0.80 ± 0.11
FTY720	6.17 ± 0.08^b	1.46 ± 0.13
Sphingosine	6.46 ± 0.13^b	0.93 ± 0.04
S1P	5.37 ± 0.16^{c†}	0.52 ± 0.06*
mCB_1-CHO		
[^{3}H]CP55,940		
WIN55,212-2	7.96 ± 0.14^a	0.79 ± 0.22
FTY720	5.84 ± 0.22^b	0.73 ± 0.09
Sphingosine	5.51 ± 0.11^b	1.04 ± 0.08
S1P	N.D.	N.D.
hCB_1-HEK		
[^{3}H]CP55,940		
WIN55,212-2	7.62 ± 0.18^a	0.96 ± 0.20
FTY720	5.06 ± 0.14^b	1.21 ± 0.31
Sphingosine	5.23 ± 0.05^b	1.36 ± 0.55
S1P	N.D.	N.D.
Cerebellum		
[^{3}H]CP55,940		
WIN55,212-2	8.36 ± 0.21^a	0.79 ± 0.04
FTY720	5.29 ± 0.07^b	0.99 ± 0.14
Sphingosine	5.34 ± 0.16^b	0.64 ± 0.07
S1P	4.82 ± 0.07^{b†}	0.45 ± 0.09*

N.D., value could not be determined.
* $p < 0.05$ different from 1 by Bonferroni-adjusted Student's *t* test.
† $p < 0.05$ different from FTY720 by Bonferroni-adjusted Student's *t* test.
§ Calculated pK_i values for S1P are only apparent values because it produced incomplete inhibition of cannabinoid ligand binding.

Sphingosine Is Not Phosphorylated by the Membrane Preparations. It was important to examine whether or not sphingosine was undergoing metabolism during the binding assays. Potential phosphorylation of sphingosine to S1P by endogenous SphKs could produce a mixture of S1P and sphingosine, which would complicate interpretation of the results. Therefore, phosphorylation of sphingosine was measured in membranes prepared from mCB_1-CHO cells and mouse cerebellum. No detectable levels of S1P were formed by any of the membrane preparations (Fig. 4). In contrast, formation of S1P from sphingosine was readily detected in membranes from HEK cells transfected with SphK1 only in the presence of added ATP, suggesting that membranes from CHO cells and cerebellum do not phosphorylate sphingosine in the binding assays. These results also suggest that it is unlikely that phospho-FTY720 is being formed in the assay because FTY720 has been reported to be a substrate for the same kinases that phosphorylate sphingosine but with 8- to 100-fold lower V_{max} values than sphingosine (Paugh et al., 2003; Allende et al., 2004).

FTY720 and Sphingosine Do Not Inhibit Binding to the CB_2 Receptor. To determine whether the interaction of FTY720 or sphingosine with the CB_1 receptor is specific to this cannabinoid receptor type, competition binding assays were performed with [^{3}H]CP55,940 in membranes from human CB_2 receptor-expressing CHO cells. As expected, WIN55,212-2 was a potent inhibitor of [^{3}H]CP55,940 binding, with a K_i value of 1.7 nM (pK_i = 8.83 ± 0.23) and a Hill coefficient of 1. However, neither FTY720 nor sphingosine significantly inhibited CP55,940 binding to hCB_2-CHO cell

