SR 144528, the First Potent and Selective Antagonist of the CB2 Cannabinoid Receptor

MURIELLE RINALDI-CARMONA, FRANCIS BARTH, JOSÉ MILLAN, JEAN-MARIE DEROCQ, PIERRE CASELLAS, CHRISTIAN CONGY, DIDIER OUSTRIC, MARTINE SARRAN, MONSIF BOUABOULA, BERNARD CALANDRA, MARIELLE PORTIER, DAVID SHIRE, JEAN-CLAUDE BRELIÈRE and GÉRARD LE FUR

Sanofi Recherche (M.R.C., F.B., J.M., J-M. D., P.C., C.C., D.O., M.S., M.B., M.P., J-C.B., G.L.F) 371 rue du Professeur Blayac, 34184 Montpellier Cedex 04 (France) and Sanofi Recherche (B.C., D.S), Labège-Innopole voie1, BP137, 31676 Labège, Cédex 04 (France)

ABSTRACT

Based on both binding and functional data, this study introduces SR 144528 as the first, highly potent, selective and orally active antagonist for the CB2 receptor. This compound which displays subnanomolar affinity ($K_i = 0.6$ nM) for both the rat spleen and cloned human CB2 receptors has a 700-fold lower affinity ($K_i = 400$ nM) for both the rat brain and cloned human CB1 receptors. Furthermore it shows no affinity for any of the more than 70 receptors, ion channels or enzymes investigated ($IC_{50} > 10$ μM). *In vitro*, SR 144528 antagonizes the inhibitory effects of the cannabinoid receptor agonist CP 55,940 on forskolin-stimulated adenylyl cyclase activity in cell lines permanently expressing the h CB2 receptor ($EC_{50} = 10$ nM) but not in cells expressing the h CB1 (no effect at 10 μM). Furthermore, SR 144528 is able to selectively block the mitogen-activated protein kinase activity induced by CP 55,940 in cell lines expressing h CB2 ($IC_{50} = 39$ nM) whereas in cells expressing h CB1 an IC_{50} value of more than 1 μM is found. In addition, SR 144528 is shown to antagonize the stimulating effects of CP 55,940 on human tonsillar B-cell activation evoked by cross-linking of surface Igs ($IC_{50} = 20$ nM). *In vivo*, after oral administration SR 144528 totally displaced the *ex vivo* [^{3}H]-CP 55,940 binding to mouse spleen membranes ($ED_{50} = 0.35$ mg/kg) with a long duration of action. In contrast, after the oral route it does not interact with the cannabinoid receptor expressed in the mouse brain (CB1). It is expected that SR 144528 will provide a powerful tool to investigate the *in vivo* functions of the cannabinoid system in the immune response.

It is now well established that Δ^9-THC, the main active component of marijuana and many synthetic cannabinoid receptor agonists as well as anandamide, an endogenous ligand (Devane *et al.*, 1992), mediate their cellular effects through specific cannabinoid receptors, members of the G protein-coupled receptor super family. To date, two human cannabinoid receptor cDNAs have been identified, designated CB1 and CB2 (Matsuda *et al.*, 1990; Munro *et al.*, 1993). Although CB1 mRNA is predominantly expressed in the brain (Matsuda *et al.*, 1990; Westlake *et al.*, 1994), it has also been detected in testis (Gérard *et al.*, 1991), spleen cells (Kaminski *et al.*, 1992) and in leukocytes (Bouaboula *et al.*, 1993). The CB2 subtype is expressed principally in immune tissue (Galiègue *et al.*, 1995; Derocq *et al.*, 1995) where it may be involved in cannabinoid-mediated immune responses, but CB2 mRNA has been detected in mouse cerebellar cells and cerebellum (Skaper *et al.*, 1996) and in rat microglial cells in

culture (Kearn and Hillard, 1997). Both receptors mediate their effects via a pertussis toxin-sensitive GTP-binding regulatory protein. Upon stimulation both CB1 and CB2 induce the inhibition of adenylyl cyclase (Howlett and Fleming, 1984; Slipetz *et al.*, 1995; Felder *et al.*, 1995) and the activation of the MAPKs (Bouaboula *et al.*, 1995a; Bouaboula *et al.*, 1996), whereas CB1, but not CB2, has been found to be associated with the inhibition of N-type (Mackie and Hille, 1992) or Q-type (Mackie *et al.*, 1995) calcium channels. In addition, it has recently been found that both CB1 and CB2 activation can induce immediate-early gene expression such as Krox-24 through a cAMP-independent pathway (Bouaboula *et al.*, 1995b; Bouaboula *et al.*, 1996). All the central cannabinoid receptor-mediated effects were prevented by the potent and selective CB1 receptor antagonist SR 141716A (Rinaldi-Carmona *et al.*, 1994, 1995, 1996). In the last few years, a number of potent synthetic cannabinoid receptor agonists have been developed (D'Ambra *et al.*, 1992; Gallant *et al.*, 1996) but until now, no antagonist for the CB2

ABBREVIATIONS: anti-Ig, anti-human surface immunoglobulins; BSA, bovine serum albumin; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; h CB1, human central cannabinoid receptor; h CB2, human peripheral cannabinoid receptor; CHO, Chinese hamster ovary; IBMX, isobutylmethylxanthine; i.c.v., intracerebral ventricular; fMAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PTX, pertussis toxin; Δ^9-THC, tetrahydrocannabinol; Tris, Tris-(hydroxymethyl)-amino-methane.

receptor has been described. Therefore, the search for potent antagonists for the CB2 cannabinoid receptor was warranted. Based on both binding and functional data, we introduce SR 144528 {N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} (fig. 1), as the first, highly potent, selective and orally active antagonist for CB2. This discovery provides a new tool to better understand the role of the CB2 receptor and to develop potential immunomodulating drugs based on the cannabinoid system.

