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SR141716A, a potent and selective antagonist of the brain cannabinoid receptor

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Abstract

SR141716A is the first selective and orally active antagonist of the brain cannabinoid receptor. This compound displays nanomolar affinity for the central cannabinoid receptor but is not active on the peripheral cannabinoid receptor. In vitro, SR141716A antagonises the inhibitory effects of cannabinoid receptor agonists on both mouse vas deferens contractions and adenylyl cyclase activity in rat brain membranes. After intraperitoneal or oral administration SR141716A antagonises classical pharmacological and behavioural effects of cannabinoid receptor agonists. This compound should prove to be a powerful tool for investigating the in vivo functions of the anandamide/cannabinoid system.

Key words: Cannabinoid receptor; Receptor antagonist; cAMP; Behavioural responses

1. Introduction

The behavioural and pharmacological actions of the active constituents of marijuana have been intensively studied and well documented [1]. Recent reports have shown that the major psychoactive component of cannabis, Δ^9-THC, as well as the putative endogenous ligand for the cannabinoid receptor, anandamide [2] mediate their pharmacological effects through a specific G protein-coupled receptor [3] which has been recently cloned both in rat [4] and human [5]. This receptor, designated CB1, is found in brain but also in lower abundance in some peripheral tissues [6,7]. Recently a novel type of cannabinoid receptor, designated CB2, that seems to be only expressed at the periphery and involved in cannabinoid-mediated immune modulation, has been described [8]. In the last few years, a number of potent synthetic cannabinoid agonists have been developed [9,10,11]. A putative cannabinoid receptor antagonist has been recently claimed [12], but no evidence has been found supporting in vivo antagonist effects of this compound [13]. Therefore, the search for more potent antagonists of the cannabinoid receptor was warranted. Based on both binding and functional data, this paper introduces SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride] (Fig. 1), as a novel, highly potent, selective and orally active antagonist for CB1. This discovery provides a new tool to better understand the mechanisms by which marijuana produces its pharmacological effects and to develop potential therapeutic agents.

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Abbreviations: Tris, Tris-[hydroxymethyl]aminomethane, Δ^9-THC, tetrahydrocannabinol; i.p., intraperitoneal; p.o., per os; i.v., intravenous; CHO, chinese hamster ovary.

2. Materials and methods

2.1. Materials

Bovine serum albumin was from Boehringer. Forskolin was from Sigma. Polyethylenenimine was purchased from Serva. Biofluor liquid scintillant and [^{3}H]CP 55,940 (111.9 Ci/mmol) were purchased from New England Nuclear Corp. A cAMP kit was from Amersham. Dimethyl sulfoxide was purchased from Prolabo. Tris was purchased from Merck-Clevenot. CP 55,940 {[1α,2-(*R*)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol} was generously provided by Pfizer. WIN 55212–2 {*R*-(+)-(2,3-dihydro-5-methyl-3-[{4-morpholinyl}methyl]pyrol [1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl)methanone monomethanesulfonate} was purchased from RBI. SR141716A {*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (Fig. 1) and anandamide {arachidonylethanolamide}were synthesised at Sanofi Recherche. Drugs were dissolved either in ethanol (anandamide, Δ^9-THC) or dimethyl sulfoxide (CP55,940, WIN55212–2, SR141716A). The concentration of solvent in assays never exceeded 0.1% (v/v). This final concentration was without effect on radioligand binding. Male Sprague–Dawley rats (180–220 g) were obtained from Charles River (France) and used for in vitro binding studies. Male Swiss mice (30–35 g) were obtained from CERJ, Le Genet St. Isle (France) and used for isolated vasa deferentia preparations. Male mice (Of1, 25–27 g) and rats (Ofa, 120–140 g) were obtained from Iffa Credo (France) and used for psycopharmacological tests.

2.2. Membrane preparations

Rat membranes were prepared from the brain, minus the cerebellum or the substantia nigra as in [14] or from the spleen as in [6]. They were suspended in 20 mM HEPES (pH 7.4) at a final protein concentration of 10 mg/ml. Membranes isolated from CHO cells expressing either

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CB1, obtained from a frontal cortex cDNA library, or CB2 isolated from a monocytic cell line U937 cDNA library [15,16], were prepared 56 h after transfection as in [17]. They were suspended in 50 mM Tris-HCl (pH 7.4) at a final protein concentration of 0.3 mg/ml. Protein concentration was determined as in [18] and membranes were stored at −80°C until use.

