ELSEVIER

European Journal of Pharmacology 401 (2000) 17–25

ejp

www.elsevier.nl/locate/ejphar

Mutational analysis and molecular modelling of the antagonist SR 144528 binding site on the human cannabinoid CB_2 receptor

Paul Gouldson [a], Bernard Calandra [a], Pascale Legoux [a], Aimée Kernéis [a], Murielle Rinaldi-Carmona [b], Francis Barth [b], Gérard Le Fur [a], Pascual Ferrara [a], David Shire [a,*]

[a] *Sanofi-Synthelabo, Centre de Labège, Labège-Innopole Voie No 1, BP 137, 31676 Labège Cedex, France*
[b] *Sanofi-Synthelabo, 371 rue du Professeur J. Blayac, 34184 Montpellier Cedex 04, France*

Received 20 March 2000; received in revised form 24 May 2000; accepted 20 June 2000

Abstract

We have investigated the binding site of the subtype specific antagonist SR 144528, (*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methoxybenzyl)-pyrazole-3-carboxamide) on the human cannabinoid CB_2 receptor based on functional studies with mutated receptors. Two serine residues in the fourth transmembrane region, Ser^{161} and Ser^{165}, were singly mutated to the cognate cannabinoid CB_1 receptor residue, alanine, and each gave receptors with wild-type properties for the cannabinoid agonists CP 55,940 (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol) and WIN 55212-2 (*R*)-(+)[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl) methanone, which SR 144528 completely failed to antagonise. Molecular modelling studies show that SR 144528 interacts with residues in transmembrane domains 3, 4, and 5 of the cannabinoid CB_2 receptor through a combination of hydrogen bonds and aromatic and hydrophobic interactions. In addition, the replacement by serine of a nearby cannabinoid CB_2 receptor-specific residue, Cys^{175} resulted in wild-type receptor properties with CP 55,940, loss of SR 144528 binding and eight-fold reduced binding and activity of WIN 55212-2, a result compatible with a recently-proposed binding site model for WIN 55212-2.

Keywords: Cannabinoid receptor; SR 144528; WIN 55212-2; Mutagenesis; Ligand docking; Molecular model

1. Introduction

The active constituent in *Cannabis sativa*, Δ^9-tetrahydrocannabinol, the endogenous cannabinoids *N*-arachidonoylethanolamine (anandamide) (Devane et al., 1992) and 2-arachidonylglycerol (Mechoulam et al., 1995) together with numerous synthetic cannabinoid agonists (Pertwee, 1997) exert their effect through the mediation of two 7-transmembrane domain G protein-coupled receptors. The cannabinoid CB_1 receptor (Gérard et al., 1991; Matsuda et al., 1990) is expressed principally in the brain, but is also found in peripheral tissues, whereas the cannabinoid CB_2 receptor (Munro et al., 1993), absent from the brain, is found predominantly in blood cells (Galiègue et al., 1995). The two subtypes share only 44% homology, which has facilitated the development of the highly-specific and potent antagonists, SR 141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride) (Rinaldi-Carmona et al., 1994) and SR 144528 (*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methoxybenzyl)-pyrazole-3-carboxamide) (Rinaldi-Carmona et al., 1998) for the cannabinoid CB_1 and cannabinoid CB_2 receptor, respectively (Fig. 1). SR 144528 has a 700-fold higher affinity for the cannabinoid CB_2 receptor than for the cannabinoid CB_1 receptor (Rinaldi-Carmona et al., 1998). Through competition binding experiments, we recently showed that residues important for the subtype specificity of SR 144528 for the cannabinoid CB_2 receptor were located somewhere within the transmembrane domain 4–extracellular loop 2–transmembrane domain 5 region (Shire et al., 1999), in the same region previously found to be important for binding of SR 141716A to the cannabinoid CB_1 receptor (Shire et al., 1996a). That report was mainly based on competition binding experiments with the tritiated bicyclic cannabinoid agonist CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-di-

* Corresponding author. Tel.: +33-5-61-00-4054; fax: +33-5-61-00-4001.
E-mail address: david.shire@sanofi-synthelabo.com (D. Shire).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00439-8

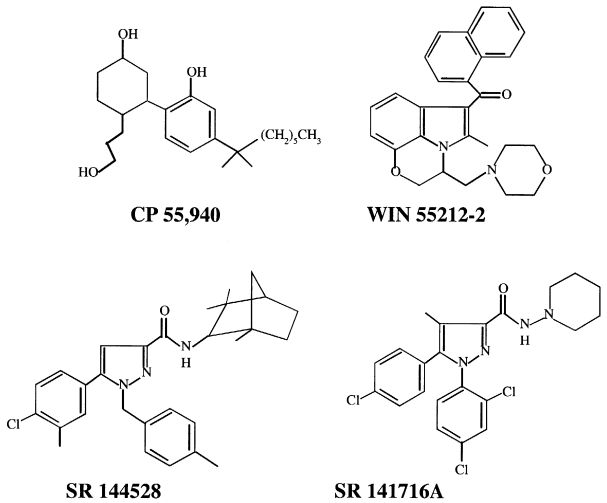

Fig. 1. Structure of cannabinoid ligands used in the present work.

methylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol) (Fig. 1) which has comparable binding affinities for the cannabinoid CB_1 and cannabinoid CB_2 receptors and, as we found, for hybrid constructs of the two receptors. Another powerful cannabinoid agonist, WIN 55212-2, (*R*)-(+)[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]-pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl) methanone (Fig. 1) has a ~20-fold higher binding affinity for the human cannabinoid CB_2 receptor than for the human cannabinoid CB_1 receptor (Shire et al., 1996b; Showalter et al., 1996). The binding site for WIN 55212-2 appeared to be in the same region of the human cannabinoid CB_2 receptor as that of the SR compounds (Shire et al., 1999) and this was recently confirmed by the discovery that Phe^{197} in transmembrane region 5 was crucial for the subtype specificity of WIN 55212-2 (Song et al., 1999).

Here, we describe experiments with monkey kidney COS-7 cells expressing point mutated cannabinoid CB_2 receptors that led to the discovery of two residues in transmembrane domain 4, Ser^{161} and Ser^{165} that are implicated in the binding of SR 144528, but not in that of either CP 55,940 or WIN 55212-2, confirmed by both binding and activity analysis. Mutation of a third residue, Cys^{175} also eliminated SR 144528 binding, diminished WIN 55212-2 binding and activity, but had no effect on CP 55,940 binding affinity or activity. From the results obtained and using molecular modelling techniques, we propose a model for SR 144528 docked in the cannabinoid CB_2 receptor.

2. Materials and methods

2.1. Drugs and chemicals

WIN 55212-2 was purchased from Sigma (Saint-Quentin-Fallavier, France). CP 55,940, SR 141716A, and SR 144528 were synthesised in the Chemistry Department of Sanofi (Montpellier, France). Drugs were dissolved in dimethyl sulfoxide, the final solvent concentration in assays never exceeding 0.1% (v/v), an amount without effect on ligand binding. Bifluor liquid scintillant and [^{3}H]CP 55,940 (111.9 Ci/mmol) were from New England Nuclear (Paris, France). Lipofectamine was from Gibco/BRL Life Technologies (Paisley, UK). Luciferase activities were determined using the Luciferase Assay System (Promega). Monkey kidney COS-7 cells were from the American Tissue Culture Collection (Reference CRL-1651) and were cycled twice weekly.

2.2. Site-directed mutagenesis

Site-directed mutagenesis was carried out using the Sculptor in vitro mutagenesis system (Amersham Life Science, Bucks, UK) according to the manufacturer's recommendations. All constructions were fused to an NH_2-terminal c-myc epitope for immunofluorescence detection, inserted into the p658 expression vector, sequenced and transfected into COS-7 cells by a modified DEAE-dextran method as described previously (Shire et al., 1996a).

2.3. Radioligand binding on COS-7 membranes

Membranes from COS-7 cells transiently expressing the wild-type or modified cannabinoid receptors were isolated 56 h after transfection and competition binding assays were carried out with [^{3}H]CP 55,940 as previously described (Rinaldi-Carmona et al., 1996).

2.4. Firefly luciferase assay

COS-7 cells (3×10^4 cells/well) were seeded into opaque 96-well plates (Packard Instruments, Rungis, France), and co-transfected with expression vectors for the wild-type cannabinoid CB_2 or mutated receptors (10 μg/plate), a cAMP response element–luciferase construct (5 μg/plate; Bouaboula et al., 1997) and corticotrophin releasing factor subtype 1 receptor (1 μg/plate) using lipofectamine according to manufacturer's instructions. After 24 h, the cells were incubated with varying concentrations of cold ligand. To assay the cAMP inhibitory agonist effects of CP 55,940 and WIN 55212-2 cells were incubated with corticotrophin releasing factor (10^{-11} M) and varying concentrations of CP 55,940 or WIN 55212-2. To assay the antagonist activity of SR 144528, cells were incubated with corticotrophin releasing factor (10^{-11} M), CP 55,940 (10^{-8} M) or WIN 55212-2 (10^{-8} M) and varying concentrations of SR 144528. Four hours after the addition of the ligands, the cells were washed once with 100 μl phosphate-buffered saline before adding luciferin

buffer (Promega, 25 μl, 1% Triton 1000). After 3–5 min incubation, light emission was measured for 30 s in a Hamamatsu MTP Reader.

2.5. Data analysis

Curves were fit to the data with the Prism non-linear least squares curve-fitting program (GraphPad Software, San Diego, CA). For binding data analyses, one- and two-site fits were tested. For all experiments, a two-site fit was considered better at $P < 0.05$. The values given in Tables 1 and 2 and in the figures are the means ± S.E.M. from three to four experiments. The significance of the values obtained was analysed using a one-way ANOVA test.