Methods

Materials. BSA was from Boehringer (Mannheim, Germany). Forskolin was from Sigma Chemical Co (St. Louis, MO). Polyethyleneneimine was purchased from Serva (St. Germain-en-Laye, France). Biofluor liquid scintillant and [³H]-CP 55,940 (111.9 Ci/mmol) were purchased from New England Nuclear Corporation (Paris, France). γ-[³³P] ATP (112.9 Ci/mmol), cAMP scintillant proximity assay and Biotrack p42/p44 MAP kinase kits were from Amersham (Les Ulis, France). Dimethyl sulfoxide was purchased from Prolabo (Paris, France). Tris was purchased from Merck-Clevenot (Nogent sur Marne, France). RO 20-1724{4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone} was purchased from Research Biochemicals Incorporated (Illkirch, France). CP 55,940 {(-)-cis-3-[2-hydroxy-4-(1,1-dimethylhepthyl) phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol} and SR 144528 {N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} were synthesized at Sanofi Recherche (Montpellier, France). Stock solutions of drugs were dissolved in dimethyl sulfoxide at 10^{-2} M and stored at -20°C. The concentration of solvent in assay never exceeded 0.1% (v/v). This final concentration was without effect on assays. The polyclonal B cell activator, rabbit anti-human surface immunoglobulin (anti-Ig) antibody coupled to polyacrylamide beads was purchased from Bio-Rad (Richmond, VA). The 3A11 (anti-CD2) and F111-409 (anti-CD3) monoclonal antibodies used for depletion of T cells from human tonsils were produced at Sanofi Recherche. Sheep anti-mouse IgG-conjugated magnetic beads (Dynabeads) were from Dynal (Oslo, Norway). Tissue culture reagents were from Gibco (Eragny, France). Culture flasks (Costar and Falcon) were purchased from Dutscher (Brumath, France) and Becton Dickinson (Le Pont de Claix, France). Male Sprague Dawley rats (180–220 g) and male mice (CD1, 20–25 g) were obtained from Charles River (France, St.-Aubin-lès-Elbeuf) and used for *in vitro* and *ex vivo* binding studies, respectively. Male Swiss mice (30–35 g) were obtained from CERJ (Le Genet St. Isle, France) and used for isolated vasa deferentia preparations.

Expression of human CB1 and CB2 receptor in CHO cells. CB1 and CB2 cDNAs were obtained by screening a cDNA library from human frontal cortex or from the human promonocytic cell line U937, respectively. The CB1 and CB2 coding sequences were amplified by PCR with sense primers bearing *Hin*dIII sites and Kozak

Fig. 1. Chemical structure of SR 144528.

consensus sequences CB1 and CB2 and a common antisense primer carrying an *Eco*RI site, 5′CCACTCGAATTCTCATCACAG AGC-CTCGGCAG. The amplicons were digested with *Hin*dIII/*Eco*RI and inserted into p658, an expression plasmid derived from p7055 (Miloux and Lupker, 1994) in which the IL-2 coding sequence was replaced by a polylinker. The vectors were transfected into CHO dihydrofolate reductase (DHFR⁻) cells by a modified $Ca_3 (PO_4)_2$ precipitation method (Graham and Van der Eb, 1973). CHO cells were treated with trypsin 48 hr after transfection and seeded at a density of 5×10^5 cells/dish onto minimum essential medium-glutamine medium containing heat-inactivated, dialysed fetal-calf serum (10%), gentamicin (20 mg/l), L-proline (40 mg/l), pyruvate sodium (0.5 mM) and anti-PPLO agent (1%). After 10 days surviving clones were recovered and cultivated in the same medium, selection being carried out by binding assays on isolated membranes (see below). For all assays, cells were seeded into 24-well cluster plates (1×10^5 cells/well) and grown to confluence. Cells were used between the 3rd and the 20th passage.

Membrane preparation. Membranes were isolated from CHO cells expressing either CB1 or CB2 (Rinaldi-Carmona et al., 1996; Shire et al., 1996) and from the rat brain, minus the cerebellum (Abita et al., 1977) or from the spleen (Bouaboula et al., 1993). Protein concentration was measured as in (Spector, 1975) and membranes were stored at -80°C until use.

Binding experiments. For *in vitro* binding assays, membranes (10–30 μg) were incubated at 30°C with 0.2 nM [³H]-CP 55,940 in 1 ml of buffer A (Tris-HCl 50 mM, pH 7.7) for 1 hr. A rapid filtration technique using Whatman GF/C filters [pretreated with polyethyleneneimine 0.5% (w/v)] and a 48-well filtration apparatus (Brandel, Paris, France) was used to harvest and rinse labeled membranes (3 × with 5 ml cold buffer A containing 0.25% BSA). The radioactivity bound to the filters was counted with 4 ml of biofluor liquid scintillant. Nonspecific binding was determined in the presence of 1 μM CP 55,940. For selectivity, binding assays were carried out using standard protocols.