2.3. Binding experiments

For in vitro binding assays membranes (7–50 μg) were incubated at 30° C with [^{3}H]CP 55,940 in 1 ml of buffer A (50 mM Tris-HCl, pH 7.7) for 1 h. A rapid filtration technique using Whatman GF/C filters (pre-treated with polyethylenenimine 0.5% (w/v)) and a 48-well filtration apparatus (Brandel) was used to harvest and rinse labelled membranes (3 × with 5 ml of cold buffer A containing 0.25% bovine serum albumin). The radioactivity bound to the filters was counted with 4 ml of biofluor liquid scintillant. Non-specific binding was determined in the presence of 1 μM CP 55,940. Data from equilibrium binding (K_d, B_{max}) and competition experiments (IC_{50}, ED_{50}) were analyzed using a non-linear least-squares method on a Compak Desk Pro 4/66i computer. All experiments were performed in duplicate and results were confirmed in at least three independent experiments.

For selectivity, binding assays were carried out using standard protocols.

2.4. Isolated mouse vasa deferentia preparations

Assays were performed as previously described [2,19]. Drugs were added once the contractile responses to electrical stimulation were reproducible. Preparations were exposed to cumulative increasing concentrations of CP 55,940 or anandamide to obtain concentration-response curves either in the absence (control) or in the presence of SR141716A added at a fixed concentration 60 min and 120 min before the first concentration of CP 55,940 and anandamide, respectively.

2.5. Adenylyl cyclase assays

Basal and forskolin-stimulated adenylyl cyclase activities were carried out in rat substantia nigra membranes (25 μg) as in [20] and in CHO cells expressing CB1 or CB2 as in [21]. The cAMP concentration was determined by radioimmunoassays.

2.6. Drug preparation and administration

For in vivo experiments, SR141716A was dissolved in two drops of Tween 80, diluted in distilled water and administered i.p. (30 min) or p.o. (1 h) in a volume of 20 ml/kg to male mice or 5 ml/kg to male rats before the i.v. injection of WIN55212–2 (dissolved in two drops of Tween 80 and diluted in saline solution). Doses are expressed as the salt.

2.7. Psychopharmacological tests, in rodents

Behavioural responses induced by WIN55212–2 were assessed either 15 min (tail flick [22], ring-immobility [23]) or 30 min (hypothermia [24]) after the administration of SR141716A. Pop corn effects in mice and barrel rotations in rats were analyzed as described in [25] and [26], respectively.

3. Results and discussion

3.1. Interaction of SR141716A with brain cannabinoid receptors in vitro

As shown in Fig. 2A, known cannabinoid drugs and SR141716A displaced in a concentration-dependent manner [^{3}H]-CP55,940 specifically bound to its high affinity receptor in rat brain synaptosomal membranes. The concentration-response curves gave K_i values of 1.37 ± 0.43, 9.94 ± 1.04, 35.30 ± 5.05 and 1.98 ± 0.36 nM ($n = 4$) for CP55,940, WIN55212–2, Δ^9THC and SR141716A, respectively. In contrast, as shown in Fig. 2B, SR141716A displayed no affinity for the cannabinoid receptor expressed in rat spleen, except at a very high concentration (38.6 ± 2.5% inhibition at 10^{-6} M, $n = 3$), whereas CP55,940, WIN55212–2 and Δ^9-THC acted as potent displacers of these sites (K_i = 1.37 ± 0.38 nM, $n = 8$, 16.2 ± 5.5 nM, $n = 4$ and 3.90 ± 0.95 nM, $n = 5$, respectively). Furthermore, in membranes isolated from CHO cells expressing human CB1, SR141716A was a potent inhibitor of [^{3}H]-CP55,940 binding sites with a K_i value of 5.6 ± 0.5 nM ($n = 4$), whereas SR141716A displayed only a very low affinity for membranes from CHO cells expressing human CB2 (38.7 ± 0.8% inhibition at 10^{-6} M, $n = 4$). These results show that SR141716A is selectif for CB1 versus CB2 and interacts with the cloned human cannabinoid receptor.

In the presence of 2 nM SR141716A, [^{3}H]CP55,940 saturation binding experiments performed in rat brain membranes showed a significant increase ($P < 0.05$) in the dissociation constant (K_d) of [^{3}H]CP55,940 (SR141716A, K_d = 0.23 ± 0.04 nM versus control, K_d = 0.09 ± 0.01 nM, $n = 3$) with no significant change ($P > 0.05$) in the maximum number of receptors (B_{max}) of [^{3}H]CP55,940 (SR141716A, B_{max} = 0.94 ± 0.10 pmol/mg of protein versus control, B_{max} = 0.95 ± 0.04 pmol/mg of protein). These results indicate that SR141716A is a competitive ligand for CB1.