2.6. Molecular modelling of the human cannabinoid CB_2 receptor and of SR 144528

To compare amino acid positions in class 1 G-protein-coupled receptors, we have used the global amino acid numbering scheme (gn) of Oliveira et al. (1993). A profile-based multiple sequence alignment of currently known cannabinoid receptor sequences with those of the β-adrenoceptor family was performed to identify the positions in the rat β_2-adrenoceptor model (Gouldson et al., 1997) that required mutation to generate the cannabinoid CB_2 receptor model. The alignment of six of the transmembrane domain regions of the cannabinoid receptors with the transmembrane domain regions from other class one 7-transmembrane domain receptors can be readily performed since they contain many of the conserved motifs associated with this class (Strader et al., 1994), i.e. a conserved asparagine (gn130) in transmembrane domain 1, an aspartic acid (gn224) in transmembrane domain 2, the DRY motif in transmembrane domain 3 (gn339–341), tryptophan in transmembrane domain 4 (gn420), a CWXP motif in transmembrane domain 6 (gn617–620), and a NPXXY motif in transmembrane domain 7 (gn729–733). However, the alignment of transmembrane domain 5 poses a problem because, unlike the majority of class one receptors, a highly conserved proline (gn520) is absent and additionally, two tyrosine residues, Tyr^{207} (gn528) and Tyr^{209} (gn530), are present in the region normally containing a single tyrosine (gn528). With no conserved proline to extrapolate from, there are two positions that transmembrane domain 5 can adopt, giving the choice of placing either Tyr^{207} or Tyr^{209} on the internal face. In the model presented here, based on hydrophobicity considerations Tyr^{207} is situated on the internal face of transmembrane domain 5.

The initial cannabinoid CB_2 receptor transmembrane domain model was energy minimised using 1000 steps of conjugate gradient minimisation followed by 10 ps molecular dynamics (backbone atoms constrained) again followed by 1000 steps of conjugate gradient minimisation to relieve any steric clashes that had arisen from the sequence changes. After this initial energy minimisation, SR 144528 was docked into the receptor model based on the experimentally-derived mutagenesis data using interactive graphics. After docking, the ligand–receptor complex was energy minimised with 1000 steps of conjugate gradient minimisation followed by 200 ps molecular dynamics (backbone atoms constrained). After the constrained molecular dynamics simulations, the model was subjected to conjugate gradient minimisation until the RMS of the energy was less than 0.05 kcal/mol Å. The sequence manipulations and model energy refinement were performed using the WHATIF (Vriend, 1990) and SYBYL (Tripos, St. Louis, MO63144) modelling packages. The AMBER 4.1 all atom force field (implemented in SYBYL)

Table 1
Competition binding of CP 55,940, SR 144528, and WIN 55212-2 with [^{3}H]CP 55,940 on membranes from COS-7 cells transiently expressing mutant human cannabinoid CB_2 receptors. The data are expressed as means ± S.E.M. of two to three independent experiments performed in duplicate

Mutant	CP 55,940 (IC_{50} nM)	SR 144528 (IC_{50} nM)	WIN 55212-2 (IC_{50} nM)
Wild-type receptor	1.1 ± 0.2	0.67 ± 0.09	2.8 ± 0.42
S161A	0.38 ± 0.2	> 1000	1.26 ± 0.1
V164I	0.58 ± 0.03	1.0 ± 0.4	1.6 ± 0.5
S165A	0.55 ± 0.3	> 1000	3.0 ± 1.5
C174S	> 1000	> 1000	> 1000
C175S	0.20 ± 0.1	> 1000	23.4 ± 11.8
R177S	0.8 ± 0.13	0.98 ± 0.18	1.6 ± 0.5
C179S	> 1000	> 1000	> 1000
S193G	1.01 ± 0.05	1.8 ± 1.33	2.2 ± 0.3

Table 2
Parameters of the effect of CP 55,940, WIN 55212-2, and SR 144528 on the luminescence induced by corticotrophin releasing factor (10^{-11} M) in COS-7 cells expressing wild-type and mutant cannabinoid CB_2 receptors. IC_{50} values for CP 55,940 and WIN 55212-2 are for the inhibition of luminescence as described in the Materials and methods. The EC_{50} values for SR 144528 are for the inhibition of the inhibitory activity of CP 55,940 (10^{-8} M). The data are expressed as means ± S.E.M. of three independent experiments performed in sextuplicate. Results were subjected to a one-way ANOVA test to estimate the significance of the results compared to wild type values

Mutant	CP 55,940 (IC_{50} nM)	WIN 55212-2 (IC_{50} nM)	SR 144528 (EC_{50} nM)
Wild-type receptor	6.4 ± 4	12.3 ± 4	3.3 ± 0.9
S161A	18 ± 6	11.6 ± 1.9	$> 10^{-5}$ M[a]
S165A	16 ± 2	8.4 ± 3.1	$> 10^{-5}$ M[a]
C175S	23 ± 8[b]	98 ± 11.8[a]	$> 10^{-5}$ M[a]

[a] Significantly different from wild type, $P < 0.001$.
[b] Significantly different from wild type, $P < 0.01$.

was used for all energy minimisation and molecular dynamics calculations. Additional force field parameters for SR 144528 were derived from existing parameters. The molecular dynamics calculations were carried out in vacuo in a distance-dependent dielectric with a time step of 0.001 ps at 298 K. The initial 3D structure of SR 144528 (S isomer) was constructed using SYBYL. A copy of the model co-ordinates has been deposited at GPCRDB.