For *ex vivo* experiments, SR 144528 was dissolved either in two drops of Tween 80 plus dimethylsulfoxide (final concentration 2%) and diluted in distilled water (p.o.) or in 100% dimethylsulfoxide (i.c.v.). It was administered in a volume of 20 ml/kg (p.o.) or 2 μl/animal (i.c.v.) to male mice before they were killed by decapitation. The brain (without the cerebellum) or the spleen were removed and homogenized in 20 ml of buffer A. Binding assays were performed as described above with 0.8-ml aliquots of homogenates. Control mice received the vehicle [two drops of Tween 80 plus dimethylsulfoxide (final concentration 2%) plus distilled water].

cAMP measurements. cAMP accumulations were carried out in CHO-CB1 or -CB2 cells grown to confluence as described previously (Matsuda et al., 1990; Rinaldi-Carmona et al., 1996). Cells were washed with PBS and incubated for 15 min at 37°C in 1 ml of PBS (containing 0.25% acid-free BSA, 0.1 mM IBMX, 0.2 mM RO20-1724) in the absence or in the presence of 3 nM CP 55,940, SR 144528 (10^{-9}–10^{-6}M), 3 nM CP 55,940 plus SR 144528 (3×10^{-9}–10^{-5}M). Forskolin (3 μM final concentration) was added and cells were incubated for another 20 min at 37°C. The reaction was terminated by rapid aspiration of the assay medium and addition of 1.5 ml of ice-cold 50 mM Tris-HCl, pH 8, 4 mM ethylenediaminetetraacetic acid. Dishes were placed on ice for 5 min and then the extracts were transferred to a glass tube. Extracts were boiled and centrifuged for 10 min at 3500 g to eliminate cell debris. Aliquots from supernatant were dried and the cAMP concentration was determined by radioimmunoassay by using the scintillant proximity assay system. The basal activity was determined in the absence of forskolin. In PTX experiments, cells were cultured in the presence of the toxin (10 ng/ml) for 24 hr before treatment with forskolin.

MAPK activity. MAP kinase activity was measured as described previously (Frodin et al., 1994; Bouaboula et al., 1995a). Briefly, cells grown to 80% confluence were maintained in culture medium containing 0.5% foetal calf serum for 24 hour prior to the application of
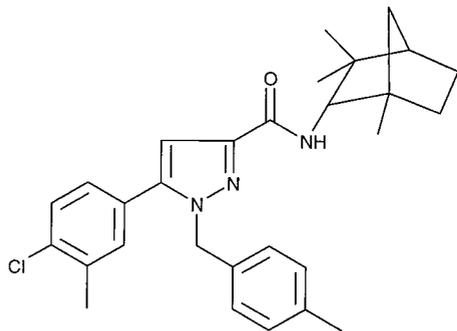
ligands. CHO-CB1 or -CB2 cells previously washed with PBS were incubated at 37°C in the absence (basal activity) or in the presence of 6 nM CP 55,940, 6 nM CP 55,940 plus SR 144528 (10^{-9}–3×10^{-6}M) for 20 min. Cells were then washed at 4°C with 0.5 ml of buffer A [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N-tetraacetic acid, 1 mM Na$_3$ VO$_4$] and lysed for 15 min in buffer A supplemented with 1% triton X-100, 10 μg/ml aprotinin, 10 μg/ml, leupeptin, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. The solubilized cell extracts were then clarified by centrifugation at 14,000 $\times$ g for 15 min at 4°C. Aliquots (15 μl) were removed and stored at -80°C until use. Phosphorylation assays were carried out at 30°C for 30 min (linear assay conditions) with γ-[^{33}P]ATP by using the Biotrack p42/p44 MAP kinase enzyme system. The radioactivity incorporated was determined by liquid scintillation counting.

Human B cell purification. B cells were isolated as previously described (Derocq *et al.*, 1995). Briefly, cells from tonsil specimens were incubated with anti-CD2 and anti-CD3 monoclonal antibodies at 2 μg/10^6 estimated target cells, for 30 min at 4°C, washed and then incubated 30 min with sheep anti-mouse IgG-conjugated magnetic beads at a bead-to-target cell ratio of 5:1. Negative selection of B cells was performed by magnetic depletion of bead-bound T cells and resulted in >95% pure B cells as determined by FACS analysis using anti-CD20, -CD4, -CD8 and -CD14 monoclonal antibodies.

Anti-Igs assay. B cell cultures were performed in RPMI 1640 supplemented with 0.5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 mM Hepes buffer. Cells were seeded in 12 replicates at 1.5×10^5 cells/well of 96-well microplates in a final volume of 0.2 ml. The cannabinoid ligand CP 55,940 was added at 10 nM after a 30-min incubation with SR 144528 (10^{-9}–10^{-7} M). Cells were then activated by cross-linking of surface Igs with the anti-Igs polyclonal B-cell activator at a final dilution of 1/5000 and incubated at 37°C for 3 days in a humidified atmosphere containing 95% air, 5% CO$_2$. DNA synthesis was determined by pulsing the cells with 1 μCi/well of [^{3}H]-thymidine for the last 16 hr of culture period.

Isolated mouse vasa deferentia preparations. Assays were performed as previously described (Ward *et al.*, 1990). Drugs were added once the contractile responses to electrical stimulation were reproducible. Preparations were exposed to cumulative increasing concentrations of CP 55,940 (3×10^{-10}–3×10^{-7}M) to obtain concentration-response curves either in the absence (control) or in the presence of SR 144528 (0.3, 1 or 3 μM) added at a fixed concentration 60 min before the first concentration of CP 55,940.

