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Inhibitory effects of SR141716A on G-protein activation in rat brain

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Abstract

N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A), a cannabinoid CB_1 receptor antagonist, has inverse agonist effects in cannabinoid CB_1 receptor-expressing cell lines, brain and peripheral organs. These studies characterized SR141716A-inhibited G-protein activity by measuring [^{35}S]GTPγS binding. Maximal inhibition of basal [^{35}S]GTPγS binding in cerebellar membranes was 50%. The EC_{50} value for inhibition of [^{35}S]GTPγS binding was 4.4 μM, whereas the K_e for inhibition of *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN 55,212-2)-stimulated [^{35}S]GTPγS binding was 0.6 nM. [^{35}S]GTPγS autoradiography was used to examine the regional specificity of SR141716A inhibition. SR141716A inhibited basal [^{35}S]GTPγS binding in all regions examined, with inhibition ranging from approximately 20% in caudate-putamen to 40% in hippocampus. These studies demonstrate that SR141716A is a competitive antagonist at nanomolar concentrations, whereas it inhibits basal receptor-mediated G-protein activity at micromolar concentrations. These data suggest that the apparent inverse agonist effect is either not cannabinoid CB_1 receptor-specific or that SR141716A is binding to different sites on the cannabinoid CB_1 receptor to produce inverse agonist versus competitive antagonist effects.

Keywords: Cannabinoid receptor; [^{35}S]GTPγS binding; G-protein-coupled receptor

1. Introduction

Cannabinoid CB_1 receptors are the most numerous G-protein-coupled receptors in the brain, with levels tenfold higher than other G-protein-coupled receptors (Devane et al., 1988; Herkenham et al., 1991). Although the role of the endogenous cannabinoid system has not yet been fully characterized, cannabinoid receptors are known to mediate the effects of Δ^9-terahydrocannabinol (Devane et al., 1988), the active ingredient in marijuana (Gaoni and Mechoulam, 1964). Functional effects associated with cannabinoid receptor activation include antinociception, decreased spontaneous activity, hypothermia and impairment of short-term memory (Dewey, 1986; Hollister, 1986). The characterization of cannabinoid receptors has been advanced by the development of selective and potent ligands, such as the cannabinoid CB_1 receptor-selective antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A) (Rinaldi-Carmona et al., 1994), which blocks cannabinoid CB_1 receptor agonist-mediated effects in both in vitro and in vivo preparations.

Many G-protein-coupled receptors exhibit spontaneous activity in the absence of agonist, which can be inhibited by inverse agonists (Costa et al., 1990; Tian et al., 1994). The possibility that SR141716A acts as an inverse agonist at cannabinoid receptors was suggested by Compton et al. (1996) in studies showing stimulation of motor activity after administration of high doses (> 3 mg/kg) of SR141716A. Subsequent studies using heterologous expression of cannabinoid CB_1 receptors in Chinese hamster ovary (CHO) cells demonstrated that SR141716A is an inverse agonist for cannabinoid-mediated effects on adenylyl cyclase and mitogen-activated protein kinase activity (Bouaboula et al., 1997). SR141716A inverse agonism has also been reported for in situ assays of brain (Gifford and Ashby, 1996) and isolated peripheral organs (Pertwee and Fernando, 1996; Izzo et al., 1998; Coutts et al., 2000). In vivo administration of SR141716A also supports its role as an inverse agonist, although these data are complicated by

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the possibility that these effects are due to antagonism of endogenous cannabinoids (Terranova et al., 1996; Richardson et al., 1997; Colombo et al., 1998a,b; Gessa et al., 1998; Rubino et al., 2000).

The inverse agonist effects of SR141716A in brain have been demonstrated using in vitro assays of G-protein and adenylyl cyclase activity (Meschler et al., 2000), as well as measures of ex vivo effector activity after in vivo administration of the drug (Rubino et al., 2000). The involvement of receptor-mediated G-protein activity in the inverse agonist response is supported by reports that SR141716A inhibits [^{35}S]GTPγS binding in cannabinoid CB_1 receptor-transfected cell lines (Bouaboula et al., 1995; Landsman et al., 1997; MacLennan et al., 1998), neuronal cells and brain (Meschler et al., 2000). Data from cannabinoid CB_1 receptor-expressing CHO cells indicate that SR141716A inhibits effector responses that are mediated by $G_i\alpha$ (i.e. adenylyl cyclase) as well as $G\beta\gamma$ (i.e. mitogen-activated protein kinase) (Bouaboula et al., 1997). Moreover, studies in purified reconstituted systems have shown that SR141716A inhibits the activation of both G_i- and G_o-type G-proteins via spontaneously active cannabinoid CB_1 receptors (Glass and Northup, 1999). Ligand-modulated [^{35}S]GTPγS binding presents the opportunity to characterize SR141716A inverse agonism in brain because: (1) this methodology is used to investigate drug actions directly at the level of the G-protein and (2) moderate levels of basal [^{35}S]GTPγS binding in many regions indicate the presence of spontaneous receptor activity. Basal [^{35}S]GTPγS binding in autoradiographic assays exhibits a specific regional distribution (Sim et al., 1995), suggesting that specific receptor populations exhibit spontaneous activity. Furthermore, cannabinoid receptor signal transduction properties are known to vary by brain region (Sim et al., 1995; Breivogel et al., 1997, 1999), but it is not clear whether this is also true for spontaneous activity and inverse agonist effects. These studies utilized ligand-modulated [^{35}S]GTPγS binding in rat brain membranes and sections to investigate the inverse agonist properties of SR141716A for G-protein activity.

2. Materials and methods

2.1. Materials

Rats (male Sprague–Dawley, 200–250 g) were obtained from Harlan. [^{35}S]GTPγS (1250 Ci/mmol) was purchased from New England Nuclear (Boston, MA). SR141716A and SR144528 were provided by the Drug Supply Program of the National Institute on Drug Abuse. Methanandamide, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN 55,212-2) and GDP (autoradiography) were purchased from Sigma/RBI. GDP (membranes) and GTPγS were purchased from Boehringer Mannheim. Kodak X-O-mat film was purchased from New England Nuclear. All other reagent grade chemicals were obtained from Sigma or Fisher.