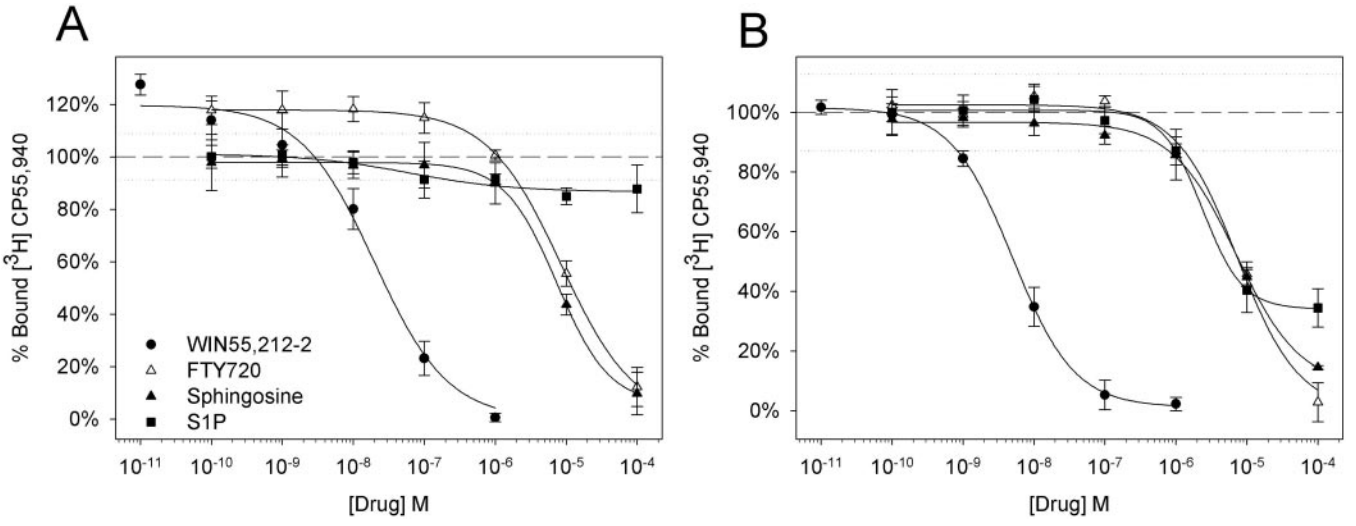

Fig. 3. FTY720 and sphingosine inhibit [^{3}H]CP55,940 binding to CB_1 receptors. Membranes from hCB_1-HEK cells (A) or mouse cerebellum (B) were incubated with 1 nM [^{3}H]CP55,940 in the absence or presence of the indicated concentrations of WIN55,212-2, FTY720, sphingosine, or S1P. Data are expressed 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 CB_1 receptors. These results also make it unlikely that this inhibition is due to nonspecific perturbation of the membrane.

FTY720 and Sphingosine Are CB_1 Receptor Antagonists. Although the experiments described above indicate that FTY720 and sphingosine interact with the CB_1 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 [^{35}S]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 CB_1 receptors, membranes from CHO cells expressing the mouse CB_1 receptor or from untransfected CHO-K1 cells were incubated with varying concentrations of FTY720, sphingosine, S1P, or WIN55,212-2. As expected, the full CB_1 agonist WIN55,212-2 stimulated [^{35}S]GTPγS binding in a concentration-dependent manner in CHO cells expressing CB_1 receptors but not untransfected CHO cells (data not shown). FTY720 and sphingosine moderately stimulated [^{35}S]GTPγS binding but only at concentrations $\geq$10 μM (data not shown). However, a similar effect was also observed in CB_2-expressing and untransfected CHO-K1 cell membranes and was mimicked by S1P at concentrations $\geq$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 CB_1 receptors.

The results described above, combined with the finding that FTY720 and sphingosine inhibited cannabinoid ligand binding to CB_1 receptors, suggest that these compounds might be CB_1 antagonists. To test this hypothesis, agonist-stimulated [^{35}S]GTPγS binding was measured in mCB_1-CHO cell membranes incubated with an EC_{90} 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 [^{35}S]GTPγS binding with K_i values of 1.0 and 1.3 μM (pK_i = 5.98 $\pm$ 0.05 and 5.92 $\pm$ 0.07, respectively). Although these are much lower potencies than those of the established CB_1 antagonist SR141716A, which had a K_i value of 0.6 nM (pK_i = 9.3 $\pm$ 0.13), they are consistent with the apparent affinities of FTY720 and sphingosine in the CB_1 competition binding assays (Table 1). Moreover, their antagonist effects were not mimicked by S1P, which, when added to mCB_1-CHO cell membranes together with WIN55,212-2, produced a concentration-dependent stimulation of [^{35}S]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 (net stimulation = 32 $\pm$ 6 versus 24 $\pm$ 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 CB_1 receptor.

FTY720 and Sphingosine Are Competitive Antagonists at CB_1 Receptors. To determine whether FTY720 and sphingosine inhibit CB_1 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 CB_1 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