Data analysis. Data from equilibrium binding (K_d, B$_{max}$) and competition experiments (IC$_{50}$, EC$_{50}$, DE$_{50}$) were analyzed using a nonlinear least-squares method on a Compaq Desk Pro 4/66i computer. All experiments were performed in duplicate and results were confirmed in at least three independent experiments. A Schild plot was constructed to estimated the pA2 and pD2 values in mouse vas deferens studies. In each estimate six isolated tissue preparations were used. Statistical significance was determined by use of Student's *t* test and P < .05 was considered significant.

Results

Interaction of SR 144528 with peripheral cannabinoid receptors *in vitro*. As shown in figure 2A, SR 144528 displaced in a concentration-dependent manner [^{3}H]-CP 55,940 specifically bound to its high affinity receptor in rat spleen microsomal membranes whereas it displayed low affinity for the cannabinoid receptor expressed in rat brain. The concentration-response curves gave K_i values of 0.30 $\pm$ 0.38 and 305 $\pm$ 44 nM (three experiments) for spleen and brain, respectively. Furthermore, in membranes isolated from CHO cells expressing human CB2, SR 144528 was a potent competitor of [^{3}H]-CP 55,940 binding sites with a K_i
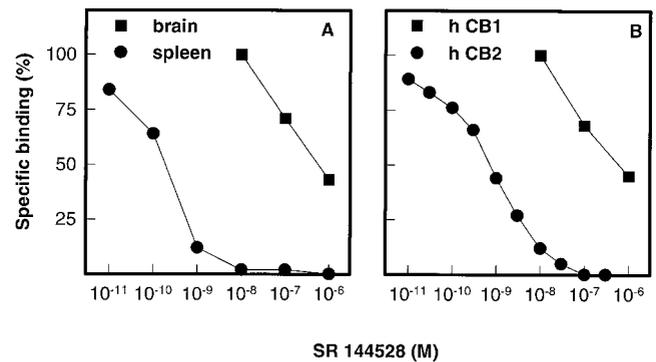
Fig. 2. Competition of [^{3}H]-CP 55,940 binding to rat (A) and CHO cell (B) membranes by SR 144528. Binding assays were carried out at 30°C as described in "Methods" using 0.2 nM [^{3}H]-CP 55,940 and increasing concentrations of SR 144528. Data are from one experiment of three performed in duplicate and are expressed as the percentage of specific binding in the absence of SR 144528 (100%).

value of 0.60 $\pm$ 0.13 nM (five experiments), whereas it displayed only low affinity for membranes from CHO cells expressing human CB1, K_i = 437 $\pm$ 33 nM (four experiments) (fig. 2B). These results show that SR 144528 is selective for CB2 versus CB1 with a selectivity ratio of 700.

As shown in figure 3 and reported in table 1, no significant changes (P < .05) in the maximum number of receptors (B$_{max}$) of [^{3}H]-CP 55,940 was observed when CHO-CB2 cell membranes were exposed to increasing concentrations of SR 144528 whereas a significant increase (P < .05) in the dissociation constant (K_d) of [^{3}H]-CP 55,940 occurred when 3 or 10 nM of SR 144528 was added in the medium. These results indicate that SR 144528 acts as a competitive ligand for CB2.

Receptor binding profile of SR 144528. SR 144528 had no affinity (IC$_{50}$ > 10 μM) for any of the other types of receptors, enzymes or channels investigated including Angiotensin II AT$_1$, endothelin (A, B), galanin, muscarinic (M$_1$, M$_2$, M$_4$), bradykinin (BK$_1$, BK$_2$), histamine (H$_1$, H$_2$, H$_3$), dopamine (D$_1$, D$_2$, D$_3$, D$_{4.2}$), adrenergic (α_1, α_2, β_1, β_2), adenosine (A$_1$, A$_2$), purinergic (P$_{2X}$, P$_{2Y}$), leukotriene B$_1$, opiate, neurotensin, cholecystokinin (A, B), benzodiazepine (central, peripheral), sigma 1, tachykinin (NK$_1$, NK$_2$, NK$_3$), neuropep-
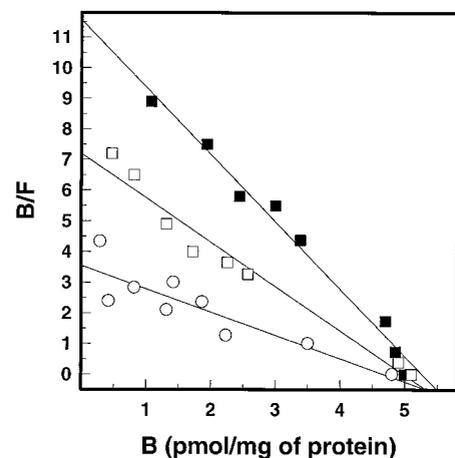
Fig. 3. Effect of SR 144528 on the equilibrium binding parameters of [^{3}H]-CP 55,940 in CHO-CB2 cell membranes. Equilibrium binding experiments were carried out at 30°C as described in "Methods" with increasing concentrations of [^{3}H]-CP 55,940 from 0.05 to 15 nM in the absence (control) (■) and in the presence of 3 (□) or 10 (○) nM SR 144528. Data are from one experiment out of two performed in duplicate.

