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European Journal of Pharmacology 377 (1999) 117–125

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Evaluation of the cannabinoid CB_2 receptor-selective antagonist, SR144528: further evidence for cannabinoid CB_2 receptor absence in the rat central nervous system

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Received 16 April 1999; received in revised form 25 May 1999; accepted 28 May 1999

Abstract

The aim of this study was to characterize the activity of the cannabinoid CB_2 receptor selective antagonist, *N*-{(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} (SR144528) in a number of biochemical assays and to look for evidence of cannabinoid CB_2 receptors in the rat central nervous system. SR144528 displaced [^{3}H]CP 55,940 ((−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol) from binding sites in CB_2- and CB_1-transfected cells ($K_i = 0.67 \pm 0.30$ and 33.0 ± 5.09 nM) and from rat cerebellum and whole brain membrane homogenates ($K_i = 54.7 \pm 9.70$ and 54.8 ± 7.86 nM). In the GTPγS binding assay, SR144528 antagonized a number of cannabinoid receptor agonists (K_B values ranging from 26.3 to 76.6 nM) in rat cerebellar membranes and in rat whole brain membranes ($K_B = 50.8$ nM). SR144528 also antagonized CP 55,940-stimulated GTPγS binding in a CB_2-expressing cell line ($K_B = 6.34$ nM). In *Xenopus* oocytes co-expressing the CB_1 receptor and G-protein coupled inwardly rectifying K^+ channels (GIRK 1/4), SR144528 antagonized WIN 55212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone)-stimulated K^+ currents ($K_B = 558$ nM). In summary, this report characterizes the cannabinoid CB_2 receptor-selective cannabinoid antagonist, SR144528, and additionally suggests an absence of cannabinoid CB_2 receptors in the rat central nervous system, an observation confirmed by Northern blot.

Keywords: Cannabinoid; Cannabinoid receptor antagonist; GTPγS binding; Radioligand binding; Cannabinoid CB_2 receptor; SR144528

1. Introduction

The pharmacological effects of cannabis and its derivative compounds, collectively known as cannabinoids, are thought to be largely mediated through two G-protein coupled receptors, CB_1 and CB_2 (Matsuda et al., 1990; Munro et al., 1993). CB_1 is found predominantly in the central nervous system, although it is also found in the periphery (Matsuda et al., 1990; Gerard et al., 1991; Glass et al., 1997), whereas CB_2 is presently thought to be located primarily in cells of the immune system and other peripheral areas and absent from the central nervous system (Galiegue et al., 1995; Griffin et al., 1997), although recent reports have suggested the presence of cannabinoid CB_2 receptors in the cerebellum (Skaper et al., 1996), and in cultured microglial cells (Kearn and Hillard, 1997). Both receptors have been shown to couple to adenylate cyclase, and to mitogen-activated protein kinase. CB_1 receptors have also been shown to couple to various ion channels (for review, see Pertwee, 1997). However, at the amino acid level, the two receptors are quite different, exhibiting only a 44% homology (Munro et al., 1993). The binding profile of various cannabinoid ligands also differentiates the two receptors (Showalter et al., 1996), and increasing research is being targeted towards the synthesis of novel ligands selective for these two subtypes. One particular aspect of this research has been the development of selective antagonists for these receptors, ideal pharmacological tools with which to investigate the differing roles which these subtypes play. In 1994, Rinaldi-Carmona et al. (1994) described an antagonist highly selective for

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00402-1

the CB_1 receptor, SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride) — a compound which has been widely used since in the assessment of CB_1-derived activities. However, until recently, such a selective antagonist for the cannabinoid CB_2 receptor has not been similarly available. In 1998, the same group announced the development of SR144528 (*N*-{(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide}), an antagonist with affinities at CB_1 and CB_2 receptors of 400 and 0.6 nM, respectively, demonstrating a 700-fold selectivity for the cannabinoid CB_2 receptor (Rinaldi-Carmona et al., 1998).

In this study, our aim was to further characterise this compound in several cannabinoid assay systems and subsequently to investigate whether or not the use of this compound may give rise to pharmacological evidence of the existence of cannabinoid CB_2 receptors in the central nervous system of the rat.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (150–250 g) were obtained from Harlan (Dublin, VA). *Xenopus laevis* frogs were purchased from Xenopus One (Dexter, MI). GDP and GTPγS were purchased from Boehringer Mannheim (Indianapolis, IN). [^{35}S]GTPγS (1000–1200 Ci/mmol) and [^{3}H]CP 55,940 ((−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol) were purchased from New England Nuclear (Boston, MA). Other reagent grade chemicals were purchased from Sigma (St. Louis, MO). Δ^9-tetrahydrocannabinol and SR144528 were obtained from National Institute of Drug Abuse (NIDA). CP 55,940 and SR141716A were generously provided by Pfizer (Groton, CT) and WIN 55212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone) was purchased from Research Biochemicals International (Natick, MA). Anandamide, 2-methylarachidonyl -(2′-fluoroethyl)amide (O-689) and dimethyl dimethyl-carboxamide delta-8-tetrahydrocannabinol (O-1125) were synthesized by Dr. Raj Razdan (Organix, Woburn, MA). Deoxy-HU-210 and 1-deoxy-delta-8-tetrahydrocannabinol dimethylheptyl (JWH-057) were synthesized by Dr. John Huffman (Clemson University, SC). All compounds were stored as 1 mg/ml solutions in ethanol at −20°C. The human cannabinoid CB_2 receptor cDNA was provided by Dr. Sean Munro (MRC, Cambridge, England). The mouse cannabinoid CB_1 receptor cDNA was cloned by this laboratory (Abood et al., 1997). G-protein-coupled inwardly rectifying K^+ channels (GIRK 1 and GIRK 4) cDNAs were generously supplied by Dr. Diomedes E. Logothetis, Mount Sinai School of Medicine, New York, NY. Human cannabinoid CB_1 receptor cDNA was provided by Dr. Marc Parmentier, Universite Libre de Bruxelles, Belgium).