3. Results

3.1. Binding characteristics of the wild-type and mutated CB_2 receptors and effect on luciferase stimulation by CP 55,940, WIN 55212-2, and SR 144528

We previously reported that the mutation of each of the conserved cannabinoid CB_2 receptor extracellular loop 2 cysteines Cys^{174} and Cys^{179} (Fig. 2) to serine resulted in receptors that failed to bind [^{3}H]CP 55,940 (Shire et al., 1996a). However, immunofluorescence examination of intact cells by means of the amino terminal c-myc epitope confirmed that the receptors were present on the cell surface (Shire et al., 1996a). In the present study, we found that the C174S and C179S mutants also failed to bind radiolabeled WIN 55212-2, Δ^9-tetrahydrocannabinol and the endogenous cannabinoid, anandamide (results not shown). Rather than conclude that they directly participate in ligand binding, elimination of these conserved cysteine residues may have resulted in an important structural perturbation, perhaps the elimination of a disulfide bridge. It can be noted that mutations of the conserved extracellular loop 2 cysteines in the cannabinoid CB_1 receptor resulted in failure of the mutated receptors to reach the cell surface (Shire et al., 1996a). To see whether mutation of the cannabinoid CB_2 receptor-specific Cys^{175} (Fig. 2) would have the same adverse effect on ligand binding as the mutations of the neighbouring Cys^{174} and Cys^{179}, we replaced it by serine. In a homologous competition binding assay with [^{3}H]CP 55,940, the C175S receptor bound CP 55,940 with comparable affinity to the wild-type receptor (Table 1), showing that the CP 55,940 binding site was largely unaffected by the replacement. In contrast, in competition with [^{3}H]CP 55,940 SR 144528 at 10^{-6} M completely failed to compete with the radioligand for the C175S receptor and WIN 55212-2 had an eight-fold reduced affinity (Fig. 3 and Table 1). We tested the mutated receptor using a convenient functional assay we have developed that avoids the use of radioelements. COS-7 cells were co-transfected with expression vectors for the receptor, corticotrophin releasing factor receptor and a reporter gene system comprised of a minimal promoter containing cAMP response element binding protein sequences fused to a luciferase gene coding region (Bouaboula et al., 1997). Activation of the corticotrophin releasing factor receptor by corticotrophin releasing factor very efficiently stimulates cAMP accumulation with downstream initiation of luciferase production, measurable by fluorescence (Calandra et al., 1999). A functional cannabinoid receptor can inhibit the fluorescence. Comparison of the fluorescence obtained with CP 55,940 or WIN 55212-2 activation of the C175S-mutated cannabinoid CB_2 receptor and the wild-type receptor showed a diminution of the fluorescence with WIN 55212-2 in line with the drop in binding affinity (Table 2). SR 144528 failed to affect the inhibitory activity of either CP 55,940 (Table 2) or WIN 55212-2.

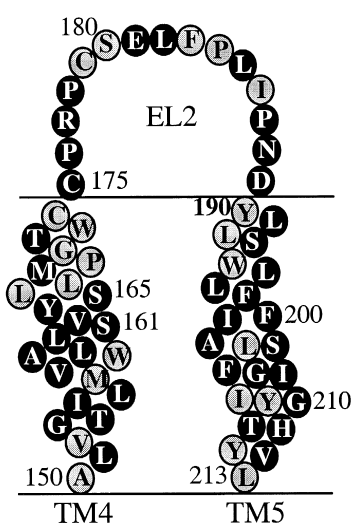

Fig. 2. The transmembrane domain 4–transmembrane domain 5 regions of the wild-type human cannabinoid CB_2 receptor. *Black circles* are cannabinoid CB_2 receptor-specific residues, *gray circles* are residues conserved in both cannabinoid CB_2 and CB_1 receptors.

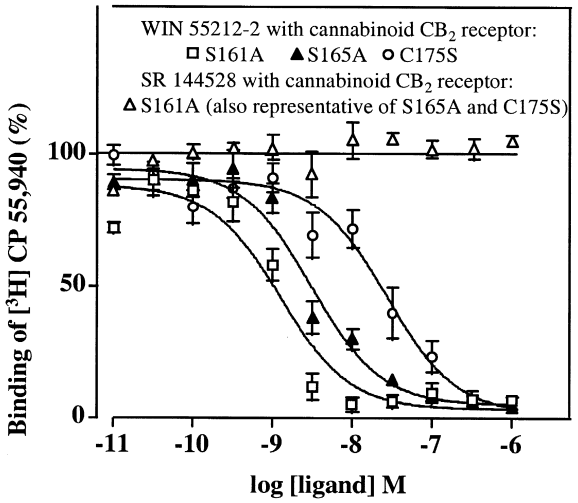

Fig. 3. Competition binding of SR 144528 and WIN 55212-2 with [^{3}H]CP 55,940 on membranes from COS-7 cells transiently expressing point-mutated cannabinoid CB_2 receptors. The results shown are from three separate experiments carried out in duplicate.