TABLE 1
Effect of SR 144528 on the equilibrium binding parameters of [³H]-CP 55,940
[³H]-CP 55,940 binding assays were performed in human CHO-CB2 cell membranes at 30°C as described under "Methods" in the absence and presence of SR 144528. Data are means ± S.E.M. from three experiments performed in duplicate. Statistical significance was determined by use of Student's t test and [a]$P < .05$ was considered significant.

Drug		K_d		B_{max}	
	nM	nM	(95% CL)	pmol/mg protein	(95% CL)
None (control)		0.49 ± 0.11	(0.37–0.76)	4.69 ± 0.58	(3.72–5.77)
SR 144528	1	0.54 ± 0.15	(0.27–0.86)	4.60 ± 0.65	(2.78–6.30)
	3	0.71 ± 0.09[a]	(0.50–0.93)	5.06 ± 0.44	(4.03–6.22)
	10	1.35 ± 0.46[a]	(0.82–3.16)	4.52 ± 0.90	(3.50–8.27)

tide Y (Y_1, Y_2), nicotinic (central), ouabain, phencyclidine, vasopressin (V_1, V_2), VIP, thromboxane A2, TRH, somatostatin, calcineurin, calpain, EGF tyrosine kinase, NO synthase (constitutive and inducible), PKC (α, β), PAF, excitatory or inhibitory amino acids [glycine (strychnine sensitive and insensitive), AMPA, kainate, NMDA, $GABA_A$, $GABA_B$], 5-hydroxytryptamine (5-$HT_{1A,B,D}$, 5-$HT_{2A,C}$, 5-HT_3, 5-HT_4), Cl^-, Na^+ (site$_1$, site$_2$), Ca^{++} (N, L) and K^+ (ATP sensitive, voltage-dependent, Ca^{++} dependent). These findings show that SR 144528 is a very selective ligand for CB2.

SR 144528 is an antagonist of the CB2 receptor *in vitro*. Activation of CB1 and CB2 cannabinoid receptors induces inhibition of cAMP accumulation via a PTX-sensitive G-binding protein (Howlett and Fleming, 1984; Felder *et al.*, 1995). As previously described (Rinaldi-Carmona *et al.*, 1996; Shire *et al.*, 1996), the cannabinoid agonist CP 55,940 inhibited in a concentration-dependent manner, the forskolin-induced accumulation of cAMP in CHO cells expressing either human CB1 ($IC_{50} = 2.0 ± 0.3$ nM, four experiments) or CB2 ($IC_{50} = 2.51 ± 0.25$ nM, four experiments). At 10^{-7} M of CP 55,940 a maximum inhibition of 75 and 90% was observed in CHO cells expressing CB1 or CB2, respectively. As shown in figure 4, SR 144528 completely blocked the inhibitory effect
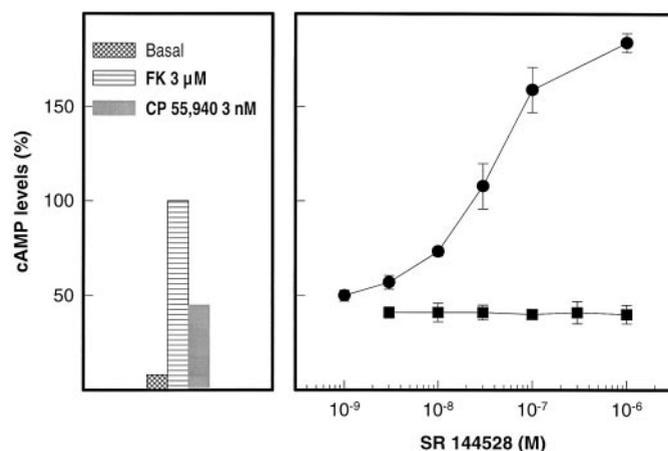

Fig. 4. Effect of SR 144528 on CP 55,940-induced inhibition of forskolin-sensitive adenylyl cyclase activity in CHO cells expressing human CB1 (■) or CB2 (●) receptors. Confluent cells were treated with vehicle (basal), forskolin (3 μM) (100%), 3 nM CP 55,940 plus forskolin (3 μM), or increasing concentrations of SR 144528 and 3 nM CP 55,940 plus forskolin (3 μM), for 30 min at 37°C. cAMP levels were measured as described in "Methods." Data are from one representative experiment of three performed in duplicate and are expressed as the percentage of forskolin effect at 3 μM (100%). cAMP levels in the absence of forskolin (basal) were: 2.77 ± 0.15 and 3.2 ± 0.3 pmol of cAMP/well in CB1 and CB2, respectively; forskolin-stimulated cAMP levels were: 32.4 ± 4.1 and 48.2 ± 2.2 pmol of cAMP/well for CB1 and CB2, respectively. In the presence of three nM CP 55,940 forskolin-stimulated cAMP levels were: 16.2 ± 1.5 and 20.7 ± 2 pmol of cAMP/well for CB1 and CB2, respectively.