2.2. Membrane preparation

Cerebella or whole brains minus the cerebella were dissected on ice from three fresh male Sprague–Dawley rats. The tissue was then homogenized in centrifugation buffer (50 mM Tris–HCl, 1 mM EGTA, 3 mM $MgCl_2$; pH 7.4) and the homogenate centrifuged at 48,000 × *g* for 20 min at 4°C. The pellet was then resuspended in assay buffer (50 mM Tris–HCl, 9 mM $MgCl_2$, 0.2 mM EGTA, 150 mM NaCl; pH 7.4), homogenized, and centrifuged as previously. The final pellet was then resuspended in assay buffer, homogenized, and diluted to a concentration of approximately 2 μg/μl with assay buffer. Membrane homogenates to be used for radioligand binding experiments were resuspended in buffer A (50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mg/ml fatty acid free bovine serum albumin, pH 7.4).

For experiments using mouse cannabinoid CB_1 receptor transfected human embryonic kidney 293 (mouse CB_1-293) cells, human cannabinoid CB_2 receptor transfected Chinese Hamster Ovary (human CB_2-CHO) cells and human cannabinoid CB_2 receptor transfected human embryonic kidney 293 (human CB_2-293) cells, the stable expression of the receptors, cell culture and membrane preparation, the methods of Abood et al. (1997) were used.

The protein concentrations of membrane preparations were determined by the method of Bradford (1976). Aliquots were then stored at −80°C.

2.3. [^{35}S]GTPγS binding

The methods for measuring agonist-stimulated [^{35}S]GTPγS binding were adapted from those of Sim et al. (1995). Rat cerebellar membranes (10 μg) or whole brain (minus cerebellum) membranes (25 μg) were incubated in assay buffer, containing 0.1% bovine serum albumin with GDP 100 μM, [^{35}S]GTPγS 0.05 nM and cannabinoids/ethanol control in siliconized glass tubes. The total assay volume was 0.5 ml which was incubated at 30°C for 30 min. The reaction was terminated by addition of 2 ml ice-cold wash buffer (50 mM Tris–HCl, 5 mM $MgCl_2$; pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass-fiber filters using a 12-well sampling manifold. The tubes were washed once with 2 ml of ice-cold wash buffer, and the filters were washed twice with 4 ml of ice-cold wash buffer. Filters were placed into 7 ml plastic scintillation vials (RPI, Mount Prospect, IL). Bound radioactivity was determined by liquid scintillation spectrophotometry after extraction in 5 ml BudgetSolve scintillation fluid, having been shaken for 1 h. Non-specific binding was determined using 10 μM GTPγS. Basal binding was assayed in the absence of agonist and in the

presence of GDP. The stimulation by agonist was defined as a percentage increase above basal levels (i.e., [(dpm (agonist) − dpm (no agonist))/dpm (no agonist)] × 100). GTPγS binding experiments using the human CB_2-293 cells was adapted from the conditions of MacLennan et al. (1998). Membranes (40 μg) were incubated (60 min) in cell assay buffer containing 10 mM HEPES, 100 mM NaCl, 32 mM $MgCl_2$, 320 μM GDP, 0.1% bovine serum albumin and 0.5 nM [^{35}S]GTPγS in a final volume of 500 μl. The cell assay buffer also contained 10 mM captopril and 0.1 mM phenylmethylsulphonyl fluoride. Filtration and counting methods were identical to those used for brain tissues.

2.4. Radioligand binding

The methods used for radioligand binding were essentially those described by Compton et al. (1993) with minor exceptions. Binding was initiated by the addition of membrane protein to siliconized tubes containing [^{3}H]CP 55,940 and a sufficient volume of buffer A (50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mg/ml fatty acid free bovine serum albumin, pH 7.4) to bring the total volume to 0.5 ml. Twenty microgram protein was used in experiments involving rat cerebellum and rat whole brain membranes, and 40 μg protein was used for CB_1- and CB_2-transfected cells. SR144528 (10^{-11}–10^{-6} M) was also included. Non-specific binding was determined in the presence of 1 μM CP 55,940. Following incubation of the tubes (30°C for 60 min), the reaction was terminated by addition of 2 ml ice-cold buffer B (50 mM Tris–HCl, 1 mg/ml bovine serum albumin; pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass-fiber (pretreated with polyethyleneimine (0.1%) for at least 4 h) using a 12-well sampling manifold. The tubes were washed once with 2 ml of ice-cold wash buffer, and the filters were washed twice with 4 ml of ice-cold wash buffer. Radioactivity was then determined as for the GTPγS binding experiments.

2.5. cRNA synthesis

Human cannabinoid CB_1 receptor in pcDNA was linearized by *Xba*I. PGEM-HE containing the cDNAs for the human homologs of GIRK1 and GIRK4 were linearized using *Nhe*I. The cRNAs were transcribed in vitro using a T7 RNA polymerase (mMESSAGE mMACHINE, Ambion; Austin, TX).

