Biochemical Pharmacology, Vol. 54, pp. 1267–1270, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00384-5

SHORT COMMUNICATION

Interaction of Brain Cannabinoid Receptors with Guanine Nucleotide Binding Protein

A RADIOLIGAND BINDING STUDY

François Petitet,‡ Bernadette Jeantaud,* Marc Capet† and Adam Doble**

*Neurochemistry Department, †Chemistry Department, Rhône-Poulenc Rorer S.A., BP14, 13 quai Jules Guesde, 94403 Vitry-sur-Seine, France

ABSTRACT. The binding of a classical cannabinoid agonist, [^{3}H]R-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-napthalenyl)methanone monomethanesulfonate ([^{3}H]WIN55212-2), and a selective cannabinoid receptor (CB_1) antagonist, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride ([^{3}H]SR141716A), to rat cannabinoid receptors was evaluated using rat cerebellar membranes. Guanine nucleotides inhibited [^{3}H]WIN55212-2 binding by approximately 50% at 10 μM and enhanced [^{3}H]SR141716A binding very slightly. In the same tissue, the binding of guanosine 5′-*O*-[γ-[^{35}S]thio]triphosphate ([^{35}S]GTP-γ-S) was characterized and the influence of cannabinomimetics evaluated on this binding. Cannabinoid receptor agonists enhanced [^{35}S]GTP-γ-S binding, whereas SR141716A was devoid of action by itself but antagonized the action of cannabinoid receptor agonists. The good correlation obtained between the half maximum efficient concentration (EC_{50}) values in [^{35}S]GTP-γ-S binding and the IC_{50} values [^{3}H]WIN55212-2 binding shows that [^{35}S]GTP-γ-S binding could be a good functional assay for brain cannabinoid receptors. BIOCHEM PHARMACOL **54**;11:1267–1270, 1997.

KEY WORDS. cannabinoid; G protein; GTP-γ-S; CP55940; WIN55212-2; SR141716A

Cannabinoid receptors have been described as G protein-coupled receptors and are sensitive to psychoactive terpenes from *Cannabis sativa* as well as to synthetic compounds such as *(cis)*-3-(2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-*(trans)*-4-(3-hydroxypropyl)cyclohexanol (CP55940)§ or R-(+)-2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol [1,2,3-de]-1,4-benzoxazin-6-yl)(1-napthalenyl)methanone monomethanesulfonate (WIN55212-2). A cDNA has been identified coding for a cannabinoid receptor (CB_1 receptor) which is present mainly in the central nervous system [1, 2]. Stimulation of the CB_1 cannabinoid receptor inhibits adenylate cyclase in cultured cells [1–3] or brain tissue [4], suggesting that the coupling partner of the CB_1 receptor is G_i. The pharmacology of the cannabinoid receptors has been studied in binding experiments using principally two agonist radioligands: [^{3}H]CP55940 and [^{3}H]WIN55212-2 [5–7]. The first cannabinoid receptor antagonist described was*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A) [8]. [^{3}H]SR141716A is now available and has been shown to label high affinity sites in rat brain [9–11].

In the present study we have examined the influence of the nonhydrolysable GTP analogue, guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S), on the binding properties of two radioligands, one agonist and one antagonist ([^{3}H]WIN55212-2 and [^{3}H]SR141716A, respectively), to rat cerebellar membranes. Moreover, receptor-stimulated [^{35}S]GTP-γ-S binding has been used in biochemical studies to measure the activation of G proteins by specific receptors [12, 13]. For cannabinoid receptors, studies using rat brain slices or membranes and [^{35}S]GTP-γ-S binding have recently been published [14, 15]. We describe here a characterization of [^{35}S]GTP-γ-S binding to rat cerebellar membranes and show that this assay could differentiate cannabinoid receptor agonists from antagonists.

MATERIALS AND METHODS

Chemicals

[^{3}H]WIN55212-2 (specific activity: 45.5 Ci/mmol) was obtained from N.E.N. and [^{3}H]SR141716A (specific activity: 40 Ci/mmol) from Amersham. WIN55212-2 and its

‡ Corresponding author: Dr. François Petitet, Neurochimie, CRVA, Rhône-Poulenc Rorer, 13 quai Jules Guesde, 94403 Vitry-sur-Seine Cedex, France. TEL. 33 1 55 71 34 78; FAX 33 1 55 71 36 53; E-mail address: francois.petitet@rp.fr

§ *Abbreviations:* CB_1, cannabinoid receptor type 1; CP55940, *(cis)*-3-(2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*(trans)*-4-(3-hydroxypropyl)cyclohexanol; PMSF, phenylmethylsulfonylfluoride; [^{35}S]GTP-γ-S, guanosine 5′-*O*-[γ-[^{35}S]thio]triphosphate; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; Δ^9-THC, Δ^9tetrahydrocannabinol; WIN-55212-2, R-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoazin-6-yl)(1-napthalenyl)methanone monomethanesulfonate.