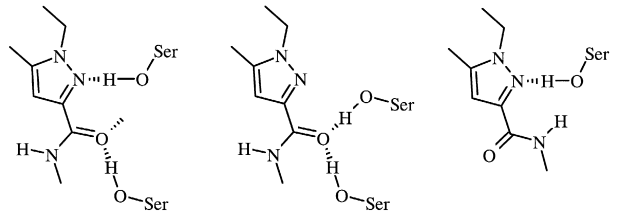

Fig. 4. Possible hydrogen bond interactions between SR 144528 and Ser^{161} and Ser^{165} in transmembrane domain 4 of the cannabinoid CB_2 receptor.

According to a structural model of the cannabinoid CB_2 receptor, the two transmembrane domain 4 serines specific to this subtype, Ser^{161} (gn423) and S^{165} (gn427), are on the internal polar face of the helix, separated by one helical turn. Their separate mutation to alanine, the cognate cannabinoid CB_1 receptor residues, resulted in receptors having wild-type affinity for CP 55,940 (Table 1). From Fig. 3, it can be seen that WIN 55212-2 competed with CP 55,940 with characteristics similar to that of the wild-type cannabinoid CB_2 receptor, but SR 144528 at 10^{-6} M failed to compete with CP 55,940. SR 144528 at 10^{-6} M also failed to compete with [^{3}H]WIN 55212-2 for binding to the S161A and S165A mutated receptors (results not shown). Since the mutation of either Ser^{161} or Ser^{165} to alanine resulted in retention of wild-type affinity for both CP 55,940 and WIN 55212-2, it can be assumed that the mutated receptors were correctly folded. This is further supported by the fact that these receptors responded with wild-type potency and efficacy to CP 55,940 and WIN 55212-2 in the functional assay (Table 2). As with the C175S mutation, SR 144528 at 10^{-5} M failed to modify the inhibitory action of CP 55,940 (Table 2) or of WIN 55212-2 (not shown). The drastic loss of affinity of SR 144528 for the S161A and S165A mutants strongly suggested that the two serines are contact points for SR 144528 with the cannabinoid CB_2 receptor.

WIN 55212-2 has about a 10-fold higher affinity for the human than for the mouse cannabinoid CB_2 receptor and 19-fold higher affinity for the human CB_2 receptor than for the human CB_1 receptor. A recent mutational study has identified Phe^{197} (gn518) in transmembrane domain 5 of the human cannabinoid CB_2 receptor as being crucial for the subtype selectivity of WIN 55212-2 (Song et al., 1999). Phe^{197} is common to human and mouse CB_2 receptors, but three residues in the human receptor situated in the transmembrane domain 4–transmembrane domain 5 region, Val^{164} (gn426), Arg^{177} (extracellular loop 2) and Ser^{193} (gn514) (Fig. 2) are not found in the mouse receptor. We singly mutated them to the corresponding mouse receptor residues, isoleucine, serine and glycine, respectively, to see whether the 10-fold difference was attributable to any of these residues. The competition binding results for WIN 55212-2 were similar to those of wild-type human cannabinoid CB_2 receptor (Table 1), showing that the 10-fold difference was not due to any single one of those residues. None of the three mutations affected SR 144528 affinity (Table 1).

3.2. Model of SR 144528 bound to the human cannabinoid CB_2 receptor

Since Ser^{161} and Ser^{165} probably form hydrogen bonds with the ligand, we considered which of the groups on SR