2.2. Ligand-modulated [^{35}S]GTPγS binding in cerebellar membranes

Animals were sacrificed by decapitation and cerebella were dissected and placed in 20 volumes cold Buffer A (50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4). Tissue was homogenized with a Polytron homogenizer and centrifuged at 48,000 × *g* at 4°C for 10 min. The tissue was 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, 60 mM NaCl, pH 7.4). Protein was determined using the method of Bradford (1976) and membranes were stored in aliquots at −80°C until use. For assays, membranes were thawed and homogenized in Buffer B, preincubated for 10 min at 30°C in 4 mU/ml adenosine deaminase to remove endogenous adenosine, then assayed for protein content (Bradford, 1976). Cerebellar membranes (10 μg protein) were incubated in Buffer B with 0.1 nM [^{35}S]GTPγS and appropriate concentrations of drugs or vehicle (50:50 ethanol/dimethylsulfoxide) in the presence of 30 μM GDP and 0.1% bovine serum albumin in a 1 ml total volume. Vehicle concentrations were adjusted to match the concentration of vehicle in the samples containing drug. In some experiments, 30 μM GDP or 60 mM NaCl was replaced by varying concentrations of GDP or NaCl, respectively. Basal binding was assessed in the absence of drug, and nonspecific binding was determined with 20 μM unlabeled GTPγS. The incubation was terminated by filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with ice-cold 50 mM Tris–HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency after extraction overnight in scintillation fluid.

2.3. Ligand-modulated [^{35}S]GTPγS autoradiography

Animals were decapitated and brains were removed and frozen in isopentane at −30°C. Twenty-micron coronal sections were cut on a cryostat at −20°C and mounted on gelatin-coated slides. Slides were desiccated at 4°C overnight and stored desiccated at −80°C until assay. Slides were brought to room temperature, then incubated in 50 mM Tris–HCl, pH 7.4, with 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl and 0.5% bovine serum albumin (Buffer C) for 10 min at 25°C. Slides were then incubated in Buffer C with 2 mM GDP and 9.5 mU/ml adenosine deaminase for 15 min at 25°C, followed by incubation in 0.04 nM [^{35}S]GTPγS, 2 mM GDP and adenosine deaminase with SR141716A (20 μM), WIN 55,212-2 (10 μM) or matching dilution of vehicle (dimethylsulfoxide) in Buffer C at 25°C for 2 h. Basal binding was determined in the absence of ligand. Slides were rinsed twice for 2 min each at 4°C in 50 mM Tris–HCl, pH 7.4, then for 30 s at 4°C in deionized H_2O. Slides were dried overnight, then

exposed to Kodak X-O-mat film for 6 days. Each cassette contained a [^{14}C] microscale for densitometric analysis and values were corrected for [^{35}S] based upon incorporation of [^{35}S] into sections of frozen brain paste (Sim et al., 1996b).

2.4. Data analysis

Data from membrane homogenate assays are reported as mean values ± standard error (S.E.) of at least three experiments, which were each performed in triplicate. Nonlinear iterative regression analyses of concentration–effect curves were performed with JMP (SAS Institute, Cary, NC). IC_{50} values for SR141716A inhibition of methanandamide-stimulated [^{35}S]GTPγS binding were determined by log–logit (Hill) analyses. For autoradiography, films were digitized with a Sony XC-77 video camera and analyzed using the NIH Image program for Macintosh computers. Data are reported as mean values ± S.E. of triplicate sections of brains from 5 animals. Net-inhibition is defined as [^{35}S]GTPγS in the presence of drug—basal [^{35}S]GTPγS binding. Percent change from basal is defined as: ([^{35}S]GTPγS in the presence of drug—basal [^{35}S]GTPγS binding/basal [^{35}S]GTPγS binding) × 100%. Statistical significance was determined by analysis of variance followed by post hoc analysis with the non-paired two-tailed Student's *t*-test using JMP (SAS Institute).

3. Results

3.1. SR141716A-inhibited [^{35}S]GTPγS binding in cerebellar membranes

To determine the effect of SR141716A on basal [^{35}S]GTPγS binding, a range of concentrations of SR-1416716A were examined in membranes prepared from rat cerebellum. Results showed that SR141716A produced a concentration-dependent and saturable inhibition of [^{35}S]GTPγS binding (Fig. 1). Non-linear regression analysis of the SR141716A concentration–effect curves showed that maximal inhibition of basal [^{35}S]GTPγS binding was 48 ± 2.8%, and half-maximal inhibition was obtained at 4.4 ± 0.6 μM. A similar concentration of SR141716A was required for half-maximal inhibition of [^{35}S]GTPγS binding in membranes from hippocampus: 6.4 ± 1.8 μM (data not shown). In contrast to the inhibitory effect of this cannabinoid CB_1 receptor-selective antagonist, the CB_2-selective antagonist SR144528 did not inhibit basal [^{35}S]GTPγS binding at concentrations up to 30 μM, but instead produced a slight (~ 10%) stimulation of [^{35}S]GTPγS binding.

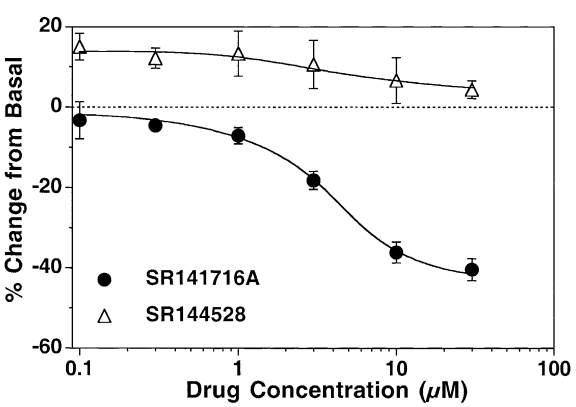

Fig. 1. Effect of SR141716A and SR144528 on basal [^{35}S]GTPγS binding in rat cerebellar membranes. Membranes were incubated as described in Section 2 with 0.1 nM [^{35}S]GTPγS, 30 μM GDP and varying concentrations of drug. Data are mean ± S.E. of percent change from basal [^{35}S]GTPγS binding.

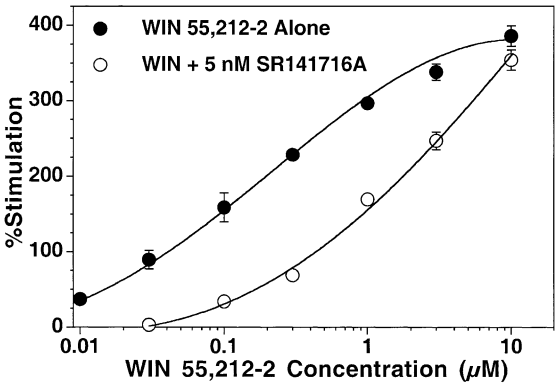

Fig. 2. Competitive inhibition of WIN 55,212-2-stimulated [^{35}S]GTPγS binding by SR141716A in rat cerebellar membranes. Membranes were incubated as described in Section 2 with 0.1 nM [^{35}S]GTPγS, 30 μM GDP and varying concentrations of WIN 55,212-2 in the absence or presence of 5 nM SR141716A. Data are mean ± S.E. of percent stimulation over basal [^{35}S]GTPγS binding.