Received 28 January 1997; accepted 20 June 1997.

inactive enantiomer WIN55212-3 and methanandamide from R.B.I. SR141716A as well as CP55940 were synthesized in the chemistry department of Rhône-Poulenc Rorer. [^{35}S]GTP-γ-S (specific activity: 1250 Ci/mmol) was obtained from N.E.N. GTP-γ-S was from Sigma as were anandamide, Δ^9-tetrahydrocannabinol (Δ^9-THC), 11-hydroxy-Δ^9-THC, cannabinol, cannabidiol, phenylmethylsulfonylfluoride (PMSF) and fatty acid-free BSA.

Binding of [^{3}H]WIN55212-2 and [^{3}H]SR141716A to Rat Cerebellar Membranes

Rat cerebellar membranes were prepared as previously described by Kuster et al. [7]. Binding to rat cerebellar membranes was carried out in a final volume of 500 μL of a 20 mM HEPES buffer (pH 7.4) containing 1 mM $MgCl_2$ and 0.5 mg/mL fatty acid-free BSA. Dilutions of WIN55212-2 and SR141716A and of the radioactive ligands were made in the incubation buffer containing 5 mg/mL fatty acid-free BSA (final concentration of fatty acid-free BSA = 1.35 mg/mL). Routinely, 15 μg of protein were added to the incubation medium for 90 min at 30° with [^{3}H]WIN55212-2 (~1 nM, 50,500 dpm) or for 90 min at 22° with [^{3}H]SR141716A (~1 nM, 44,400 dpm) with or without the unlabelled ligand. Nonspecific binding was determined in the presence of 1 μM of WIN55212-2 or SR141716A, respectively. The incubation was terminated by filtration (12 mL of cold buffer) through Whatman GF/B filters pretreated for 3–4 hr with a solution of fatty acid-free BSA (5 mg/mL). Membrane-bound radioactivity was estimated by liquid scintillometry using Ready Solv® (Beckman). All assays were run in triplicate.

Binding of [^{35}S]GTP-γ-S to Rat Cerebellar Membranes

Rat cerebellar membranes (15 μg per tube) prepared as above were incubated with [^{35}S]GTP-γ-S (0.2 nM) in a final volume of 500 μL 20 mM HEPES buffer (pH 7.4) containing 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM GDP and 0.5 mg/mL fatty acid-free BSA with or without the compound to be tested. After a 90-min incubation at 30°, the medium was filtered (12 mL of cold buffer) through pretreated Whatman GF/B filters. Membrane-bound radioactivity was estimated as above. All assays were run in triplicate. Nonspecific binding was determined in the presence of 100 μM GTP-γ-S.

RESULTS AND DISCUSSION

New pharmacological tools for studying cannabinoid receptors have recently been described, including the synthetic agonist [^{3}H]WIN55212-2 [7] and the Sanofi antagonist [^{3}H]SR141716A [8]. The radiolabelled form of these two compounds provides the opportunity to study the modulation of agonist and antagonist binding to cannabinoid receptor by guanine nucleotides. The specific binding of