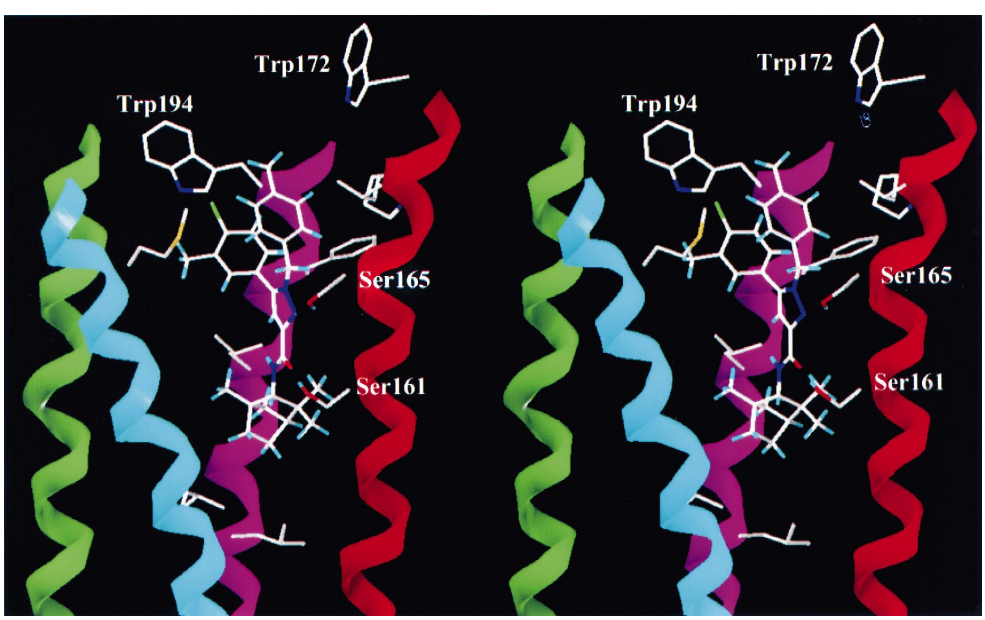

Fig. 5. Stereo diagram of SR 144528 docked in an energy minimised representation of the human cannabinoid CB_2 receptor. Transmembrane domains 3–6 are shown in cyan, red, purple and green, respectively.

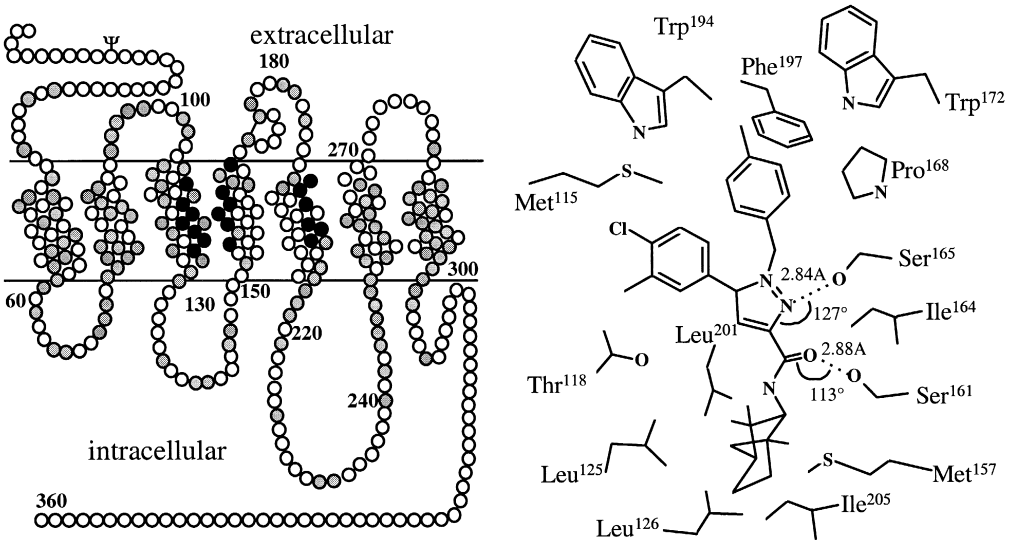

Fig. 6. Residues involved in the SR 144528 binding site in the cannabinoid CB_2 receptor. Left, serpentine representation of the human cannabinoid CB_2 receptor. *White circles*, cannabinoid CB_2-specific residues; *gray circles*, conserved residues; *black circles*, residues contacting SR 144528. Ψ represents the potential glycosylation site. Right, schematic representation of the SR 144528 binding site in the cannabinoid CB_2 receptor. According to the model SR 144528 contacts residues in transmembrane domain 3, transmembrane domain 4, and transmembrane domain 5.

144528 could potentially participate in hydrogen bonding. Three potential hydrogen bonding groups exist in the structure of SR 144528, one hydrogen bond donor, the amide nitrogen hydrogen and two hydrogen bond acceptors, the free pyrazole nitrogen and the carboxyl oxygen (Fig. 1). Through the rotation of the pyrazole ring–amide bond two conformers that maintain the planarity of the molecule are observed. In the first, the free pyrazole nitrogen is on the same face of the molecule as the carboxyl oxygen (Fig. 4a) and in the second, the amide hydrogen is on the same face as the pyrazole nitrogen (Fig. 4c). We hypothesised that the most energetically favourable orientation would be with SR 144528 forming a hydrogen bond with both Ser^{161} and Ser^{165} (Fig. 4a) since the alternative conformations of SR 144528 result in the interaction with one hydrogen bonding group of SR 144528 or only one hydrogen bond (Fig. 4b and c). Serine has three major groups of preferred side chain rotamers when situated in an α-helix. Of the three rotamer groups available, the pairing of rotamer 1 and rotamer 2, for Ser^{161} and Ser^{165}, respectively, gave acceptable hydrogen bond interactions with the most likely conformation of SR 144528 (Fig. 4a). Other pairings either allowed for good interaction with only one of the hydrogen bonding groups of SR 144528 (Fig. 4b and c) or generated steric clashes with other residues in transmembrane domain 4. Because of the inherent structural flexibility of the receptor, it is possible that SR 144528 interacts with the receptor through an ensemble of hydrogen bonding arrangements (Fig. 4a–c) with Ser^{161} and Ser^{165}, but this does not change the overall position of SR 144528 drastically.