Because the inhibitory effect of SR141716A on basal [^{35}S]GTPγS binding was only observed at μM concentrations of the drug, its ability to antagonize cannabinoid agonist-stimulated [^{35}S]GTPγS binding was also examined. At a concentration of 5 nM, SR141716A produced a rightward shift in the WIN 55,212-2 concentration–effect curve (Fig. 2), yielding a calculated K_e value of 0.57 ± 0.21 nM. Similarly, the K_e value for SR141716A antagonism of methanandamide-stimulated [^{35}S]GTPγS binding was 0.32 ± 0.07 nM (data not shown). The ability of SR141716A to antagonize methanandamide-stimulated [^{35}S]GTPγS binding was also examined by varying the concentration of the antagonist in the presence of 5 μM methanandamide. As shown in Fig. 3, SR141716A inhibited methanandamide-stimulated [^{35}S]GTPγS binding in a concentration-dependent manner, with an IC_{50} value of 6.6 ± 0.7 nM ($n_H = 1.0 \pm 0.1$; $r = 0.98$). Concentrations of SR141716A above those required to completely abolish stimulation by methanandamide resulted in inhibition of [^{35}S]GTPγS binding below basal levels. Interestingly, half-maximal inhibition of basal [^{35}S]GTPγS binding by SR141716A in the presence of 5 μM methanandamide was obtained at 2.8 ± 0.8 μM, which was not significantly

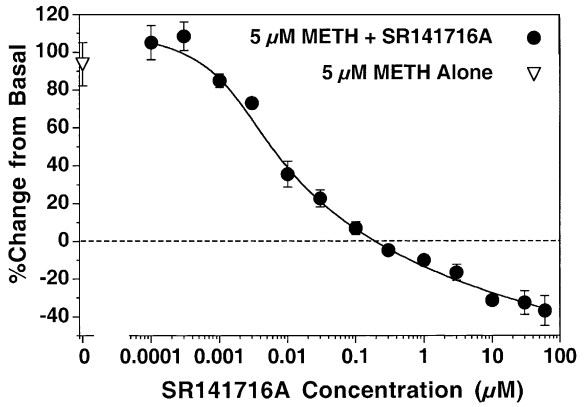

Fig. 3. Effect of SR141716A on methanandamide-stimulated [^{35}S]GTPγS binding in rat cerebellar membranes. Membranes were incubated as described in Section 2 with 0.1 nM [^{35}S]GTPγS, 30 μM GDP and 5 μM methanandamide in the absence or presence varying concentrations of SR141716A. Data are mean ± S.E. of percent change from basal [^{35}S]GTPγS binding.

different from the SR141716A EC_{50} value obtained in the absence of cannabinoid agonist (see above). Thus, the presence of methanandamide reduced the potency of SR141716A for competitive antagonism by over tenfold, but had no effect on its potency for inhibition of basal [^{35}S]GTPγS binding.

GDP is required for the optimal modulation of [^{35}S]GTPγS binding by receptors in both membranes (Hilf et al., 1989) and tissue sections (Sim et al., 1995). Thus, the GDP-dependence of SR141716A inhibited [^{35}S]GTPγS binding was examined in rat cerebellar membranes. Results (Fig. 4) showed that, similar to stimulation of [^{35}S]GTPγS binding by WIN 55,212-2, inhibition of [^{35}S]GTPγS binding by SR141716A required the presence of μM GDP concentrations. There were, however, quantitative differences in the GDP-dependence of agonist-stimulated compared to SR141716A-inhibited [^{35}S]GTPγS binding. Although the percent stimulation of [^{35}S]GTPγS binding by WIN 55,212-2 increased as a function of GDP concentration up to 30 μM GDP, the percent inhibition by SR141716A remained constant from approximately 3 to 30 μM GDP. This is probably due to the fact that net-stimulated [^{35}S]GTPγS binding by WIN 55,212-2 was maximal at 1–3 μM GDP, whereas net-inhibited [^{35}S]GTPγS binding by SR141716A was maximal 0.1–0.3 μM GDP (data not shown).

The GDP dependence of SR141716A-inhibited [^{35}S]GTPγS binding suggested that SR141716A was inhibiting spontaneous receptor activity. The spontaneous activity of many $G_{i/o}$-coupled receptors is inhibited by Na^+ ions (Costa et al., 1990; Hilf and Jakobs, 1992; Tian et al., 1994). Therefore, the effect of Na^+ on the inhibition of [^{35}S]GTPγS binding by SR141716A was investigated in cerebellar membranes. As expected, increasing NaCl concentrations inhibited basal [^{35}S]GTPγS binding (Fig. 5A), and the difference between basal and SR141716A-inhibited [^{35}S]GTPγS binding (net inhibition) was also reduced by increasing NaCl concentrations. However, it is evident that although the percent inhibition was also decreased by NaCl (Fig. 5B), a significant percentage of

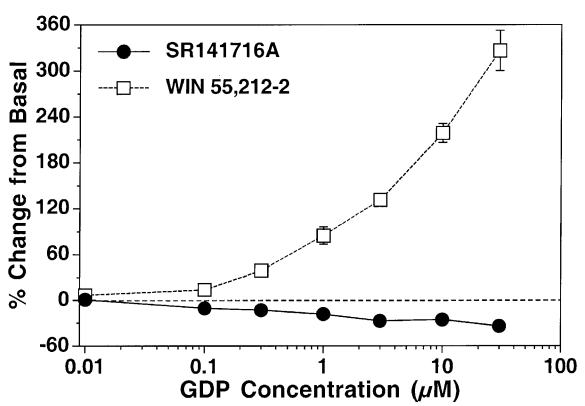

Fig. 4. Effect of GDP on WIN 55,212-2-stimulated and SR141716A-inhibited [^{35}S]GTPγS binding in rat cerebellar membranes. Membranes were incubated as described in Section 2 with 0.1 nM [^{35}S]GTPγS, and varying concentrations of GDP in the absence or presence of 20 μM WIN 55,212-2 or 20 μM SR141716A. Data are mean ± S.E. of percent change from basal [^{35}S]GTPγS binding (measured at each GDP concentration).