3.2. Receptor binding profile of SR141716A

SR141716A had no affinity ($IC_{50} > 1$ μM) for any of the other types of receptors or channels investigated [histamine (H_1, H_2, H_3), dopamine (D_1, D_2), adrenergic (α_1, α_2, β_1, β_2), adenosine (A_1, A_2), purinergic (P_{2Y}), opiate, neurotensin, cholecystokinin (A, B), benzodiazepine, sigma, tachykinin (NK_1, NK_2, NK_3), excitatory or inhibitory amino acids (glycine, AMPA, kainate, NMDA, $GABA_A$, $GABA_B$), 5-hydroxytryptamine (5-HT_1, 5-HT_2, 5-HT_3) receptors and Cl^-, Na^+ (site1, site2), Ca^{2+} (N, L) and K^+ channels. These findings show that SR141716A is a very selective ligand for CB1.

3.3. Cannabinoid receptor antagonism properties of SR141716A in vitro

Cannabinoid agonists have been shown to inhibit neuronally stimulated smooth muscle contractions [19]. As

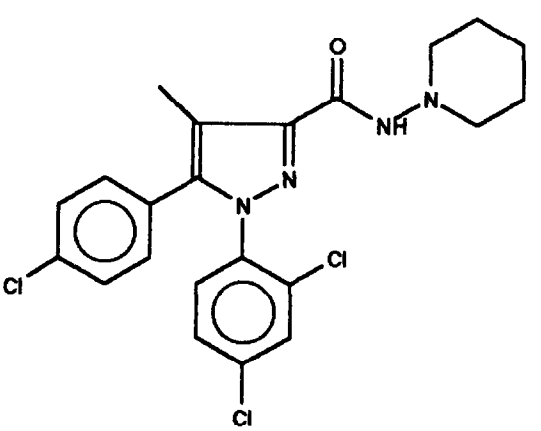

Fig. 1. Structure of SR141716A.

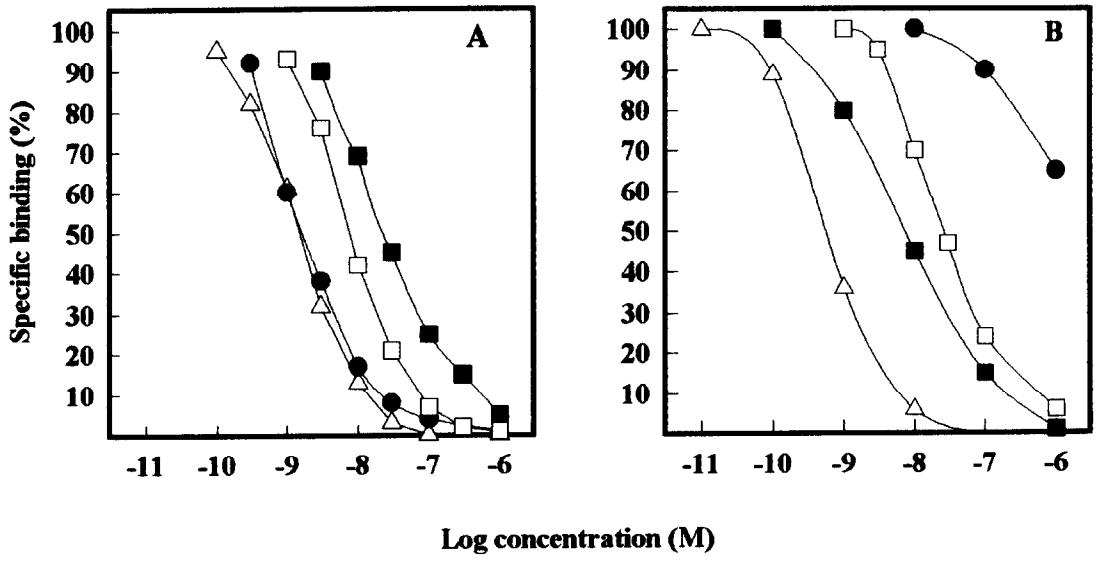

Fig. 2. Inhibition of [^{3}H]CP55,940 binding to rat brain (A) and spleen (B) membranes by various cannabinoid drugs and SR141716A. Binding assays were carried out at 30°C as described in section 2 using 0.1 nM [^{3}H]CP55,940 and increasing concentrations of CP 55,940 (△), WIN 55,212–2 (□), Δ^9THC (■) and SR141716A (●). Data are from one experiment out of 3 performed in duplicate and are expressed as the percentage of specific binding in the absence of competitor.

shown in Fig. 3, in the mouse vas deferens CP 55,940 and anandamide induced a concentration-dependent inhibition of the twitch contractions with pD_2 values of 8.41 ± 0.03 and 7.8 ± 0.04, respectively. SR141716A produced a concentration-dependent rightward and almost parallel shift of the concentration–response curve for both compounds, showing that it behaved as a competitive antagonist versus the synthetic cannabinoid agonist CP55,940 and the putative endogenous ligand anandamide with pA_2 values of 7.98 ± 0.03 and 8.17 ± 0.06, respectively.