The next question concerned the orientation of SR 144528. Clearly, the most energetically favoured interactions for the phenyl groups in the molecule are those implicating an aromatic stacking arrangement. The region

Table 3
Contacts of SR 144528 with the human cannabinoid CB_2 receptor in the model shown in Fig. 5. The table gives the residue position, type of contact, and the cognate residue in the CB_1 receptor subtype. The global number is according to that described by Oliveira et al. (1993)

Contact (global number)	Type of contact made with SR 144528	Residue in the cannabinoid CB_1 receptor
Met^{115} (324)	Hydrophobic/sulfur–π	alanine
Thr^{118} (327)	Hydrophobic/H-bond	threonine
Ala^{119} (328)	Hydrophobic	alanine
Gly^{122} (331)	Hydrophobic	glycine
Leu^{125} (334)	Hydrophobic	phenylalanine
Leu^{126} (335)	Hydrophobic	leucine
Met^{157} (419)	Hydrophobic	methionine
Ser^{161} (423)	H-bond	alanine
Val^{164} (426)	Hydrophobic	isoleucine (also in mouse and rat cannabinoid CB_2 receptors)
Ser^{165} (427)	H-bond	alanine
Pro^{168} (430)	Hydrophobic	proline
Leu^{169} (431)	Hydrophobic	leucine
Trp^{172} (434)	Aromatic/hydrophobic	tryptophan
Ser^{193} (514)	Undefined contact	phenylalanine (glycine in mouse and rat cannabinoid CB_2 receptors)
Trp^{194} (515)	Aromatic/hydrophobic	tryptophan
Phe^{197} (518)	Aromatic/hydrophobic	valine
Leu^{201} (522)	Hydrophobic	leucine
Gly^{204} (525)	Hydrophobic	phenylalanine
Ile^{205} (526)	Hydrophobic	isoleucine

encompassed by transmembrane regions 3 to 5 is relatively rich in aromatic residues, particularly near the extracellular side of the cannabinoid receptors. Two tryptophan residues at the extracellular extremities of transmembrane domain 4 and transmembrane domain 5, Trp^{172} (gn434) and Trp^{194} (gn515) (Fig. 2) are well situated to form aromatic interactions with SR 144528. The molecule was therefore orientated with the phenolic side chains at the extracellular side of the receptor to facilitate potential $\pi-\pi$ interactions with aromatic residues. This placed the hydrophobic fenchyl group near hydrophobic residues in transmembrane domain 3, Gly^{122} (gn331), Leu^{125} (gn334), Leu^{126} (gn335) in transmembrane domain 4, Met^{157} (gn419) and in transmembrane domain 5, Leu^{201} (gn522), Gly^{204} (gn525) and Ile^{205} (gn526). The overall position of SR 144528 with respect to transmembrane domains 3, 4, and 5 after energy minimisation is shown in Fig. 5. The positions of the contacts with respect to the entire cannabinoid CB_2 receptor sequence together with a schematic representation of the binding site are shown in Fig. 6, showing a penetration of the antagonist deep into the helix bundle. A comprehensive list of the contacts made by SR 144528 with the cannabinoid CB_2 receptor in the final energy minimised model structure are listed in Table 3. We note that it is likely that water molecules are present in the binding site. These water molecules may act as bridges replacing the direct interactions between the receptor and ligand.

4. Discussion

In the model for SR 144528 docked into the cannabinoid CB_2 receptor presented here, we propose that the specificity of the molecule relies on crucial contacts with subtype specific residues in transmembrane domain 4. Other contacts are with mainly conserved residues in transmembrane domains 3 and 5. This corroborates previous work in which we used chimeric cannabinoid CB_1/CB_2 receptor to show that an important contribution to the high affinity binding sites for SR 144528 and SR 141716A was situated somewhere in the transmembrane domain 4–transmembrane domain 5 region of their respective receptors (Shire et al., 1996a, 1999). At the same time, we found that the same cannabinoid CB_2 receptor region appeared to contain residues necessary for the subtype and species specificity for WIN 55212-2. This agonist has an affinity for the human cannabinoid CB_2 receptor > mouse cannabinoid CB_2 receptor > human cannabinoid CB_1 receptor. In the present work, we showed that none of several non-conserved residues in transmembrane domains 4 and 5 were accountable for the selectivity of WIN 55212-2 for the human over the mouse cannabinoid CB_2 receptor. However, following the results obtained with the chimeric receptors (Shire et al., 1999), the Reggio laboratory recently identified Phe^{197} (gn518) in transmembrane domain 5 of the human receptor as being important for the specificity of WIN 55212-2 for the cannabinoid CB_2 receptor subtype (Song et al., 1999). Phe^{197}, in concert with an extensive aromatic cluster in the transmembrane 3–5 region of the cannabinoid CB_2 receptor, provided the principal contacts for WIN 55212-2 in a docking model for the interaction (Song et al., 1999).