2.6. Expression in oocytes and recordings

Adult oocyte positive *X. laevis* frogs were housed in distilled, de-chlorinated water (18–20°C) with 12/12 h light/dark lighting cycle and fed twice weekly. Frogs were anesthetized using 0.25% MS-222 and a portion of the eggs were removed. The eggs were defolliculated with 1 mg/ml Collagenase type1A for 60–90 min. Human cannabinoid CB_1 receptor cRNA, 14–25 ng, and 0.1–0.3 ng of GIRK1/4 channel cRNA were co-injected per oocyte. Recordings were performed after 7–9 days of incubation in 0.5 × L-15 media supplemented with L-glutamine and antibiotics. For initial recordings, the eggs were placed in a chamber (total volume 200 ml) and perfused at 4 ml/min with low potassium containing (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES: pH 7.5), high potassium containing (2 mM NaCl, 96 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES pH 7.5), or high potassium + WIN 55212-2. Bovine serum albumin (3 μM) was added to all drug solutions to minimize adsorption of cannabinoid compounds to the perfusion system. Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.5–1 MW) and were voltage-clamped at reported voltages using an Axon GeneClamp amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 10 Hz, collected and analyzed using a Macintosh Centris 650 containing a 16-bit analog-digital interface board and voltage-clamp software running under the IGOR graphics environment (Wavemetrics, Lake Oswego, OR). Exchange of solutions was accomplished using a manual 6-port stream selection valve. In the presence of low potassium, high potassium and high potassium containing increasing concentrations of WIN 55,212-2, pulses of −80 mV were periodically applied an oocyte in order to monitor the current. When the inward current produced by perfusion with activating high potassium (I_{HK}) reached a stable steady-state, drug was added. In studies used to determine the K_e for SR144528, oocytes were pretreated with SR144528, at a concentration of 2 μM, for 5 min before addition of WIN 55,212-2.

2.7. Northern analysis

RNA was prepared from rat tissues using Trizol Reagant (Gibco BRL, Grand Island, NY). Equivalent amounts of RNA were denatured by heating at 65°C for 5 min in loading buffer and loaded onto 0.8% formaldehyde-agarose horizontal gels (Sambrook et al., 1989). Loading buffer consisted of 50% formamide, 6% formaldehyde, 20 mM boric acid, 10% glycerol, 0.2 mM EDTA, 0.1% bromphenol blue and 0.1% xylene cyanol. After denaturation, 1 μl ethidium bromide (1 mg/ml) was added to aid visualisation of the RNA samples. The gels were made with 0.8% agarose in 200 mM boric acid, pH 8.3, 0.2 mM EDTA and 3% formaldehyde and run in a buffer of the same composition at 100 mA. The gels were transferred to nylon membranes by capillary transfer, then UV-irradiated to fix the RNA onto the blot. The hybridization buffer for the Northern blots consisted of 0.5 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulphate (SDS), 1% bovine serum albumin and 1% EDTA, pH 8.0. The blots were prehybridized for 4 h, then 10^7 dpm/ml probe added and hybridization continued overnight at 60°C. The blots were

Table 1
Comparison of [^{3}H]CP 55,940 K_D and B_{max} values and SR144528 K_i values in CB_1- and CB_2-transfected cells, rat cerebellum and rat whole brain (minus cerebellum) membrane homogenates. Values are presented as means ± S.E. derived from 4–6 experiments, each performed in triplicate

	mCB_1-293	hCB_2-CHO	Cerebellum	Whole Brain (minus cerebellum)
[^{3}H]CP 55,940 K_D (nM)	1.50 ± 0.12	1.11 ± 0.09	1.08 ± 0.01	1.45 ± 0.95
B_{max} (pmol/mg protein)	2.06 ± 0.51	1.65 ± 0.71	3.79 ± 0.46	1.07 ± 0.37
SR144528 K_i (nM)	33.0 ± 5.09	0.67 ± 0.30	54.6 ± 9.70	54.8 ± 7.86

washed to a final stringency of 20 mM sodium phosphate, pH 7.4, 2 mM EDTA, pH 8.0, 1% SDS at 60°C for 1 h. A random primed probe was prepared from the rat cannabinoid CB_2 receptor gene (Abood and Tao, unpublished results) and labeled to a specific activity of $> 5 \times 10^8$ dpm/μg (Sambrook et al., 1989).

2.8. Data analysis

Data are reported as means ± standard error of the means of four to eight experiments, performed in triplicate. Non-linear regression analysis of concentration–response data was performed using Prism 2.0 software for the Macintosh (GraphPad Software, San Diego, CA) in order to calculate and compare E_{max} and EC_{50} values. The equilibrium dissociation constant (K_B) for the interaction of the antagonist and the receptor has been calculated from the equation $K_B = [B]/(\text{dose ratio} - 1)$, where [B] is the concentration of the antagonist used in the experiment (Schild, 1949). In experiments involving multiple concentrations of antagonist, the K_B value was calculated from Schild and/or Tallarida plots of the data. B_{max} and K_d