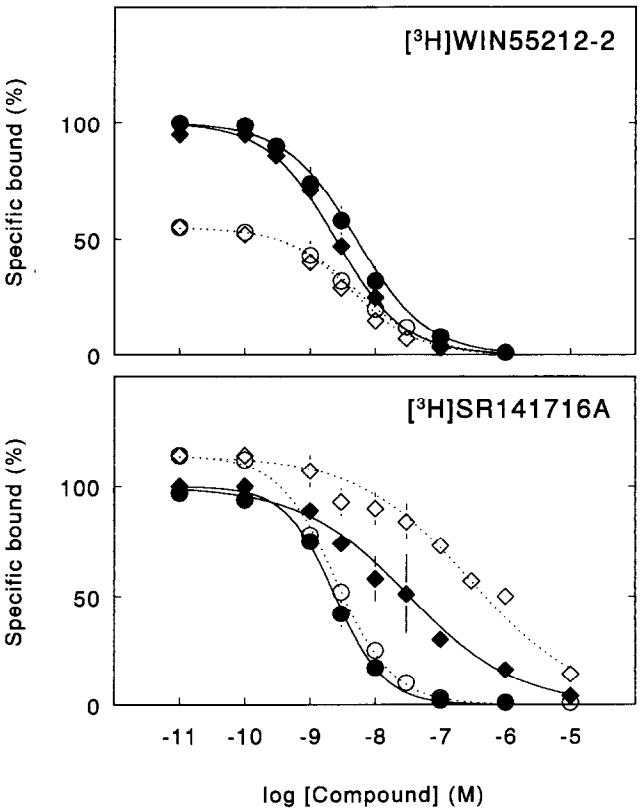

FIG. 1. Inhibition by WIN55212-2 and SR141716A of the binding of [^{3}H]WIN55212-2 and [^{3}H]SR141716A in the absence or presence of GTP-γ-S. Competition curves of ◆, ◇ WIN55212-2, and ●, ○ SR141716A, for [^{3}H]WIN55212-2 (upper panel) and [^{3}H]SR141716A (lower panel). The dashed lines and open symbols represent competition curves obtained in the presence of 10 μM GTP-γ-S. Each point is the mean ± SEM of three to five separate experiments performed in triplicate. Under our conditions, total binding of [^{3}H]WIN55212-2 represents 2814 ± 117 dpm and nonspecific binding 339 ± 34 dpm; for the binding of [^{3}H]SR141716A: total binding = 3824 ± 127, and nonspecific binding 529 ± 42 dpm.

[^{3}H]WIN55212-2 was reduced by GTP-γ-S (46% at 10^{-5} M), whereas the specific binding of [^{3}H]SR141716A was not reduced by GTP-γ-S but rather slightly increased (24% at 10^{-5} M). WIN55212-2 and SR141716A dose dependently inhibited the binding of [^{3}H]WIN55212-2, with an IC_{50} value of $3.10 \pm 0.50 \times 10^{-9}$M ($n_H = 0.85 \pm 0.14$) and of $5.20 \pm 1.80 \times 10^{-9}$M ($n_H = 0.87 \pm 0.03$), respectively. In the presence of GTP-γ-S (10 μM), the IC_{50} values were $5.20 \pm 1.00 \times 10^{-9}$M ($n_H = 0.94 \pm 0.19$) and $5.40 \pm 2.80 \times 10^{-9}$M ($n_H = 0.71 \pm 0.04$) for WIN55212-2 and SR141716A, respectively (Fig. 1). At this concentration of GTP-γ-S (10 μM), [^{3}H]WIN55212-2 binding capacity was decreased by approximately 50%. When binding experiments were performed with [^{3}H]SR141716A, a small increase in binding capacity could be noted in the presence of 10 μM GTP-γ-S (see above). WIN55212-2 and SR141716A dose dependently inhibited the binding of [^{3}H]SR141716A, with IC_{50} values of $3.40 \pm 1.90 \times 10^{-8}$ M

TABLE 1. Comparison between the IC_{50} value obtained in the inhibition of [^{3}H]WIN55212-2 binding and the EC_{50} value obtained in the stimulation of [^{35}S]GTP-γ-S binding

	[^{3}H]WIN55212-2	[^{35}S]GTP-γ-S	
	IC_{50}	EC_{50}	E_{max}
CP55940	$2.0 \pm 1.0 \times 10^{-10}$M	$9 \pm 3 \times 10^{-9}$M	200 ± 2%
WIN55212-2	$3.1 \pm 0.5 \times 10^{-9}$M	$9.9 \pm 4.5 \times 10^{-8}$M	175 ± 10%
WIN55212-3	$>1 \times 10^{-5}$M	$>1 \times 10^{-5}$M	—
Cannabinol	$2.0 \pm 0.3 \times 10^{-7}$M	$1.7 \pm 0.3 \times 10^{-7}$M	130 ± 2%
Δ^9-THC	$4.8 \pm 0.5 \times 10^{-8}$M	$5.3 \pm 3.1 \times 10^{-7}$M	154 ± 8%
11-hydroxy-Δ^9-THC	$3.1 \pm 0.5 \times 10^{-8}$M	$1.1 \pm 0.5 \times 10^{-7}$M	162 ± 6%
11-*nor*-Δ^8-THC-9-carboxylic acid	1×10^{-5}M	$>1 \times 10^{-5}$M	—
11-*nor*-Δ^9-THC-9-carboxylic acid	1×10^{-5}M	$>1 \times 10^{-5}$M	—
Anandamide	$1.5 \pm 0.2 \times 10^{-6}$M	$2.3 \pm 1.1 \times 10^{-6}$M	186 ± 12%
Anandamide (+PMSF)	$1.5 \pm 0.5 \times 10^{-8}$M	$5.4 \pm 1.3 \times 10^{-7}$M	173 ± 12%
Methanandamide	$1.6 \pm 0.1 \times 10^{-8}$M	$1.8 \pm 0.8 \times 10^{-7}$M	204 ± 17%
Methanandamide (+PMSF)	ND	$3.0 \pm 1.4 \times 10^{-7}$M	179 ± 14%
SR141716A	$5.2 \pm 1.8 \times 10^{-9}$M	$>1 \times 10^{-5}$M	—
CP55940 + SR141716A (1 μM)	—	$> \times 10^{-5}$M	—
WIN55212-2 + SR141716A (1 μM)	—	$>1 \times 10^{-5}$M	—
Δ^9-THC + SR141716A (1 μM)	—	$>1 \times 10^{-5}$M	—