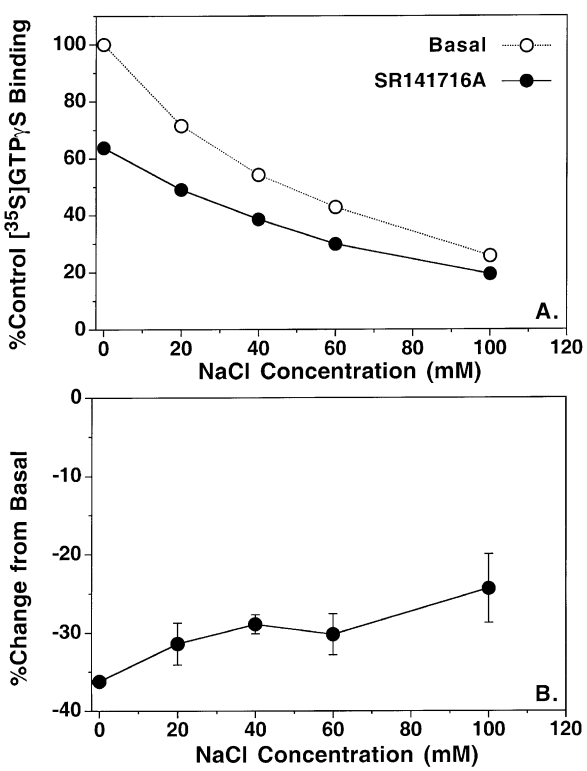

Fig. 5. Effect of NaCl on inhibition of [^{35}S]GTPγS binding by SR141716A in rat cerebellar membranes. Membranes were incubated as described in Section 2 with 0.1 nM [^{35}S]GTPγS, 30 μM GDP and varying concentrations of NaCl in the absence or presence of 20 μM SR141716A. Data are mean ± S.E. of (A) percent of control [^{35}S]GTPγS binding (defined as basal binding in the absence of NaCl) or (B) percent change from basal [^{35}S]GTPγS binding (measured at each NaCl concentration).

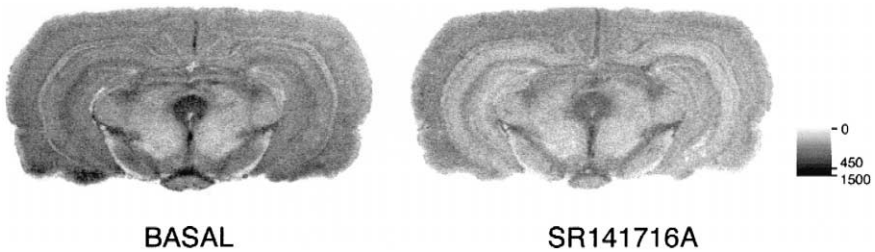

Fig. 6. Effect of SR141716A on [^{35}S]GTPγS binding in coronal sections of rat brain. Sections were incubated as described in Section 2 with 0.04 nM [^{35}S]GTPγS and 2 mM GDP in the absence or presence of 20 μM SR141716A. Densitometric contrast is enhanced in the autoradiograms to accentuate inhibition of basal [^{35}S]GTPγS binding by SR141716A, which is seen in regions including the substantia nigra, hippocampus and periaqueductal gray.

basal [^{35}S]GTPγS binding was inhibited by SR141716A even at 100 mM NaCl.

3.2. SR141716A-inhibited [^{35}S]GTPγS autoradiography

Coronal sections at several levels of the rat brain were processed for [^{35}S]GTPγS autoradiography to determine the regional distribution of SR141716A inverse agonism. Adjacent sections were processed for WIN 55,212-2-stimulated [^{35}S]GTPγS binding to confirm the distribution of cannabinoid receptor-activated G-proteins in regions of interest (data not shown). As previously reported (Sim et al., 1995, 1996a), WIN 55,212-2-stimulated [^{35}S]GTPγS binding was highest in basal ganglia, cerebellum and hippocampus, although stimulation of [^{35}S]GTPγS binding was found in most brain regions examined. Comparison of autoradiograms processed for SR141617A-inhibited [^{35}S]GTPγS binding compared to basal showed visible decreases in [^{35}S]GTPγS binding. These differences were most obvious when the contrast was maximally enhanced to accentuate basal [^{35}S]GTPγS binding (Fig. 6). Reductions in basal [^{35}S]GTPγS binding by SR141716A were visible in most regions, including hippocampus, hypothalamus, thalamus, amygdala, cortex, substantia nigra and periaqueductal gray.

Quantitative densitometry was performed to measure SR141716A-inhibited [^{35}S]GTPγS binding. Data are expressed as either net inhibition of basal [^{35}S]GTPγS binding [basal—SR141716A-inhibited [^{35}S]GTPγS binding] or percent inhibition [(net/basal) × 100]. In most regions, net SR141716A-inhibited [^{35}S]GTPγS binding was significantly different from basal ($P < 0.005$) (Table 1). The one exception was the red nucleus, where SR141716A-inhibited [^{35}S]GTPγS binding did not significantly differ from basal. Densitometric analysis also showed low levels of WIN 55,212-2-stimulated [^{35}S]GTPγS binding in the red nucleus, with net WIN 55,212-2-stimulated [^{35}S]GTPγS binding of 35 ± 8 nCi/g ($P \leq 0.001$ different from basal [^{35}S]GTPγS binding).

Net SR141716A-inhibited [^{35}S]GTPγS binding ranged from -36 nCi/g in the lateral thalamus to -97 nCi/g in the amygdala (Table 1). Linear regression analysis showed a correlation ($r = 0.884$, $P < 0.0002$) between the levels of basal [^{35}S]GTPγS binding and net inhibition produced by SR141716A. Data were also calculated as percent inhibition of [^{35}S]GTPγS binding (Fig. 7). These values

Table 1
Densitometric analysis of basal and SR141716A-inhibited [^{35}S]GTPγS autoradiography in rat brain sections

Region	[^{35}S]GTPγS bound (nCi/g tissue)		
	Basal	+ SR141716A	Net-inhibited
Cingulate cortex	196 ± 5	150 ± 8[a]	−52 ± 4
Caudate-putamen	212 ± 7	167 ± 6[a]	−41 ± 9
Globus pallidus	243 ± 10	172 ± 15[a]	−56 ± 16
Hippocampus	184 ± 3	113 ± 3[a]	−73 ± 4
Amygdala	387 ± 12	277 ± 13[a]	−97 ± 2
Hypothalamus	404 ± 10	314 ± 13[a]	−91 ± 7
Medial thalamus	255 ± 11	179 ± 6[a]	−79 ± 6
Lateral thalamus	150 ± 7	105 ± 4[a]	−36 ± 5
Substantia nigra	200 ± 5	138 ± 8[a]	−62 ± 8
Periaqueductal gray	336 ± 4	257 ± 12[a]	−89 ± 11
Cerebellum	124 ± 6	88 ± 6[a]	−41 ± 4
Red nucleus	96 ± 2	89 ± 5	−7 ± 4

Sections were processed for [^{35}S]GTPγS autoradiography and densitometric analysis was performed as described in Section 2.