exerted by CP 55,940 (3 nM) with an EC_{50} value of 10.0 ± 2.7 nM (three experiments) in CHO cells expressing h CB2 but not in cells expressing CB1 (no blockade up to 10 μM). These results show that SR 144528 is a functional antagonist of the peripheral cannabinoid receptor CB2. Interestingly, we observed that in the presence of 10^{-7} M SR 144528 plus CP 55,940 (3 nM), the cAMP levels reached a value (150%) superior to that obtained in the presence of the forskolin alone (100%). In fact, SR 144528 alone was able to stimulate in a concentration-dependent manner ($EC_{50} = 26 ± 6$ nM, two experiments) the forskolin-sensitive adenylyl cyclase activity in CHO-CB2 cells with a maximum effect at 1 μM (4-fold stimulation) whereas at this concentration it had no significant effect on CHO-CB1 cells (15% inhibition) (not shown). No stimulation of cAMP by SR 144528 was observed in the absence of forskolin, excluding a direct stimulation of GTP-binding protein Gs by SR 144528 (data not shown). Both effects (the antagonism and the stimulation) exerted by SR 144528 were completely prevented by PTX pretreatment of the cells, indicating that these two processes were CB2 receptor mediated. These effects of SR 144528 are consistent with the blockage of adenylyl cyclase inhibition mediated by autoactivated CB2 receptor.

We found in previous work that treatment of CHO cells expressing h CB1 or -CB2 receptors with cannabinoid agonists activated the 42- and 44-kDa MAPK in a concentration-dependent manner and that the activation could be inhibited by pertussis toxin (Bouaboula *et al.*, 1995a, 1996). In both cell lines half-maximal MAPK stimulation occurred at about 6 nM CP 55,940 and this concentration was chosen to study the effect of SR 144528. As shown in figure 5, SR 144528 was able to produce a concentration-dependent inhibition of MAPK activity stimulated by CP 55,940 in CHO-CB2 cells with an IC_{50} value of 39.02 ± 2.96 nM (three experiments) whereas in cell line expressing CB1 an IC_{50} value superior to 1 μM was found. These results confirmed that in CHO-CB2 cells SR 144528 behaved as an antagonist *vs.* the cannabinoid agonist CP 55,940.

We have recently described that exposure of human tonsillar B cells to physiologically relevant concentrations (in the nanomolar concentration range) of cannabinoid receptor agonists (under conditions of low level of serum) induced a significant enhancement of the rate of DNA synthesis evoked by cross-linking of their surface immunoglobulins by anti-Igs (Derocq *et al.*, 1995). Because B cells naturally express functional cannabinoid receptors, this model was chosen to test the effect of SR 144528. As shown in figure 6, the effect of 10 nM CP 55,940 (39 ± 8% of stimulation, four experiments) was completely blocked by the presence of increasing concentrations of SR 144528 in the culture medium. The concentra-
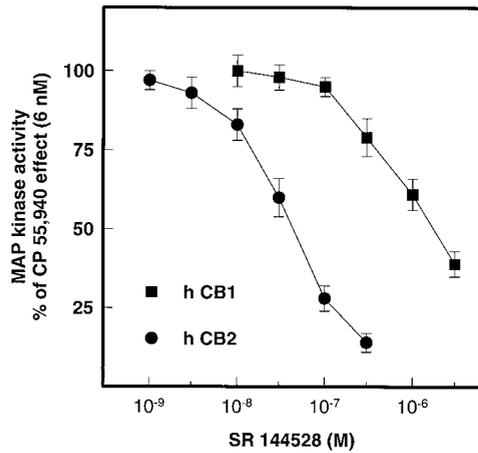
Fig. 5. Effect of SR 144528 on CP 55,940-induced MAPK kinase stimulation in CHO cells expressing human CB1 (■) or CB2 (●) receptors. Growth-arrested CHO-CB1 (■) or CHO-CB2 (●) cells were treated with vehicle, (basal activity), 6 nM CP 55,940 or various concentrations of SR 144528 in the presence of 6 nM CP 55,940 for 15 min. MAPK activity (42- and 44 KDa MAPK) was measured in cell lysates as described in "Methods." Data are from one representative experiment of three performed in triplicate and are expressed as the percentage of CP 55,940 effect at 6 nM in the absence of SR 144528 (100%).
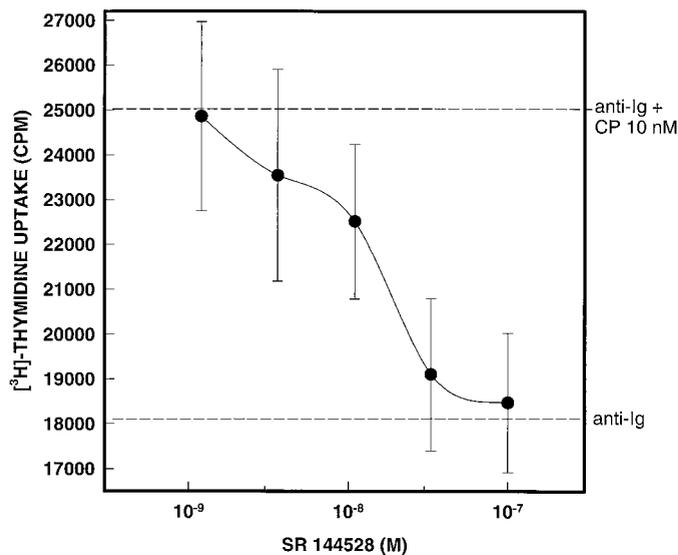

Fig. 6. Effect of SR 144528 on CP 55,940-induced B cell stimulation. Purified human tonsil B cells were costimulated for 72 hr with antisurface Ig antibody at 1/5000 dilution in the absence or presence of 10 nM CP 55,940, in the presence of 10 nM CP 55,940 plus increasing concentrations of SR 144528. Data are from one representative experiment out of four performed in 12 replicates. Data are means ± S.E.M. of twelve values and are expressed in dpm.

tion-response curve gave an IC$_{50}$ value of 20 ± 5 nM (four experiments).