Cannabinoid receptor activation has been shown to inhibit cAMP accumulation [20]. As previously described [12], the cannabinoid agonist WIN55212–2 inhibited in a concentration-dependent manner (IC_{50} = 40.0 ± 3.6 nM, n = 3), the forskolin-stimulated adenylyl cyclase activity in rat substantia nigra synaptosomal preparations. A maximum inhibition of

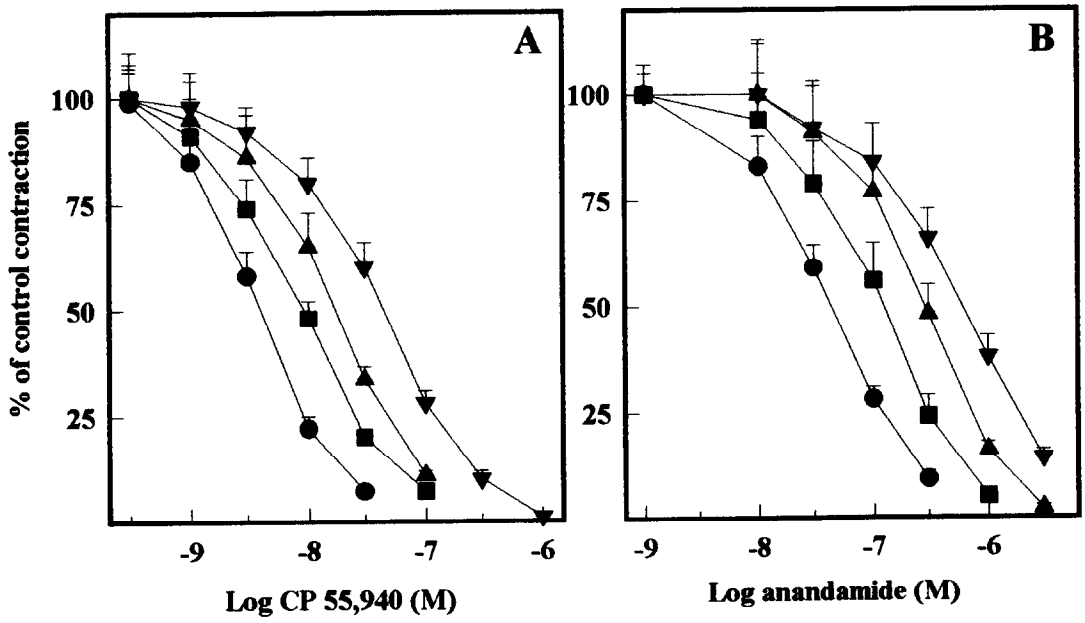

Fig. 3. Cumulative concentration-response curves for CP 55,940 (A) or anandamide (B) on the amplitude of twitch contractions elicited by electrical field stimulation of the mouse vas deferens obtained in the absence (●) (control) and in the presence of SR141716A at 10^{-8} M (■), 3×10^{-8} M (▲) and 10^{-7} M (▼). Assays were performed as described in section 2. Data are expressed as a percentage of control values after incubation with SR141716A. Each point is the mean value ± S.E.M. of 6 determinations.

35.0 ± 1.5% ($n = 8$) was observed at 10^{-5} M of WIN55212–2. SR141716A, which produced no significant effect on either basal or forskolin-stimulated adenylyl cyclase activity completely reversed the inhibition exerted by WIN55212–2 (10^{-5} M) with an IC_{50} value of 48.0 ± 2.7 nM ($n = 3$). In CHO cells expressing either human CB1 or CB2, CP55,940 exerted an inhibitory effect on the forskolin-stimulated adenylyl cyclase with IC_{50} values of 1.27 ± 0.37 nM ($n = 3$) and 2.51 ± 0.25 nM ($n = 3$), respectively. SR141716A which produced no effect by itself, completely antagonised the inhibition elicited by CP55,940 (3×10^{-9} M) in CHO cells expressing CB1 (IC_{50} = 5.6 nM) but not in cells expressing CB2 ($IC_{50} > 1$ μM). Taken together, these in vitro results indicate that SR141716A is a potent and selective antagonist of the brain cannabinoid receptor.