[a] $P \leq 0.01$ different from basal binding.

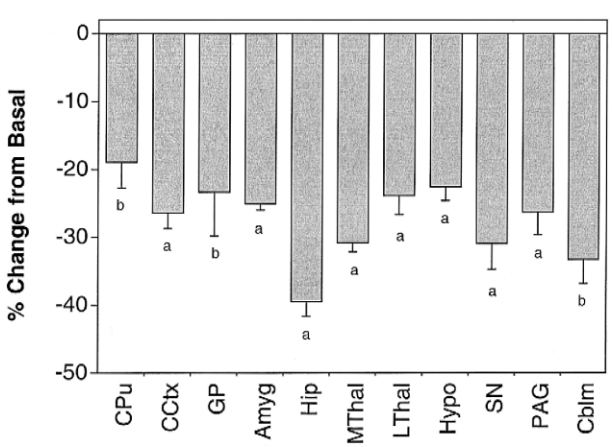

Fig. 7. Regional distribution of SR141716A-inhibited [^{35}S]GTPγS binding. Brain sections were processed as described in Section 2 and resulting autoradiograms were analyzed densitometrically. Data are mean ± S.E. of SR141716A-inhibited [^{35}S]GTPγS binding expressed as percent change from basal [^{35}S]GTPγS binding. ([a] $P = 0.001$; [b] $P = 0.005$, different from basal).

ranged from −19% in the caudate-putamen to −39% in the hippocampus, with no significant correlation between the levels of basal [^{35}S]GTPγS binding and percent inhibition.

The receptor specificity of SR141716A-inhibited [^{35}S]GTPγS binding was investigated in autoradiographic assays by incubating brain sections with the cannabinoid CB_2 receptor inverse agonist SR144528 (Portier et al., 1999). No visible differences were seen between basal and SR144528-modulated [^{35}S]GTPγS binding (data not shown). This was confirmed by densitometric analysis of the caudate-putamen and hippocampus, where SR144528-modulated [^{35}S]GTPγS binding did not significantly differ from basal (11 ± 22 and -7 ± 8 net nCi/g, respectively).

4. Discussion

The results of the present study indicate that the cannabinoid CB_1 receptor-selective antagonist SR141716A inhibits basal [^{35}S]GTPγS binding in rat brain, in agreement with previous reports of inverse agonist effects of this compound in cannabinoid receptor-containing systems. However, much higher concentrations of SR141716A were required to produce inverse agonism than antagonism of cannabinoid-mediated [^{35}S]GTPγS binding: the EC_{50} value for inhibition of basal [^{35}S]GTPγS binding (4.4 μM) was over 7000-fold greater than its K_e value (0.57 nM) for competitive antagonism of WIN 55,212-2-stimulated [^{35}S]GTPγS binding. Moreover, the potency of SR141716A for inhibition of basal [^{35}S]GTPγS binding was not affected by the presence of 5 μM methanandamide, even though the IC_{50} value of SR141716A for antagonism of 5 μM methanandamide-stimulated [^{35}S]GTPγS binding was increased by over tenfold compared to its K_e value. These results suggest that the apparent inverse agonist effect of SR141716A may be produced by binding to a site other than the agonist binding site on the cannabinoid CB_1 receptor. Three explanations for the inhibitory effect of SR141716A on basal [^{35}S]GTPγS binding are most probable: (1) a non-receptor-mediated effect of the drug; (2) SR141716A acting as an inverse agonist at a G-protein-coupled receptor other than the cannabinoid CB_1 receptor, to which it binds with much lower affinity; or (3) SR141716A acting as an inverse agonist by binding with lower affinity to a different site on, or a specific conformation of, the cannabinoid CB_1 receptor.

The possibility that SR141716A is inhibiting [^{35}S]GTPγS binding by a non-receptor-mediated mechanism is unlikely for two reasons. First, the effect is concentration-dependent and saturable. Second, the effect is only observed at concentrations of GDP that support receptor-mediated effects on [^{35}S]GTPγS binding, such as stimulation by agonist. Although the inhibitory effect of SR141716A was maximal at a lower GDP concentration than stimulation by agonist, this is to be expected because lower concentrations of GDP would favor spontaneous receptor-mediated G-protein activation (Onaran et al., 1993). The second possibility, that SR141716A inhibits the activity of non-CB_1 G-protein-coupled receptors seems plausible. Although the inhibitory effect of SR141716A showed a regional distribution consistent with cannabinoid CB_1 receptor localization in brain, the effect did not show a perfect correlation with reported cannabinoid CB_1 receptor density (see below). Nonetheless, the fact that the inhibitory effect of SR141716A was relatively insensitive to the Na^+ concentration agrees with previous findings that cannabinoid agonist-stimulated G-protein activation in cerebellar membranes was also insensitive to Na^+ (Pacheco et al., 1994; Selley et al., 1996). Thus, the possibility that SR141716A is inhibiting basal receptor-mediated activity in brain by binding to a site on the cannabinoid CB_1 receptor that is distinct from the agonist binding site seems to be the most likely explanation. However, the possibility that it may also inhibit the basal activity of other $G_{i/o}$-coupled receptors by acting at a homologous site cannot be eliminated.