Taken together, these *in vitro* results indicate that SR 144528 is a potent and selective antagonist of the CB2 versus the CB1 receptor. In addition, cannabinoid receptor agonists have been shown to inhibit neuronally stimulated smooth muscle contractions such as those in the mouse vas deferens (Ward *et al.*, 1990). As shown in figure 7, in this model, SR 144528, which had no effect by itself up to 10^{-6} M, produced a significant (P < .05) concentration-dependent rightward and almost parallel shift of the concentration response-curve for CP 55,940 (0.3 μM SR 144528, pD2 value of CP 55,940 =
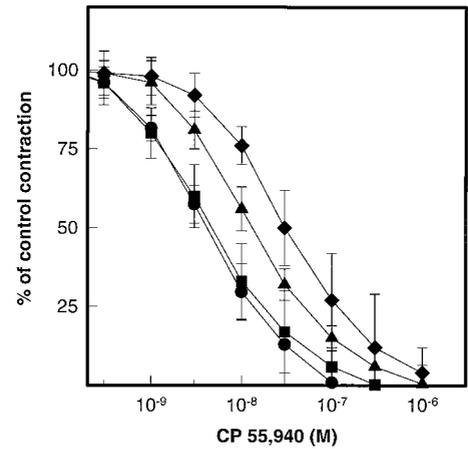

Fig. 7. Cumulative concentration-response curves for CP 55,940 on the amplitude of twitch contractions elicited by electrical field stimulation of the mouse vas deferens obtained in the absence (●) (control) and in the presence of SR 144528 at 0.3 (■), 1 (▲) or 3 (◆) μM. Assays were performed as described in "Methods." Data are expressed as the percentage of control values after incubation with SR 144528. Each point is the mean value of six determinations.

8.32 ± 0.09, 1 μM SR 144528, pD2 value of CP 55,940 = 7.85 ± 0.04*, 3 μM SR 144528, pD2 value of CP 55,940 = 7.50 ± 0.05* *vs.* control pD2 value of CP 55,940 = 8.38 ± 0.05, six experiments). From these curves, a pA$_2$ value of 6.3 could be determined for SR 144528.

***In vivo* interaction of SR 144528 with peripheral cannabinoid receptors.** As shown in figure 8A, after oral administration, SR 144528 totally displaced in a dose-dependent manner the specific binding of [^{3}H]-CP 55,940, measured *ex vivo*, to mouse spleen homogenates. A median effective dose value (ED$_{50}$) of 0.36 ± 0.06 mg/kg (three experiments) was found. In contrast, no effect on the binding of [^{3}H]-CP 55,940 to its specific sites in the brain was observed after either oral (up to 10 mg/kg) or i.c.v. (10 μg/animal) administration of SR 144528 in mice. In the same experimental conditions the CB1 receptor antagonist SR 141716 interacted with the brain CB1 sites with an ED$_{50}$ value of 0.4 μg/animal. These results showed that SR 144528 did not interact with the cannabinoid receptor expressed in the mouse brain (CB1). The occupancy by SR 144528 of the spleen cannabinoid receptor was time-dependent and significant for at least 18 hours after oral administration at 3 mg/kg (fig. 8B). These results indicated that SR 144528 is orally active, has long duration of action and confirmed its specificity for the CB2 receptor.

Discussion

The discovery that Δ^9-THC can act through two subtypes of cannabinoid receptors, sharing only 44% overall identity, has presented us with the opportunity of searching for drugs that are highly specific for each subtype. We describe for the first time a potent, selective and orally effective CB2 cannabinoid receptor antagonist, SR 144528, that has a 700-fold higher affinity for the CB2 receptor than for the CB1 receptor.

In vitro, SR 144528 has a very high affinity for the rat spleen (K$_i$ = 0.3 nM) and the human cloned CB2 (K$_i$ = 0.6 nM) receptors labeled by [^{3}H]-CP 55,940 (Devane *et al.*, 1988). In the same experimental conditions, the cannabinoid receptor agonist CP 55,940 was shown to act as a potent
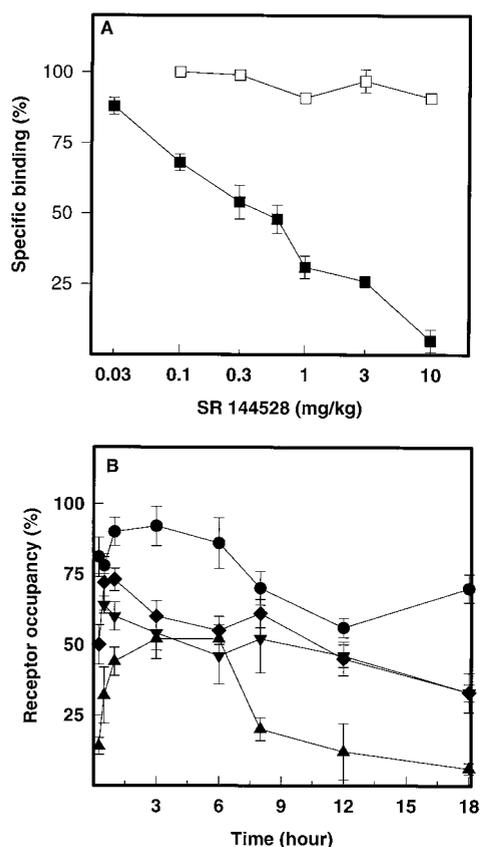
Fig. 8. Competition of the specific [³H]-CP 55,940 binding to its sites by SR 144528 after oral administration. A, Mice were administered with increasing doses of SR 144528. They were then sacrificed 1 hr after these administrations. The brain (□) or the spleen (■) were then removed and *ex vivo* binding assays were performed using [³H]-CP 55,940 as described in "Methods." Data are from one representative experiment of three performed in six replicates obtained from three animals. Data are expressed as percentage of the [³H]-CP 55,940 specific binding compared to control tissue of untreated mice (100%). B, Time course of the spleen cannabinoid receptor occupancy by SR 144528 after oral administration. Mice were administered with 0.3 (▲), 1 (▼), 3 (◆) or 10 (●) mg/kg of SR 144528. They were sacrificed at different times after drug administration. The spleen was removed and binding studies were performed as described in "Methods." Data are means ± S.E.M. of six values obtained from three animals. They are expressed as percentage of inhibition of the [³H]-CP 55,940 specific binding compared to control tissue of untreated mice.