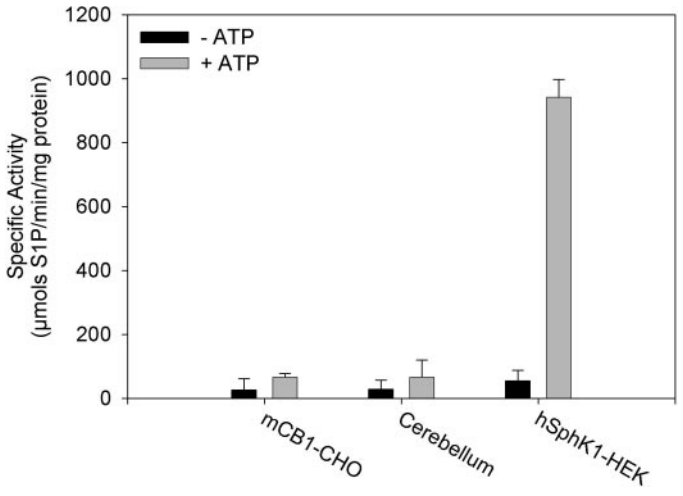

Fig. 4. Lack of sphingosine phosphorylating activity in membrane preparations. Membrane fractions prepared from mCB_1-CHO cells and cerebellum were incubated with 100 μM [^{3}H]sphingosine with or without 1 mM ATP, and the formation of [^{3}H]S1P was determined. Cell lysate from HEK-293 cells transfected with SphK1 was used as a positive control. Data are mean picomoles per milligram per minute of [^{3}H]S1P formed $\pm$ S.E. (n = 3).

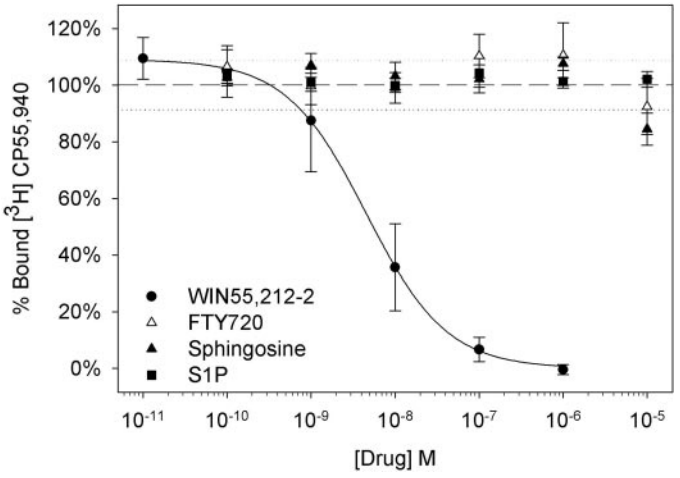

Fig. 5. FTY720 and sphingosine do not inhibit [^{3}H]CP55,940 binding to CB_2 receptors. Membranes from hCB_2-CHO cells were incubated with 1 nM [^{3}H]CP55,940 in the absence and presence of the indicated concentrations of WIN55,212-2, FTY720, sphingosine, or S1P. Data are expressed as mean $\pm$ S.E. of the percentage of maximal binding in the absence of unlabeled competitor (n = 4).

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 [^{35}S]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 pK_i values for these compounds to inhibit CB_1 binding and CB_1-mediated G-protein activation, as described above. These results indicate that FTY720 and sphingosine act as competitive antagonists of CB_1 receptors.

WIN55,212-2-Stimulated Activation of ERK1/2 and Akt Is Inhibited by FTY720 and Sphingosine. To determine whether FTY720 and sphingosine antagonize CB_1 receptor signaling in intact cells, their ability to inhibit CB_1-mediated activation of downstream signaling was examined in mCB_1-CHO cells. In agreement with previous studies in CB_1-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 mCB_1-CHO cells. These findings further support the hypothesis that FTY720 and sphingosine are functional CB_1 antagonists.

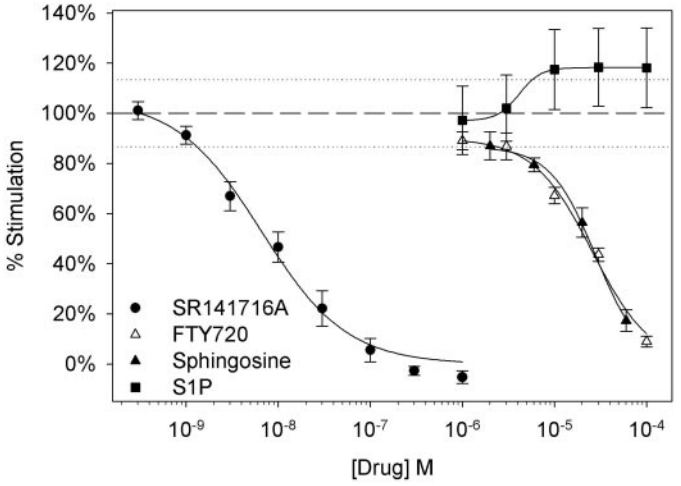

Fig. 6. FTY720 and sphingosine are CB_1 receptor antagonists. Membranes from mCB_1-CHO cells were incubated with 3 μM WIN55,212-2, 10 μM GDP, and 0.1 nM [^{35}S]GTPγS in the absence and presence of the indicated concentrations of SR141716A, FTY720, sphingosine, or S1P. Results are expressed as the percentage of the stimulation produced by 3 μM WIN55,212-2 alone (net stimulation = 159 ± 32 pmol/mg). Data are mean values ± S.E. (n = 5).