Information from mutational analyses of the cannabinoid CB_1 receptor may help to clarify this question. Studies using cannabinoid CB_1/CB_2 chimeric receptors have shown that transmembrane regions IV and V are particularly important for binding of SR141716A to the cannabinoid CB_1 receptor (Shire et al., 1996). However, mutational analysis of cannabinoid-modulated Ca^{2+} currents indicates that SR141716A may require different sites on the cannabinoid CB_1 receptor for antagonist versus inverse agonist activity. A cannabinoid CB_1 receptor mutant in which lysine-192 in the third transmembrane domain was replaced with alanine (K192A) does not bind CP 55940 or anandamide, but retains binding for WIN 55212-2 (Song and Bonner, 1996). WIN 55212-2 also inhibits Ca^{2+} currents in the K192A mutant when expressed in neuronal cultures, and this effect is reversed by SR141716A (Pan et al., 1998). However, SR141716A fails to produce inverse agonism at the K192A mutant receptor, even though it facilitates Ca^{2+} currents under conditions where endogenous cannabinoids are not involved. These data suggest that residues in the transmembrane IV/V region may be important for high affinity binding and antagonist activity of SR141716A, whereas residues in the transmembrane III region are important for inverse agonism. This apparent difference in the structural requirements of the cannabinoid CB_1 receptor for antagonist versus inverse agonist activity of SR141716A may be the molecular basis for the different concentrations required to produce antagonism versus inhibition of receptor-mediated G-protein activity. Therefore, decreased basal receptor activity in brain produced by high concentrations of SR141716A may be due to allosteric modulation rather than true inverse agonism.

There are also two less plausible explanations for the inhibitory effect of SR141716A on [^{35}S]GTPγS binding in brain. Although the affinity of SR141716A for cannabi-

noid CB_1 versus CB_2 receptors differs by over 100-fold (Rinaldi-Carmona et al., 1994; Showalter et al., 1996; MacLennan et al., 1998) SR141716A also acts as an inverse agonist at cannabinoid CB_2 receptors (MacLennan et al., 1998), so it is possible that SR141716A-induced inhibition of [^{35}S]GTPγS binding is mediated via inverse agonism of cannabinoid CB_2 receptors. However, this is unlikely because the cannabinoid CB_2 receptor-selective antagonist SR144528 did not inhibit basal [^{35}S]GTPγS binding in brain sections or membranes, even though it is a potent inverse agonist at cannabinoid CB_2 receptors (Portier et al., 1999). These data agree with previous reports that demonstrated the absence of cannabinoid CB_2 receptors in brain (Griffin et al., 1999). It is also possible that the effects of SR141716A are due to antagonism of endogenous cannabinoid receptor ligands, as suggested for in vivo assays (Compton et al., 1996; Terranova et al., 1996; Richardson et al., 1997; Strangman et al., 1998). Results from the present studies do not appear to be due to antagonism of endogenous cannabinoids in the tissue because competitive antagonism of endogenous ligands should require only nanomolar concentrations of SR141716A, whereas the EC_{50} value for inhibition of basal [^{35}S]GTPγS binding was 4.4 μM. Furthermore, these experiments did not include enzyme inhibitors, so that any endogenous ligands present in the tissue would probably have been metabolized during incubation (Childers et al., 1994). Finally, [^{35}S]GTPγS binding is conducted under low affinity agonist binding conditions (i.e. in the presence of high concentrations of NaCl and GDP), which should promote dissociation of endogenous cannabinoid agonist (Devane et al., 1988; Griffin et al., 1999).

The high concentration of SR141716A required for inhibition of [^{35}S]GTPγS binding explains why previous studies failed to show a saturable inhibitory effect of this compound in rat cerebellar membranes (Breivogel et al., 1998). In fact, SR141716A inverse agonism in brain has only been demonstrated under conditions that favor increased basal receptor activity (i.e. replacement of Na^+ with K^+) (Meschler et al., 2000). Approximately 25% inhibition of basal [^{35}S]GTPγS binding was seen in those studies. However, SR141716A was used only at 1 μM and regional differences in inhibition were not examined. In contrast, studies of inverse agonist effects of SR141716A in cell lines heterologously expressing the cannabinoid CB_1 receptor have found EC_{50} values in 1–5 nM range for inhibition of [^{35}S]GTPγS binding (Landsman et al., 1997; MacLennan et al., 1998) or modulation of effector activity (Bouaboula et al., 1997). One possible explanation for this discrepancy is the different species of origin of the receptor: all of the previous studies in transfected cell systems used the human cannabinoid CB_1 receptor. However, recent work showing inverse agonist effects of SR141716A for human cannabinoid CB_1 receptor-mediated regulation of K^+ channel conductance in an oocyte expression system found an EC_{50} value of 200 nM (McAllister et al., 1999). Similarly, SR141716A was reported to stimulate Ca^{2+} channel conductance with an EC_{50} value of 32 nM via the rat cannabinoid CB_1 receptor heterologously expressed in neurons (Pan et al., 1998). Thus, the potency of SR141716A for inhibition of basal cannabinoid CB_1 receptor activity does not appear to be related to a species difference. Most of these previous studies (with the exception of Landsman et al., 1997) reported EC_{50} values for inverse agonism that were greater than the reported receptor binding affinity of SR141716A in brain, which is in the 0.3–2 nM range (Rinaldi-Carmona et al., 1994; Breivogel et al., 1997; Hillard et al., 1999; Meschler et al., 2000). Moreover, the antagonist potency of SR141716A ($\sim$ 8–20 nM) against cannabinoid receptor-mediated inhibition of smooth muscle contractions in isolated organ preparations (Pertwee and Fernando, 1996; Izzo et al., 1998; Coutts et al., 2000) or inhibition of acetylcholine release in hippocampal slices (Gifford and Ashby, 1996) is at least tenfold higher than its potency as an inverse agonist in these preparations ($\geq$ 100 nM). Thus, although the potency of SR141716 for inhibition of [^{35}S]GTPγS binding in rat brain is lower than expected, it has generally been reported that the inverse agonist potency of SR141716A is lower than its affinity for the cannabinoid CB_1 receptor. These findings are in agreement with in vivo studies showing that the locomotor stimulant (Compton et al., 1996), gastrointestinal propulsive (Colombo et al., 1998b), and acetylcholine-releasing (Gessa et al., 1998) effects of SR141716A occur at higher doses than those required to antagonize cannabinoid agonist effects. Thus, both in vitro and in vivo data indicate that antagonist versus apparent inverse agonist effects of SR141716A in brain can be differentiated on the basis of potency.