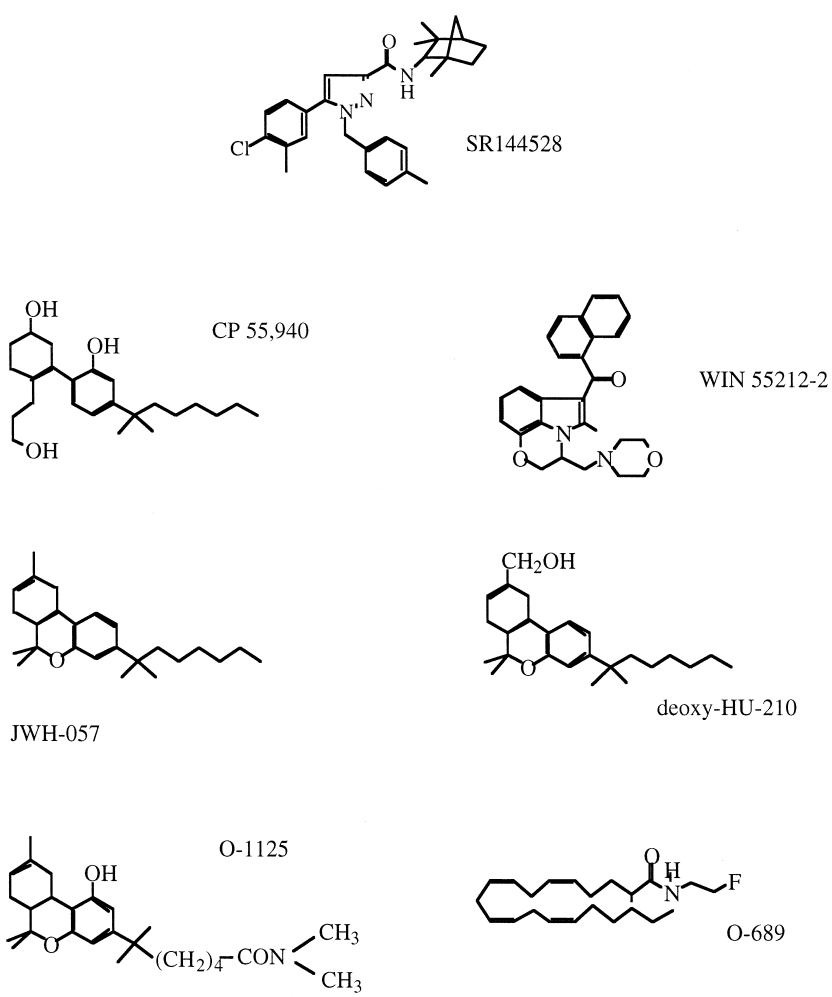

Fig. 1. Chemical structures of the compounds used in this study.