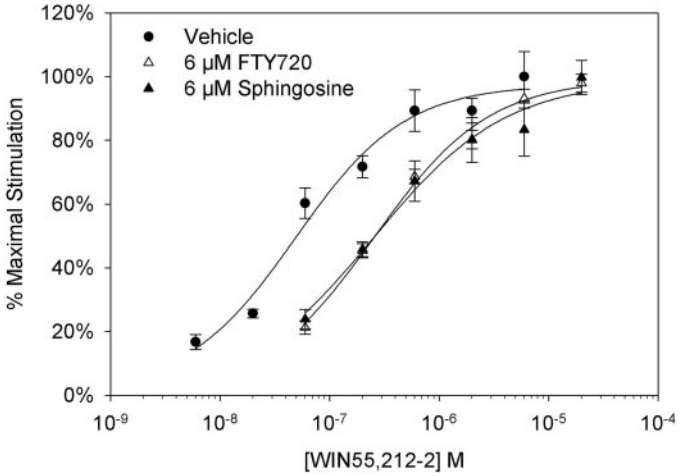

Fig. 7. FTY720 and sphingosine are competitive CB_1 receptor antagonists. Membranes from mCB_1-CHO cells were incubated with varying concentrations of WIN55,212-2, 10 μM GDP, and 0.1 nM [^{35}S]GTPγS with and without a fixed concentration (6 μM) of FTY720 or sphingosine. Results are expressed as the percentage of maximal stimulation by 10 μM WIN55,212-2. Data are means ± S.E. (n = 4).

CB_1 Internalization Induced by WIN55,212-2 Is Inhibited by FTY720 and Sphingosine. It is established that activation of CB_1 receptors is followed by rapid internalization (Hsieh et al., 1999). To determine whether FTY720 and sphingosine inhibit agonist-stimulated CB_1 receptor internalization, CHO cells were transfected with a green fluorescent protein-tagged rat CB_1 receptor (rCB_1-GFP) that has been used previously to examine intracellular trafficking of the CB_1 receptor (Leterrier et al., 2004). Results showed that in the absence of agonist, rCB_1-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 rCB_1-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 rCB_1-GFP, they inhibited WIN55,212-2-stimulated internalization of rCB_1-GFP (Fig. 9, D and F). These results provide further evidence of functional antagonism of CB_1 receptors by FTY720 and sphingosine.

Discussion

The results of this study demonstrate that FTY720 and sphingosine are competitive antagonists of CB_1 but not CB_2 cannabinoid receptors. In contrast, S1P did not antagonize agonist-mediated CB_1 receptor activation, although it demonstrated partial inhibition of CB_1 ligand binding in a subset of tissues examined. The affinities of FTY720 and sphingosine for CB_1 receptors are clearly lower than those of the CB_1 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 CB_1 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 $S1P_1$ and $S1P_5$, respectively, and >5 μM for $S1P_{2-4}$ (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 phsophorylation by a maximally effective WIN55,212-2 concentration in intact mCB_1-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 $S1P_1$ 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 CB_1 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 CB_1 receptors are found in immune tissue, CB_2 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 CB_1 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, CB_1 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 CB_1 receptors. This is the first evidence that an endogenous CB_1 receptor antagonist exists, and this finding could reveal an important endogenous regulator of CB_1 receptor function. Although CB_1 receptors are expressed in a number of tissues, the predominant expression is in the CNS. Four of the five known S1P receptor subtypes ($S1P_{1,2,3,5}$) 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 [^{35}S]GTPγS autoradiography using S1P has revealed S1P receptor-mediated G-protein activity throughout the brain (Waeber and Chiu, 1999). Of interest in the present study is the apparent overlap in the distribution of S1P and CB_1 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 CB_1 receptor function via the modulation of levels of the endogenous CB_1 antagonist sphingosine. Moreover, CB_1 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 CB_1 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 CB_1 signaling and trafficking in some cell types, suggesting that a portion of CB_1 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 CB_1 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