The binding assays were performed with [^{3}H]WIN55212-2 and [^{35}S]GTP-γ-S as described in "Materials and Methods." IC_{50} and EC_{50} data correspond to mean values ± SEM obtained from three to eight independent experiments. Binding data were analyzed with GraphPad Prism™ software. ND: not determined. E_{max} was expressed in % of basal [^{35}S]GTP-γ-S binding (in absence of cannaboid receptor agonist). In a typical experiment, total [^{35}S]GTP-γ-S represent 5300 dpm and non-specific binding (in the presence of 100 μM GTP-γ-S) 135 dpm. When the IC_{50} were compared with the EC_{50}, the correlation coefficient and the slope of the correlation curve were 0.88 and 1.24, respectively.

($n_H = 0.53 \pm 0.01$) for WIN55212-2 and of $2.50 \pm 0.40 \times 10^{-9}$M ($n_H = 1.14 \pm 0.14$) for SR141716A, in the absence of GTP-γ-S. When GTP-γ-S (10 μM) was added to the incubation medium, the affinity of SR141716A did not change ($IC_{50} = 2.60 \pm 1.00 \times 10^{-9}$ M) ($n_H = 0.84 \pm 0.10$), but the affinity of WIN55212-2 decreased ($IC_{50} = 4.00 \pm 1.00 \times 10^{-7}$ M) ($n_H = 0.56 \pm 0.07$) (Fig. 1). The resolution of [^{3}H]SR141716A binding inhibition curves obtained with WIN55212-2 was also performed with a two-site model (GraphPad Prism™). IC_{50} values for WIN55212-2 were 2.80×10^{-9}M and 1.25×10^{-7}M without GTP-γ-S, whereas when 10 μM of GTP-γ-S was present the IC_{50} values obtained were 3.00×10^{-9}M and 1.07×10^{-6}M.

The situation could be interpreted by the existence of multiple affinity states of the cannabinoid receptor [16, 17], and further work should provide a tentative model concerning the affinities of agonists and antagonists for both high and low affinity states of the receptor.

One of the first biochemical events after agonist occupation of G protein-linked receptors is guanine nucleotide exchange. The validity of [^{35}S]GTP-γ-S binding as a functional measure of response has been demonstrated for other G protein-linked receptors [12, 13]. Cannabinoid receptor agonists are able to stimulate the binding of [^{35}S]GTP-γ-S to membranes from rat cerebellum [15]. This provides a functional correlate of ligand-binding experiments. The most potent agonist was CP55940, which increased [^{35}S]GTP-γ-S binding by *ca.* 100%. The rank order of potency reveals the classical pharmacology of a brain cannabinoid receptor; CP55940 > WIN55212-2 > cannabinol > Δ^9-THC > anandamide. Interestingly, the enantiomer of WIN55212-2, WIN55212-3, which is devoid of affinity, was also devoid of activity in [^{35}S]GTP-γ-S binding assay. Methanandamide is a stable analogue of anandamide and was thus more active. In the presence of PMSF (50 μM), the degradation of anandamide was reduced, resulting in an increase in activity. There was a good overall correlation ($r = 0.88$; $s = 1.24$) between the ability of different cannabinoids to compete with [^{3}H]WIN55212-2 binding on rat cerebellar membranes and to stimulate [^{35}S]GTP-γ-S binding on the same tissue (Table 1). SR141716A, described as a CB_1 selective antagonist, was devoid of action when used alone (no effect on [^{35}S]GTP-γ-S binding at 0.1 and 1 μM, and only a slight diminution of [^{35}S]GTP-γ-S binding at 10 μM). When SR141716A (1 μM) was used with various concentrations of WIN55212-2, CP55940, anandamide or Δ^9-THC, the effect of the agonist was antagonized. [^{35}S]GTP-γ-S binding could thus be a suitable method to distinguish between agonists and antagonists at the brain cannabinoid receptors.