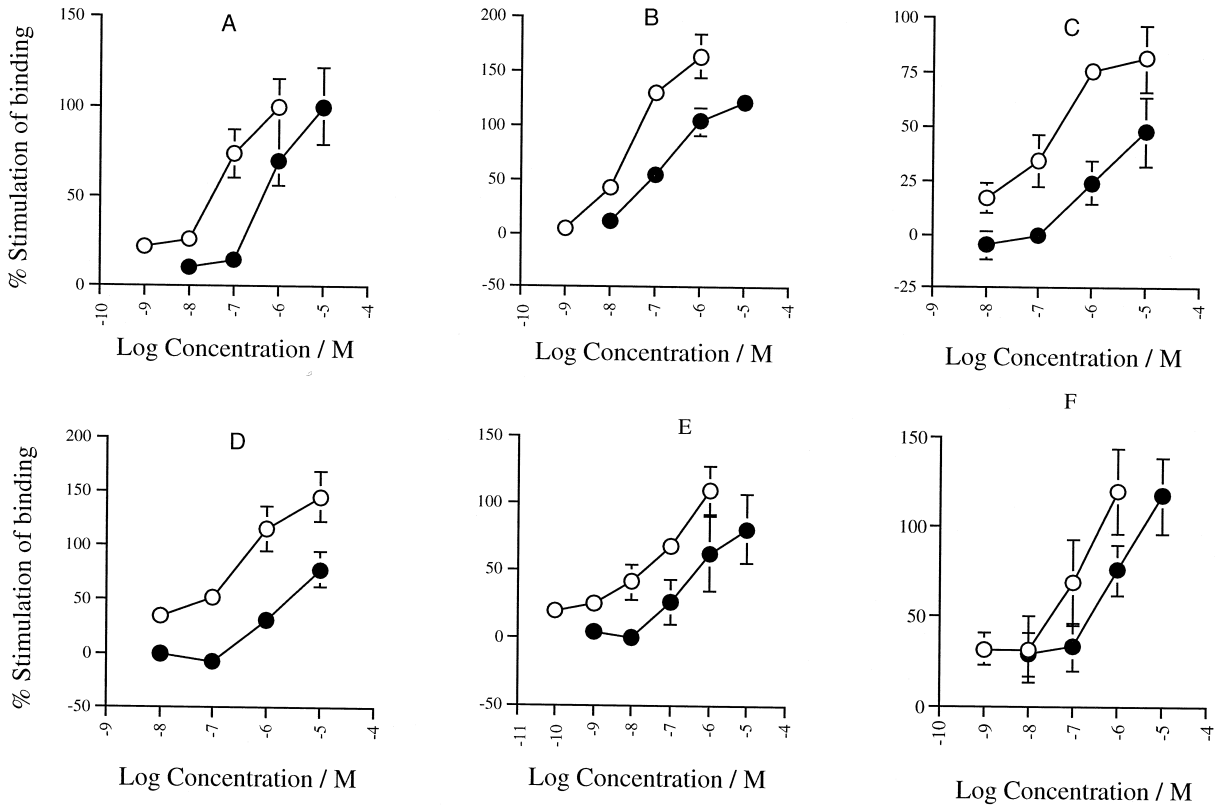

Fig. 2. Effect of SR144528, at a concentration of 1 μM (closed circles) on the ability of CP 55,940 (A), O-1125 (B), O-689 (C), WIN 55212-2 (D), Deoxy-HU-210 (E) and JWH-057 (F) to stimulate [^{35}S]GTPγS binding in rat cerebellar membrane homogenates. Concentration–response curves constructed in the absence of antagonist are shown with open circles. Data represent percentage stimulation over basal levels. Results are presented as means ± S.E. for 4–7 experiments, performed in triplicate.

values obtained from Scatchard analysis of saturation binding curves were determined by the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ). Displacement IC_{50} values were determined originally by unweighted least-squares non-linear regression of log concentration-percentage of displacement data and then converted to K_i values using the method of Cheng and Prusoff (1973). One-way analysis of variance using Bonferroni's post-hoc was used for comparison of K_i values ($P < 0.05$).

3. Results

3.1. Radioligand binding

In order to compare the affinity of SR144528 at cannabinoid CB_1- and CB_2-receptors and in the central nervous system, radioligand binding studies were carried out. K_d and B_{max} values for [^{3}H]CP 55,940 did not differ significantly between the cell lines and the two brain membrane tissues (Table 1). In contrast, the ability of SR144528 to displace [^{3}H]CP 55,940 was not identical for all of the membrane homogenates while the K_i value of SR144528 did not differ significantly between the CB_1 cell line, the rat cerebellum and the whole brain (minus cerebellum), all three differed significantly from the much higher affinity at cannabinoid CB_2 receptors (One-way ANOVA, Bonferroni's post-hoc, $P < 0.05$) (Table 1). These results confirm the observation that SR144528 is a cannabinoid CB_2 receptor-selective compound, and furthermore suggest the absence of substantial levels of cannabinoid CB_2 receptors in the rat central nervous system.

3.2. Antagonism of agonist-induced stimulation of GTPγS binding

In order to assess the ability of SR144528 to antagonise cannabinoid receptor agonists, GTPγS binding experi-

Table 2
Equilibrium dissociation constants (K_B values) of SR144528. Values are presented as means ± S.E. derived from 4–6 experiments performed in triplicate

Agonist	GTPγS assay (Cerebellum)
CP 55,940	76.6 (53.6–109) nM
O-1125	76.4 (59.0–98.7) nM
O-689	26.3 (12.0–48.7) nM
WIN 55212-2	36.1 (27.6–46.3) nM
deoxy-HU-210	50.5 (13.5–145) nM
JWH-057	56.7 (33.5–92.0) nM

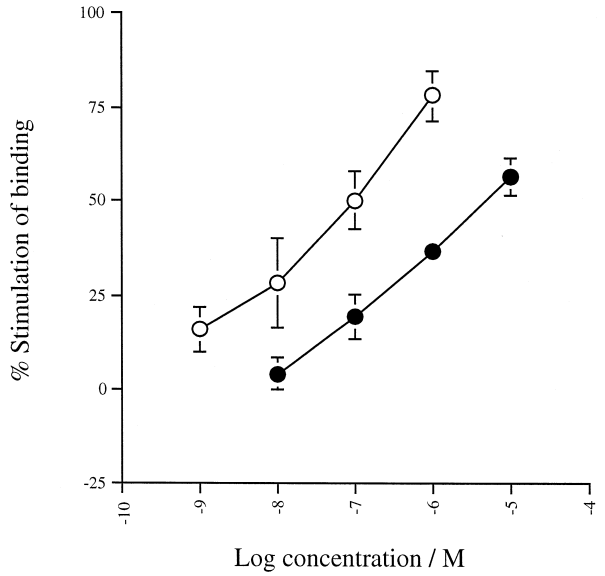

Fig. 3. Effect of SR144528, at a concentration of 1 μM (closed circles) on the ability of O-1125 to stimulate [^{35}S]GTPγS binding in rat whole brain (minus cerebellum) membrane homogenates. A concentration–response curve constructed in the absence of antagonist is shown with open circles. Data represent percentage stimulation over basal levels. Results are presented as means ± S.E. for five experiments, performed in triplicate.

ments were carried out in rat cerebellar membrane homogenates. The cannabinoid receptor agonists used in this study were chosen for both their high efficacies in the GTPγS binding assay and their range of selectivities for