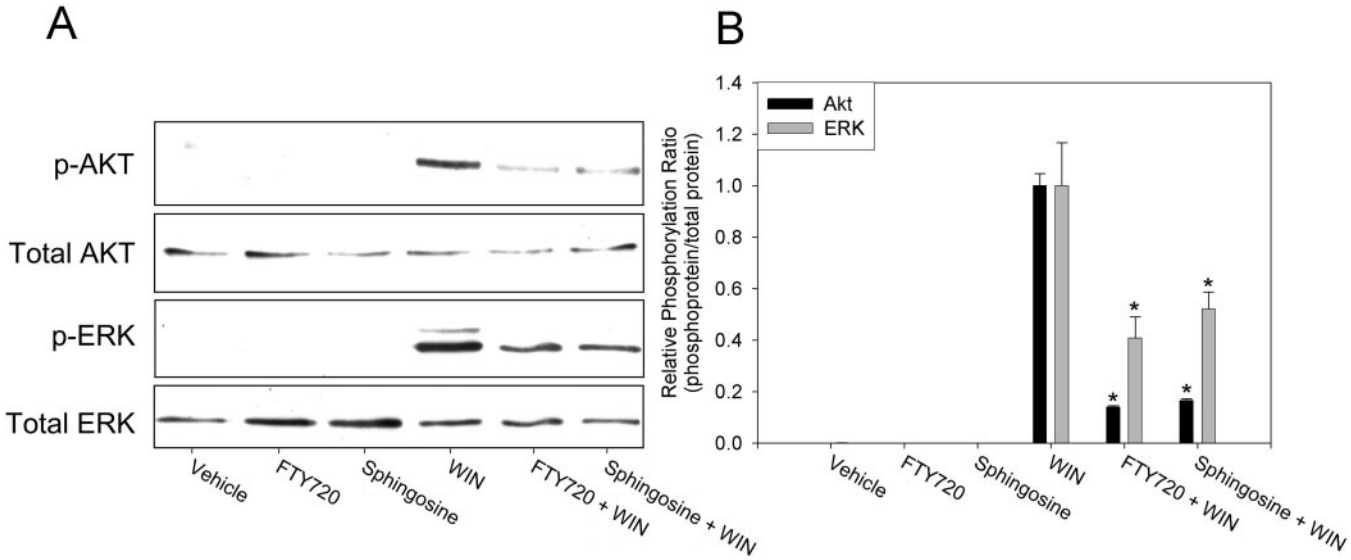

Fig. 8. FTY720 and sphingosine inhibit WIN55,212-2-induced ERK1/2 and Akt activation. mCB_1-CHO cells were serum-starved for 3 h and then incubated with vehicle (4 mg/ml BSA), FTY720 (6 μM), or sphingosine (6 μM) for 10 min. Cells were then treated without or with WIN55,212-2 (WIN, 5 μM) for 8 min, lysed, 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.

endocannabinoids are produced by neurons, they could activate CB_1 receptors on adjacent glial cells to produce ceramide. Ceramide could then be metabolized to sphingosine, which acts as an endogenous CB_1 antagonist. Whether sphingosine could be released from glial cells to affect neuronal CB_1 receptors is unclear, but this possibility could be tested in future studies.

Unphosphorylated FTY720 could mimic the CB_1 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 CB_1 receptors could result from clinical use of FTY720.

In summary, the present study demonstrates that sphingosine and its analog FTY720 are competitive antagonists of CB_1 but not CB_2 receptors. This property is not shared by S1P, indicating that phosphorylated sphingosine analogs do not significantly interact with CB_1 receptors. Sphingosine, an endogenous metabolite of membrane sphingomyelin, could act as an endogenous antagonist of CB_1 receptors. Because CB_1 receptor activation in glia has been shown to stimulate sphingomyelin catabolism, it is possible that endocannabinoid-mediated CB_1 receptor activation could stimulate the formation of sphingosine, which could then feed back and antagonize CB_1 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 (dihydrosphingosine), 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.

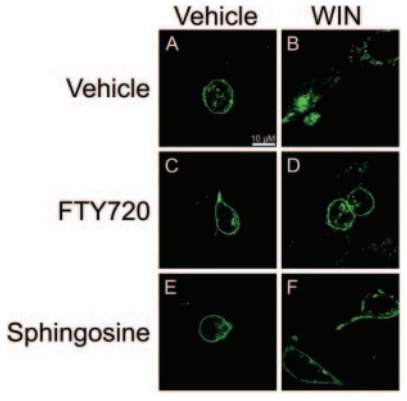

Fig. 9. Sphingosine and FTY720 inhibit WIN55,212-2-induced internalization of CB1 receptors. CHO-K1 cells transfected with rCB_1-GFP were pretreated with vehicle (A and B), 10 μM sphingosine (C and D), or 50 μM FTY720 (E and F) for 10 min and then stimulated without (A, C, and E) or with 2 μM WIN55,212-2 (B, D, and F) for 30 min. Cells were then fixed and examined by confocal fluorescence microscopy. Data shown are representative images from more than 20 cells examined per group.

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