Although inhibition of [^{35}S]GTPγS binding by SR141716A was detected in virtually all brain areas, the magnitude of inhibition varied by region. Interestingly, the regions in which the highest net SR141716A-inhibited [^{35}S]GTPγS binding was seen differed from those exhibiting the greatest percent inhibition of basal [^{35}S]GTPγS binding. Net inhibition was modestly correlated with basal [^{35}S]GTPγS binding levels ($r = 0.88$), indicating that greater absolute inhibition occurred in areas with higher levels of basal [^{35}S]GTPγS binding, such as amygdala, hypothalamus and periaqueductal gray. In contrast, the percent inhibition produced by SR141716A did not correlate with basal levels of [^{35}S]GTPγS binding, suggesting that spontaneous activity of SR141716A-inhibited receptors contributes differentially to basal [^{35}S]GTPγS binding in different brain areas. The relative levels of SR141716A inhibition were also compared with cannabinoid receptor density, by determining whether the levels of net SR141716A-inhibited [^{35}S]GTPγS binding measured autoradiographically were correlated with [^{3}H]CP55,940 binding density reported by Herkenham et al. (1991). However, neither net nor percent inhibition by SR141716A significantly correlated with cannabinoid CB_1 receptor density.

These data suggest that SR141716A inverse agonism may not be due to inhibition of cannabinoid CB_1 receptors alone. Nevertheless, regional differences in SR141716A-inhibited [^{35}S]GTPγS binding are consistent with previous studies showing regional differences in Na^+ sensitivity, receptor efficiency and desensitization of cannabinoid receptors (Pacheco et al., 1994; Sim et al., 1995; Breivogel et al., 1997, 1999).

In conclusion, the cannabinoid CB_1 receptor antagonist SR141716A inhibits basal [^{35}S]GTPγS binding in rat brain in a GDP and brain region-dependent manner, suggesting that it inhibits the spontaneous activity of one or more G-protein-coupled receptors. The high concentrations of the drug required to produce this effect preclude the determination that it is exclusively mediated by cannabinoid CB_1 receptors. These findings, as well as previous studies showing apparent inverse agonist effects of SR141716A at high concentrations, and mutational studies showing that different residues on the cannabinoid CB_1 receptor may be required for antagonism versus inverse agonism by SR141716A indicate that different mechanisms mediate competitive antagonism versus inhibition of basal receptor activity. Therefore, we propose that SR141716A may inhibit basal receptor activity by binding to an additional site on the cannabinoid CB_1 receptor that is not the agonist binding site, and that this site may be common to cannabinoid CB_1, CB_2 and possibly other G-protein-coupled receptors.

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References

Bouaboula, M., Poinot-Chazel, C., Bourrié, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Casellas, P., 1995. Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB_1. Biochem. J. 312, 637.

Bouaboula, M., Perrachon, S., Milligan, L., Canat, X., Rinaldi-Carmona, M., Portier, M., Barth, F., Calandra, B., Pecceu, F., Lupker, J., Maffrand, J.-P., Le Fur, G., Casellas, P., 1997. A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. J. Biol. Chem. 272, 22330.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248.

Breivogel, C.S., Sim, L.J., Childers, S.R., 1997. Regional differences in cannabinoid receptor/G-protein coupling in rat brain. J. Pharmacol. Exp. Ther. 282, 1632.

Breivogel, C.S., Selley, D.E., Childers, S.R., 1998. Cannabinoid receptor agonist efficacy for stimulating [^{35}S]GTPgS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J. Biol. Chem. 273, 16865.

Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E., Vogt, L.J., Sim-Selley, L.J., 1999. Chronic delta^9-tetrahydrocannabinol treatment produces a time-dependent loss of cannabinoid receptor-activated G-proteins in brain. J. Neurochem. 73, 2447.

Childers, S.R., Sexton, T., Roy, M.B., 1994. Effects of anandamide on cannabinoid receptors in rat brain membranes. Biochem. Pharmacol. 47, 711.

Colombo, G., Agabio, R., Diaz, G., Lobina, C., Reali, R., Gessa, G.L., 1998a. Appetite suppression and weight loss after the cannabinoid antagonist SR141716A. Life Sci. 63, 113.

Colombo, G., Agabio, R., Lobina, C., Reali, R., Gessa, G.L., 1998b. Cannabinoid modulation of intestinal propulsion in mice. Eur. J. Pharmacol. 344, 67.

Compton, D.R., Aceto, M.D., Lowe, J., Martin, B.R., 1996. In vivo characterization of a specific cannabinoid receptor antagonist (SR141716A): inhibition of delta 9-tetrahydrocannabinol-induced responses and apparent agonist activity. J. Pharmacol. Exp. Ther. 277, 586.

Costa, T., Lang, J., Gless, C., Herz, A., 1990. Spontaneous association between opioid receptors and GTP-binding proteins in native membranes: specific regulation by antagonists and sodium ions. Mol. Pharmacol. 37, 383.

Coutts, A.A., Brewster, N., Ingram, T., Razdan, R.K., Pertwee, R.G., 2000. Comparison of novel cannabinoid partial agonists and SR141716A in the guinea-pig small intestine. Br. J. Pharmacol. 129, 645.

Devane, W.A., Dysarz, F.A.I., Johnson, M.R., Melvin, L.S., Howlett, A.C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 34, 605.

Dewey, W.L., 1986. Cannabinoid pharmacology. Pharmacol. Rev. 38, 151.

Gaoni, Y., Mechoulam, R., 1964. Isolation, structure, and partial synthesis of an active constituent of hashish. J. Am. Chem. Soc. 86, 1646.

Gessa, G.L., Casu, M.A., Carta, G., Mascia, M.S., 1998. Cannabinoids decrease acetylcholine release in the medial-prefrontal cortex and hippocampus, reversal by SR 141716A. Eur. J. Pharmacol. 355, 119.

Gifford, A.N., Ashby Jr., C.R., 1996. Electrically evoked acetylcholine release from hippocampal slices is inhibited by the cannabinoid receptor agonist, WIN 55212-2, and is potentiated by the cannabinoid antagonist, SR 141716A. J. Pharmacol. Exp. Ther. 277, 1431.

Glass, M., Northup, J.K., 1999. Agonist selective regulation of G-proteins by cannabinoid CB_1 and CB_2 receptors. Mol. Pharmacol. 56, 1362.

Griffin, G., Wray, E.J., Tao, Q., McAllister, S.D., Rorrer, W.K., Aung, M., Martin, B.R., Abood, M.E., 1999. Evaluation of the cannabinoid CB_2 receptor-selective antagonist, SR144528: further evidence for cannabinoid CB_2 receptor absence in the rat central nervous system. Eur. J. Pharmacol. 377, 117.

Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., de Costa, B.R., Rice, K.C., 1991. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J. Neurosci. 11, 563.