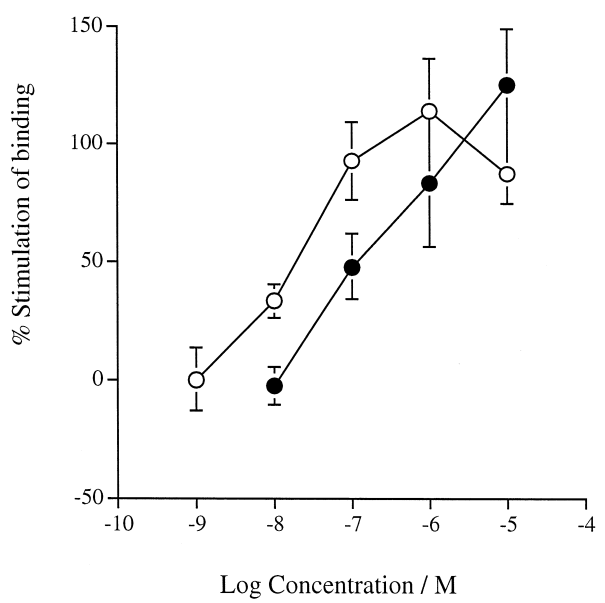

Fig. 4. Effect of SR144528, at a concentration of 50 nM (closed circles) on the ability of CP 55,940 to stimulate [^{35}S]GTPγS binding in membrane homogenates prepared from a human CB_2-293 cell line. A concentration–response curve constructed in the absence of antagonist is shown with open circles. Data represent percentage stimulation over basal levels. Results are presented as means ± S.E. for six experiments, performed in triplicate.

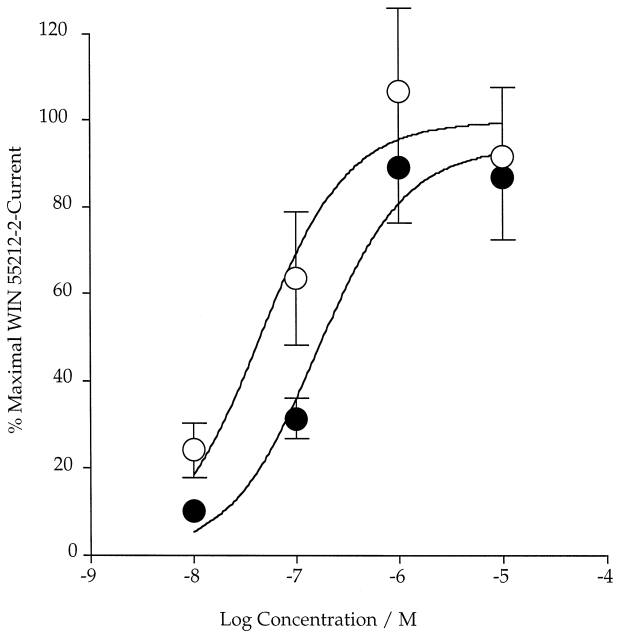

Fig. 5. Effect of SR144528, at a concentration of 2 μM (closed circles) on the ability of WIN 55212-2 to enhance the inward potassium current (I_{ag}) in *Xenopus* oocytes co-transfected with CB_1 receptors and GIRK 1/4 channels. A concentration–response curve constructed in the absence of antagonist is shown with open circles. Data represent percentage maximal current. Results are presented as means ± S.E. for six experiments.

the two cannabinoid receptors (ranging from a 35-fold cannabinoid CB_2 receptor selectivity (Deoxy-HU-210) to a 30-fold selectivity for CB_1 receptor (O-689). Compounds equally selective for both CB_1 and CB_2 receptors, such as O-1125, were also chosen (Showalter et al., 1996; Griffin et al., 1998). The structures of the compounds are shown in Fig. 1. Fig. 2 shows the ability of SR144528, at a concentration of 1 μM, to antagonise the ability of CP 55,940, O-1125, 2-methylarachidonyl-(2′-fluoroethyl)-amide, WIN 55212-2, deoxy-HU-210 and JWH-057 to stimulate GTPγS binding. JWH-057 is the only compound in this series whose activity in the GTPγS binding assay has not previously been described. Fig. 2F shows JWH-057 to be a full agonist, with an EC_{50} of 135 (30.0–612) nM. The equilibrium dissociation constants (K_B) for the antagonism of the various agonists ranged from 26.0 (12.0–48.7) to 76.6 (53.6–108.8) nM, and did not differ significantly in the presence of different agonists (Table 2).

SR144528, at concentrations of 0.1, 1 and 5 μM, produced a concentration-dependent antagonism of the ability of O-1125 to stimulate GTPγS binding in rat cerebellar membranes without affecting the E_{max} of the agonist (Results not shown). The unitary nature of the slope of the Schild plot confirmed the competitive and reversible nature of the antagonism as previously described (Rinaldi-Carmona et al., 1998). SR144528, at a concentration of 1 μM, also antagonised the stimulatory effects of O-1125 on GTPγS binding in the rat whole brain (minus

cerebellum) membrane homogenates (Fig. 3). The K_B value obtained was 50.8 (29.1–84.3) nM. As previously described, one of the aims of this study was to investigate whether the use of this antagonist would provide evidence of cannabinoid CB_2 receptors in the central nervous system. The results of the radioligand binding studies suggested the absence of these receptors in both the rat cerebellum and in the rat whole brain, a conclusion which is supported by the similarity of the K_B values obtained in the GTPγS binding experiments.

SR144528, at a concentration of 50 nM, also antagonised the stimulatory effects of CP 55,940 on GTPγS binding in membrane homogenates prepared from HEK-293 cells expressing the human cannabinoid CB_2 receptor (Fig. 4). The K_B value obtained was 6.34 (3.26–13.3) nM.

Alone, SR144528, at concentrations ranging from 0.01 to 10 μM, did not produce any evidence of agonist or inverse agonist effects in any of the receptor systems used in this study (data not shown).