competitor for both subtypes with the same potency in rodent (Rinaldi-Carmona *et al.*, 1994, Jung *et al.*, 1997) and human cell lines, including CHO cells transfected with cannabinoid receptors (Bouaboula *et al.*, 1993, 1995b; Rinaldi-Carmona *et al.*, 1996, Shire *et al.*, 1996). A detailed analysis of the inhibitory effect of SR 144528 on the equilibrium binding parameters of [³H]-CP 55,940 (decrease of the affinity and no change in the maximal binding capacity) indicates a competitive nature of the interaction with the CP 55,940 binding sites in CHO cells expressing the human cloned CB2. Finally, SR 144528 was shown to be strictly restricted to CB2 receptors inasmuch as no significant interaction was observed in screening studies carried out on a panel of more than 70 different receptors, ion channels or enzymes.

At a functional level, inhibition of forskolin-stimulated adenylyl cyclase and activation of the MAPKs induced by the "bispecific" agonist CP 55,940 in CHO-cells expressing either h CB1 or -CB2 receptors indicated that SR 144528 potently antagonized the responses mediated through the CB2 receptors without affecting those induced via the CB1 receptors. In these tests the IC_{50} for SR 144528 appears to be 10-fold higher than the K_i for SR 144528 in binding studies. This discrepancy could be explained by the fact that binding competition experiments were performed with a 10- to 20-fold lower CP 55,940 concentration than cAMP and MAPK assays. The effect observed for SR 144528 on forskolin-sensitive adenylyl cyclase activity in CHO-CB2 cells is consistent with the blockade of adenylyl cyclase mediated by autoactivated CB2 receptors present in these cells.

In the last few years, a new class of antagonist molecules designated as inverse agonist has been identified. The first to be characterized were the β carbolines acting toward the inotropic γ aminobutyric acid receptor. These molecules contrast with classical antagonists in that they exhibit a biological activity by blocking the signal transduction mediated by constitutively activated receptors. Most of the other inverse agonist molecules identified so far are ligands for receptors of the GPCR superfamily. Our results provided an additional example of such autoactivated receptors and showed that SR 144528 functions as an inverse agonist.

In addition, SR 144528 demonstrated also a potent antagonistic effect against the stimulating action of CP 55,940 on human tonsillar B cell proliferation induced by cross-linking of Igs (Derocq *et al.*, 1995). This data, added to the fact that human B cells express much higher level of CB2 than CB1 receptor (Galiègue *et al.*, 1995) and to the lack of blocking effect of the specific CB1 antagonist SR 141716, allowed to conclude that the growth enhancing activity observed on B cells is mainly mediated through the peripheral CB2 receptor.

Based on the *ex vivo* [³H]-CP 55,940 binding studies, SR 144528 appears to be effective in blocking the CB2 but not the CB1 receptors (ED_{50} value of 0.36 ± 0.06 mg/kg in the spleen *vs.* no interaction in the brain up to 10 mg/kg, p.o. or 10 μg/mouse, i.c.v.) with a long duration of action after oral administration, in mice. In addition we found that SR 144528 does not have any behavioral effect following either oral (10 mg/kg) or i.c.v. (10 μg/animal) administration in mice (data not shown).

In contrast, in the model of the mouse vas deferens, SR 144528 was shown to be a weak antagonist unlike SR 141716 that strongly blocks the cannabinoid-induced inhibition of smooth muscle contractions (Pertwee *et al.*, 1995; Rinaldi-Carmona *et al.*, 1994, 1995). This data suggested that CB1 rather than CB2-receptors are involved in this model and brought an additional indication of the selectivity of SR 144528.

In vitro and *ex vivo* studies, carried out at both binding and functional levels, have clearly demonstrated that SR 144528 is a highly selective and potent CB2-receptor antagonist. This new compound represents a valuable tool that associated to its CB1 antagonist counterpart SR 141716, will contribute to the deciphering of the cannabinoid system.

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Send reprint requests to: Dr. Murielle Rinaldi-Carmona: Sanofi Recherche, 371 rue du Professeur J Blayac, 34184 Montpellier Cédex, France.