Using the same preparation, we describe here the characteristics of the binding of an agonist ([^{3}H]WIN55212-2) and an antagonist ([^{3}H]SR141716A) and how these bindings are modulated by guanine nucleotides. A multiple state of affinity for the cannabinoid receptor seems to exist, but further studies are needed in order to determine how agonists and antagonists could interfere with the different states of the receptor. Finally, we demonstrate that cannabinoid receptor agonists are able to stimulate the binding of [^{35}S]GTP-γ-S to G proteins in rat cerebellar membranes. This assay provides a simple functional response for cannabinoid receptor agonists which could distinguish compounds with different intrinsic activities and, in combination with classical binding studies, compounds with variable efficacies.

The authors thank J.-C. Beaujouan, M.-C. Dubroeucq, A. Imperato and L. Pradier for helpful discussions and G. Le Fur for the original gift of SR141716A.

References

1. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI, Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346:** 561–564, 1990.
2. Gérard CM, Mollereau C, Vassart G and Parmentier M, Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* **279:** 129–134, 1991.
3. Howlett AC and Fleming RM, Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. *Mol Pharmacol* **26:** 532–538, 1984.
4. Bidaut-Russell M, Devane WA and Howlett AC, Cannabinoid receptors and modulation of cyclic AMP accumulation in the rat brain. *J Neurochem* **55:** 21–26, 1990.
5. Devane WA, Dysarz FA, Johnson MR, Melvin LS and Howlett AC, Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34:** 605–613, 1988.
6. Compton DR, Rice KC, de Costa BR, Razdan RK, Melvin LS, Johnson MR and Martin BR, Cannabinoid structure-activity relationships: correlation of receptor binding and *in vivo* activities. *J Pharmacol Exp Ther* **265:** 218–226, 1993.
7. Kuster JE, Stevenson JI, Ward SJ, D'Ambra TE and Haycock DA, Aminoalkylindole binding in rat cerebellum: selective displacement by natural and synthetic cannabinoids. *J Pharmacol Exp Ther* **264:** 1352–1363, 1993.
8. Rinaldi-Carmona M, Barth F, Héaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Néliat G, Caput D, Ferrara P, Soubrié P, Brelière JC and Le Fur G, SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350:** 240–244, 1994.
9. Petitet F, Marin L and Doble A, Biochemical and pharmacological characterization of cannabinoid binding sites using [^{3}H]SR141716A. *Neuro Report* **7:** 789–792, 1996.
10. Rinaldi-Carmona M, Pialot F, Congy C, Redon E, Barth F, Bachy A, Brelière JC, Soubrié P and Le Fur G, Characterization and distribution of binding sites for [^{3}H]-SR 141716A, a selective brain (CB_1) cannabinoid receptor antagonist, in rodent brain. *Life Sci* **58:** 1239–1247, 1996.
11. Hirst RA, Almond SL and Lambert DG, Characterisation of the rat cerebella CB1 receptor using SR141716A, a central cannabinoid receptor antagonist. *Neurosci Lett* **220:** 101–104, 1996.
12. Lorenzen A, Fuss M, Vogt H and Schwabe U, Measurement of guanine nucleotide-binding protein activation by A_1 adenosine receptor agonists in bovine brain membranes: stimulation of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding. *Mol Pharmacol* **44:** 115–123, 1993.
13. Traynor JF and Nahorski SR, Modulation by μ-opioid agonists of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* **47:** 848–854, 1995.
14. Sim LJ, Selley DE and Childers SR, *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 USA* **92:** 7242–7246, 1995.
15. Selley DE, Stark S, Sim LJ and Childers SR, Cannabinoid receptor stimulation of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding in rat brain membranes. *Life Sci* **59:** 659–668, 1996.
16. Houston DB and Howlett AC, Effector modulation of agonist and antagonist interactions with the rat brain CB_1 cannabinoid receptor. *International Cannabinoid Research Symposium* p. 66, 1996.
17. Kearn CS and Hillard CJ, Agonist affinity states of the brain cannabinoid receptor studied using [^{3}H]SR141716A. *International Cannabinoid Research Symposium* p. 37, 1996.