3.3. Antagonism of agonist-enhancement of GIRK currents

In order to assess the activity of SR144528 in a functional assay known to contain solely CB_1 receptors, its activity in the *Xenopus* oocyte system was investigated. Application of increasing concentrations of WIN 55,212-2 led to a concentration-dependent enhancement of inward current (I_{ag}). The maximal response to WIN 55,212-2 was considered 100%. All responses are expressed as percent maximal WIN 55212-2 current: (I_{ag}/maximal I_{Ag} produced with WIN 55,212-2) × 100. SR144528, at a concentration of 2 μM, antagonised the effects of WIN 55,212-2 in oocytes expressing human CB_1/GIRK1/4, producing a parallel rightward shift of the concentration–response curve (Fig. 5). The equilibrium dissociation constant (K_B) of SR144528, calculated in the presence of WIN 55,212-2, was 0.56 (0.32–1.17) μM.

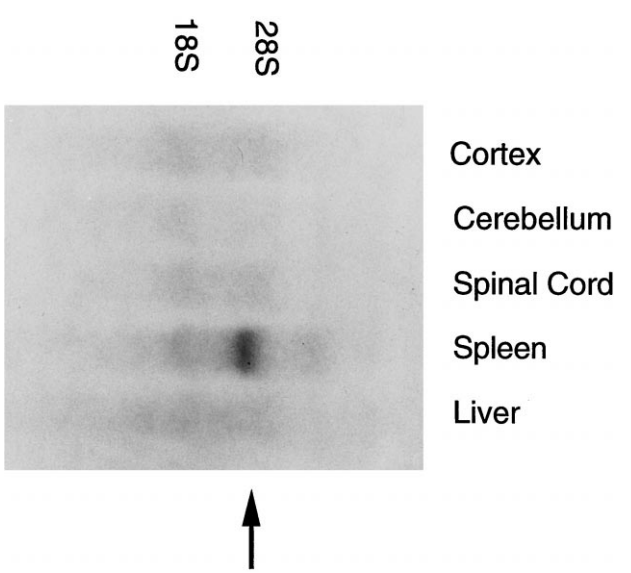

Fig. 6. Northern blot analysis of rat CB_2 RNA. Each lane contains 5 μg of total RNA. The arrow indicates the approximately 5.0 kb mRNA detected in spleen using rat CB_2. The blot was exposed for overnight. Longer exposure of the blot (1 week) did not produce the appearance of the signal in any other sample.

3.4. Northern blot analysis

The distribution of rat cannabinoid CB_2 receptor mRNA was assessed by Northern blot analysis. Under the conditions used, a single mRNA band of approximately 5.0 kb was detected in the spleen. However, there was no cannabinoid CB_2 receptor mRNA detected in samples prepared from central nervous system tissues, including the cerebellum, or from the liver (Fig. 6). Longer exposure of the blot did not result in the appearance of a signal in any of the other samples. This result is in accordance with the pharmacological data obtained using the GTPγS binding assays with centrally-derived tissues, which also suggested an absence of cannabinoid CB_2 receptors in the central nervous system.

4. Discussion

The purpose of this study was to further characterize the recently announced cannabinoid CB_2 receptor selective antagonist SR144528 by investigating its activities in radioligand binding assays and in two functional assays. The results of the binding assays confirm the CB_2 receptor selectivity of this compound, displaying a 40-fold higher affinity for human cannabinoid CB_2 receptors than mouse cannabinoid CB_1 receptors when stably expressed in cell lines. The affinity at cannabinoid CB_2 receptors observed in this study, 0.67 ± 0.30 nM, closely correlates with that of Rinaldi-Carmona et al. (1998) who reported a value of 0.60 nM at stably expressed human cannabinoid CB_2 receptors. However, the affinity of SR144528 for the CB_1 receptor is an order of magnitude higher in this study than that of Rinaldi-Carmona et al. (1998). As a result of this difference, SR144528 displays a markedly lower selectivity in this study than previously reported, 50-fold rather than >1000-fold. The reasons for this discrepancy are presently unknown. One question that is yet to be satisfactorily answered in the field of cannabinoid pharmacology is the possible presence of cannabinoid CB_2 receptors in the central nervous system. By using a strongly cannabinoid CB_2 receptor selective compound, such as SR144528, it may be possible to observe a significant difference in the affinity of this compound between a homologous (CB_1 or CB_2) receptor population and a heterologous one, possibly brain tissue. The experiments with membranes prepared from rat cerebellum and rat whole brain minus the cerebel-

lum provided K_i values that did not differ significantly from that obtained in the mouse CB_1 cell line. This suggests that, on the basis of this evidence, the rat brain does not express a numerically significant population of cannabinoid CB_2 receptors, although the possibility of a distinct difference between the rat cannabinoid CB_2 receptor and the human cannabinoid CB_2 receptor cannot be discounted. Interestingly, the amino acid composition of the rat cannabinoid CB_2 receptor has an approximately 81% homology with the sequence of the human receptor (Abood and Tao, unpublished results). A further possible explanation could be that any CB_2 receptors present in the brain are present in such a low relative number to the CB_1 receptors that their presence is being masked. A compound with a high cannabinoid CB_2 receptor affinity and a negligible affinity for the CB_1 receptor may provide a more definitive answer. A further, speculative, possibility is that any cannabinoid CB_2 receptors present in the region may not be membrane-bound and therefore lost in the preparation of the cerebellar homogenates used in this study.