3.4. *In vivo antagonism of SR141716A at central cannabinoid receptors*

The in vivo antagonism of SR141716A for the brain cannabinoid receptors was investigated in several animal models classically used to study cannabinoid drug effects. As previously described [13], WIN55212–2 injected i.v. (0.8 mg/kg) produced hypothermia (−3.5°C), ring immobility (85% immobility) and increased tail-flick latency (12 s) in mice. In addition, WIN55212-2 (1.6 mg/kg, i.v.) induced 'pop corn' effects in mice, hypothermia (−3°C) and barrel rotations in rats. As shown in Table 1, SR141716A administered i.p. or p.o. potently and dose-dependently antagonised the responses elicited by WIN55212–2 in all evaluation tests. Interestingly, SR141716A alone failed to produce any effect on these pharmacological and behavioural models, suggesting that control of body temperature as well as behaviour responses to noxious heat are not under the tonic influence of an endogenous CB1 ligand. Furthermore, SR141716A administered i.p. did not antagonise the hypothermia produced by reserpine (2 mg/kg, i.p.), oxotremorine (0.1 mg/kg, p.o.) or apomorphine (1 mg/kg, subcutaneous) in mice. As shown in Fig. 4, the reversal by SR141716A of WIN55212–2-induced hypothermia

Table 1
Antagonism by SR141716A of pharmacological and behavioural responses induced by WIN55212-2, in rodents

Test	Species	WIN55212-2 (mg/kg, i.v.)	ED_{50} of SR141716A (mg/kg [95% CL])
Hypothermia	mouse	0.8	0.28 [0.12–0.65], i.p. 0.38 [0.21–0.71], p.o.
	rat	1.6	0.11 [0.03–0.38], i.p. 0.15 [0.03–0.36], p.o.
Antinociception	mouse	0.8	1.62 [0.24–10.9], i.p.
Ring-immobility	mouse	0.8	1.70 [0.70–4.30], i.p.
Popcorn effect	mouse	1.6	0.62 [0.46–0.84], i.p.
Barrel rotations	rat	1.6	0.54 [0.31–0.90], i.p.

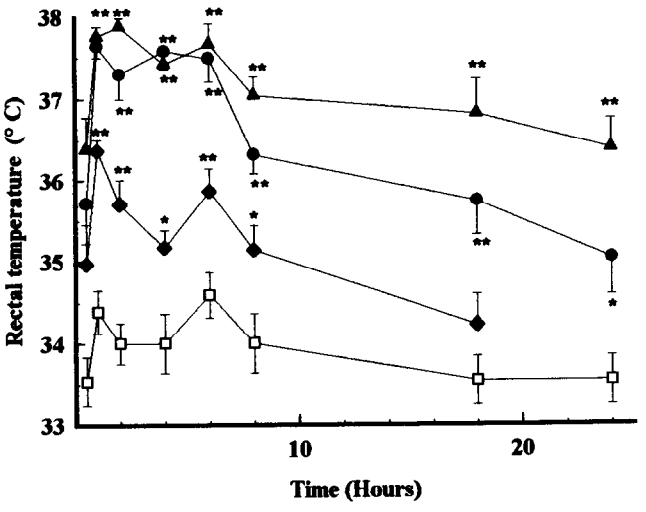

Fig. 4. Time course of the antagonism of WIN55212–2 induced hypothermia by SR141716A, in mice. Groups of mice (10 per group) were administered orally with vehicle (□), 1 (◆), 3 (●) or 10 mg/kg (▲) of SR141716A. At different times after the administration of SR141716A the effect of WIN55212–2 injected intravenously (0.8 mg/kg) on colonic temperature was evaluated. All data are the mean ± S.E.M. The Student *t*-test was used to obtain a measure of significance between control and SR141716A treated groups (*$P < 0.05$, **$P < 0.01$).

was time-dependent and significant for at least 18 h after administration at 3 mg/kg (p.o.). These results show that SR141716A is a functional antagonist of the brain cannabinoid receptor (CB1) with good oral bioavailability and a long duration of action.

4. Conclusion

In this report we describe for the first time a potent, selective and orally effective cannabinoid receptor antagonist, SR141716A, that has a 1000-fold higher affinity for the CB1 receptor than for the recently described CB2 receptor. Functional studies in vitro and in vivo show that SR141716A is able to antagonise the pharmacological effects, involving CB1, induced by both the known cannabinoid receptor agonists and the putative endogenous receptor ligand anandamide. Thus SR141716A may be considered as a useful tool to elucidate the respective physiological or pathophysiological roles of central (CB1) versus peripheral (CB2) cannabinoid receptors.

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