Hilf, G., Jakobs, K.H., 1992. Agonist-independent inhibition of G protein activation by muscarinic acetylcholine receptor antagonists in cardiac membranes. Eur. J. Pharmacol. 225, 245.

Hilf, G., Gierschik, P., Jakobs, K.H., 1989. Muscarinic acetylcholine receptor-stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) to guanine-nucleotide-binding proteins in cardiac membranes. Eur. J. Biochem. 186, 725.

Hillard, C.J., Muthian, S., Kearn, C.S., 1999. Effects of CB(1) cannabinoid receptor activation on cerebellar granule cell nitric oxide synthase activitym. FEBS Lett. 459, 277.

Hollister, L.E., 1986. Health aspects of Cannabis. Pharmacol. Rev. 38, 1.

Izzo, A.A., Mascolo, N., Borrelli, F., Capasso, F., 1998. Excitatory transmission to the circular muscle of the guinea-pig ileum: evidence

for the involvement of cannabinoid CB_1 receptors. Br. J. Pharmacol. 124, 1363.

Landsman, R.S., Burkey, T.H., Consroe, P., Roeske, W.R., Yamamura, H.I., 1997. SR141716A is an inverse agonist at the human cannabinoid CB_1 receptor. Eur. J. Pharmacol. 334, R1.

MacLennan, S.J., Reynen, P.H., Kwan, J., Bonhaus, D.W., 1998. Evidence for inverse agonism of SR141716A at human recombinant cannabinoid CB_1 and CB_2 receptors. Br. J. Pharmacol. 124, 619.

McAllister, S.D., Griffin, G., Satin, L.S., Abood, M.E., 1999. Cannabinoid receptors can activate and inhibit G protein-coupled inwardly rectifying potassium channels in a xenopus oocyte expression system. J. Pharmacol. Exp. Ther. 291, 618.

Meschler, J.P., Kraichely, D.M., Wilken, G.H., Howlett, A.C., 2000. 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. Biochem. Pharmacol. 60, 1315.

Onaran, H.O., Costa, T., Rodbard, D., 1993. βγ Subunits of guanine nucleotide-binding proteins and regulation of spontaneous receptor activity: Thermodynamic model for the interaction between receptors and guanine nucleotide-binding protein subunits. Mol. Pharmacol. 43, 245.

Pacheco, M.A., Ward, S.J., Childers, S.R., 1994. Differential requirements of sodium for coupling of cannabinoid receptors to adenylyl cyclase in rat brain membranes. J. Neurochem. 62, 1773.

Pan, X., Ikeda, S.R., Lewis, D.L., 1998. SR 141716A acts as an inverse agonist to increase neuronal voltage-dependent Ca^{2+} currents by reversal of tonic CB_1 cannabinoid receptor activity. Mol. Pharmacol. 54, 1064.

Pertwee, R.G., Fernando, S.R., 1996. Evidence for the presence of cannabinoid CB_1 receptors in mouse urinary bladder. Br. J. Pharmacol. 118, 2053.

Portier, M., Rinaldi-Carmona, M., Pecceau, F., Combes, T., Poinot-Chazel, C., Calandra, B., Barth, F., LeFur, G., Casellas, P., 1999. SR144528, an antagonist for the peripheral cannabinoid receptor that behaves as an inverse agonist. J. Pharmacol. Exp. Ther. 288, 582.

Richardson, J.D., Aanonsen, L., Hargreaves, K.M., 1997. SR 141716A, a cannabinoid receptor antagonist, produces hyperalgesia in untreated mice. Eur. J. Pharmacol. 319, R3.

Rinaldi-Carmona, M., Barth, F., Heaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Neliat, G., Caput, D., Ferrara, P., Soubrie, P., Breliere, J.C., Le Fur, G., 1994. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. 350, 240.

Rubino, T., Vigano, D., Zagato, E., Sala, M., Parolaro, D., 2000. In vivo characterization of the specific cannabinoid receptor antagonist, SR141716A: behavioral and cellular responses after acute and chronic treatments. Synapse 35, 8.

Selley, D.E., Stark, S., Sim, L.J., Childers, S.R., 1996. Cannabinoid receptor stimulation of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding in rat brain membranes. Life Sci. 59, 659.

Shire, D., Calandra, B., Delpech, M., Dumont, X., Kaghad, M., LeFur, G., Caput, D., Ferrara, P., 1996. Structural features of the central cannabinoid CB_1 receptor involved in the binding of the specific CB_1 antagonist SR141716A. J. Biol. Chem. 271, 6941.

Showalter, V.M., Compton, D.R., Martin, B.R., Abood, M.E., 1996. Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB_2): identification of cannabinoid receptor subtype selective ligands. J. Pharmacol. Exp. Ther. 278, 989.

Sim, L.J., Selley, D.E., Childers, S.R., 1995. In vitro autoradiography of receptor-activated G-proteins in rat brain by agonist-stimulated guanylyl 5′-[γ-[^{35}S]thio]-triphosphate binding. Proc. Natl. Acad. Sci. U. S. A. 92, 7242.

Sim, L.J., Hampson, R.E., Deadwyler, S.A., Childers, S.R., 1996a. Effects of chronic treatment with Δ^9-tetrahydrocannabinol on cannabinoid-stimulated [^{35}S]GTPγS autoradiography in rat brain. J. Neurosci. 16, 8057.

Sim, L.J., Selley, D.E., Dworkin, S.I., Childers, S.R., 1996b. Effects of chronic morphine administration on mu opioid receptor-stimulated [^{35}S]GTPγS autoradiography in rat brain. J. Neurosci. 16, 2684.

Song, Z.H., Bonner, T.I., 1996. A lysine residue of the cannabinoid receptor is critical for receptor recognition by several agonists but not WIN55212-2. Mol. Pharmacol. 49, 891.

Strangman, N.M., Patrick, S.L., Hohmann, A.G., Tsou, K., Walker, J.M., 1998. Evidence for a role of endogenous cannabinoids in the modulation of acute and tonic pain sensitivity. Brain Res. 813, 323.

Terranova, J.P., Storme, J.J., Lafon, N., Perio, A., Rinaldi-Carmona, M., LeFur, G., Soubrie, P., 1996. Improvement of memory in rodents by the selective CB_1 cannabinoid receptor antagonist, SR141716. Psychopharmacology 126, 165.

Tian, W.-N., Duzic, E., Lanier, S.M., Deth, R.C., 1994. Determinants of α_2-adrenergic receptor activation of G proteins: evidence for a precoupled receptor/G protein state. Mol. Pharmacol. 45, 524.