The results of the experiments with the GTPγS binding assays confirm the antagonist nature of SR144528. SR144528, at concentrations ranging from 316 to 3160 nM, antagonized the stimulatory effects of several cannabinoid receptor agonists on GTPγS binding in cerebellar and whole brain membrane homogenates, without having any effect of its own. This antagonism was consistent with a competitive reversible nature. the rightward shifts of agonist concentration–response curves produced by the antagonist did not significantly deviate from parallelism nor involve a reduction in the E_{max} of the agonist. Furthermore, when several concentrations of antagonist were used in the presence of a single agonist, a Schild plot was produced with a slope of unity. The K_B values of SR144528 did not differ significantly between the various agonists used (each selected because of their own differing selectivities at cannabinoid CB_1 and CB_2 receptors), nor from the K_i values observed at cannabinoid CB_1 receptors and in brain tissues. These results taken together provide two conclusions. Firstly, they further support the observation that there is not a significant population of cannabinoid CB_2 receptors in the rat cerebellum. Secondly, they also suggest that SR144528 acts as an antagonist at cannabinoid CB_1 receptors.

SR144528, at a concentration of 50 nM, also demonstrated an ability to antagonize CP 55,940-stimulated GTPγS binding in membranes prepared from a cannabinoid CB_2 receptor expressing cell line without affecting the level of GTPγS binding itself. Similarly to its activity in the brain, SR144528 caused a parallel rightward shift of the CP 55,940 concentration response curve without significantly affecting the E_{max} of the agonist. The K_B of SR144528 was calculated to be 6.34 (3.26–13.29) nM, demonstrating that SR144528 acts as an antagonist at the cannabinoid CB_2 receptor. Interestingly, this value is significantly higher than the K_i of SR144528 at cannabinoid CB_2 receptors. The reasons for this remain to be established.

Using membranes prepared from rat cerebellum, rat whole brain minus the cerebellum and a human cannabinoid CB_2 receptor cell line, SR144528 was inactive and neither stimulated GTPγS binding nor caused a significant inhibition of GTPγS binding over basal levels. This is in contrast to several studies, including the original report by Rinaldi-Carmona et al. (1998). However, as reported by MacLennan et al. (1998), the observation of inverse agonism by SR141716A at CB_1 receptors in the GTPγS binding assay is sensitive to the assay conditions used. Under the conditions used in this study no evidence of inverse agonism was obtained.

In the other functional assay system used in this study, the co-expression of CB_1 receptors and GIRK1/4 channels in *Xenopus* oocytes, SR144528 also antagonized the ability of WIN 55212-2 to produce an enhancement of the GIRK current. The antagonism again produced a parallel rightward shift of the agonist concentration response curve without affecting the E_{max}. This result again demonstrates the ability of SR144528 to act as an antagonist of CB_1 receptors. Interestingly, the K_B obtained in this experiment was significantly higher (approximately 10-fold) than that obtained in the brain tissue GTPγS binding experiments and also the K_i at CB_1 receptors. The value was much closer to the CB_1 K_i reported by Rinaldi-Carmona et al. (1998). However, this experimental model has also been used to test the CB_1-selective antagonist SR141716A, and the K_B obtained for that antagonist was again approximately 10-fold higher than comparable experiments using the GTPγS binding assay (Griffin et al., 1998; McAllister et al., 1998). This discrepancy may be the result of the method of drug administration. The GTPγS assay involves the direct incubation of the receptor with the ligand, whereas the oocyte model uses a constant perfusion system of the appropriate concentration of drug. It may be that by using this constant flow of drug over the tissue does not allow sufficient time for the drug to equilibrate with the receptor, and that the actual concentration of drug reaching the active site is lower than anticipated.

In order to further test the hypothesis that there is an absence of cannabinoid CB_2 receptors in the central nervous system, a Northern blot was constructed using RNA prepared from rat cerebellum, cortex, spinal cord, liver and spleen and a probe constructed from the rat cannabinoid CB_2 receptor sequence (Abood and Tao, unpublished results). It was found that although a strong signal was detected in the spleen, no cannabinoid CB_2 receptor mRNA was detected in the cerebellum, cortex, spinal cord or liver. This further supports the pharmacological evidence, derived from the GTPγS binding assays in the cerebellum and whole brain tissues, that the rat central nervous system does not appear to contain a substantial population of cannabinoid CB_2 receptors.

In summary this report confirms the findings of Rinaldi-Carmona et al. (1998) that SR144528 is indeed a cannabinoid CB_2 receptor-selective antagonist, although the degree of selectivity found in this study is considerably lower than that previously reported. This finding is highly relevant for future studies that may use this compound in order to attribute a cannabinoid CB_2 receptor-based mechanism of action to a particular observation. The higher CB_1 receptor affinity and lower CB_2 receptor selectivity observed in this study indicate care should be taken to use antagonist concentrations at which significant CB_1 binding would not be expected to occur. The study also presents evidence to suggest the absence of cannabinoid CB_2 receptors from the central nervous system of the rat. Since the discovery of the cannabinoid CB_2 receptor (Munro et al., 1993), progress in isolating the roles of this receptor in the body has been hampered by the lack of selective compounds, both agonists and antagonists, and this compound, along with others that may be produced as a result, or in addition, to these should assist in the understanding of this subtype of cannabinoid receptor.

Acknowledgements

This work was supported in part by National Institute on Drug Abuse Grants DA-09978, DA-05274, DA-09789 and the Council for Tobacco Research Grant CTR-4482.

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