The docking model we present here for SR 144528 in the cannabinoid CB_2 receptor has several features in common with the WIN 55212-2 docking model. In the model, Phe^{197} is also implicated in an interaction with SR 144528, together with Trp^{172} (gn434) and Trp^{194} (gn515), two residues also present in the WIN 55212-2 docking model (Song et al., 1999). However, the SR 144528–receptor interaction differs from that of WIN 55212-2 in that hydrogen bonds with Ser^{161} (gn423) or Ser^{165} (gn427) in transmembrane domain 4 are important, since replacement by alanine of either of the serine residues eliminates both the binding and the activity of SR 144528, while not affecting those of WIN 55212-2. This is the first report of the role played by the transmembrane domain 4 serines in a ligand–receptor interaction. Interestingly, they are particularly well conserved in the class 1 heptahelical receptor superfamily ($\sim$65%), with the alanine residues found in the cannabinoid CB_1 receptor being the usual alternatives ($\sim$30%).

The overall position of SR 144528 (and of WIN 55212-2) in the receptor is different from the ligand binding sites normally found for small nonpeptide molecules. Many models place the ligand in the general binding site described for the rhodopsin and monoamine receptors, i.e. between transmembrane domains 3, 5, 6 and 7 (Gouldson et al., 1997). The model for SR 144528 bound to the cannabinoid CB_2 receptor presented here differs in that the majority of the interactions are with transmembrane domains 3, 4, and 5, and none with transmembrane domains 6 or 7 (Fig. 6). Up to now, the few studies that have implicated transmembrane domain 4 in ligand–receptor interactions have all concerned neuropeptide receptors. All these interactions involve residues on the same face of the helix: Met^{208} (gn434) in the neurotensin type 1 receptor with another diarylpyrazole, SR 48692 (Labbé-Jullié et al., 1998); Phe^{173} in the same position in the neuropeptide Y subtype 1 receptor (gn434) with a small molecule antagonist; in the substance P receptor, Gly^{166} (gn431) was found to modulate the affinity for the natural peptide agonist (Ciucci et al., 1997).

The general topology of the CB_2 receptor we have adopted in the present work is based on the refined crystal structure of bovine rhodopsin (Krebs et al., 1998). However, it cannot be excluded that the cannabinoid receptors have a somewhat different topology to other class 1 heptahelical G protein-coupled receptors. Unexpected structures are coming to light, as exemplified by an unusual tertiary structure recently suggested for the bradykinin B2 receptor in which the loop region joining transmembrane domain 1 and transmembrane domain 2 was situated extracellularly,

not intracellularly (Quiza et al., 1999). In particular, the role of cysteine residues in the cannabinoid receptors remains to be clarified. A pair of cysteine residues in extracellular regions 1 and 2 has been shown in several G protein-coupled receptors to be linked by a disulfide bridge, which would clearly impose a certain structural constraint on the receptors. Both cannabinoid receptors lack a cysteine in extracellular loop 1, but extracellular loop 2 contains two and three cysteines in the cannabinoid CB_1 and CB_2 receptors, respectively (Fig. 2). In common with several other class 1 G protein-coupled receptors (Zeng et al., 1999), replacement of the extracellular loop 2 cysteines in the cannabinoid CB_1 receptor prevented translocation of the mutated receptors to the plasma membrane (Shire et al., 1996a). Although cannabinoid CB_2 receptors carrying mutations in the conserved extracellular loop 2 cysteines are translocated, they fail to bind ligands, for reasons that remain unclear. Cysteine residues in extracellular loops have also been implicated in receptor dimerisation (Zeng and Wess, 1999), but we have no evidence at present that the cannabinoid receptors dimerise.

The role played by the non-conserved Cys^{175} in the cannabinoid CB_2 receptor is also unclear. The inability of SR 144528 to displace CP 55,940 in the C175S mutant or to inhibit its activity would at first suggest that Cys^{175} is a contact residue for SR 144528. It is possible that the extracellular loop 2 dips back down towards the binding site of SR 144528 to form a sulfur–π interaction with the pyrazole group of SR 144528. The wild-type binding value for CP 55,940 with the C175S mutant and the relatively moderate reduction in WIN 55212-2 binding to the mutant would imply that the binding sites of these ligands were not drastically affected by the replacement. The effects of the mutation on SR 144258 binding may also be attributable to an alternative disulfide bridge formation, but further work is required in order to clarify this interaction.

Given the high level of structural similarity of SR 144528 to SR 141716A, it is conceivable that hydrogen bonding is also important for the binding of the latter to the cannabinoid CB_1 receptor subtype. Potential hydrogen bonding partners for the amide group in SR 141716A have to be found elsewhere in this subtype. Two possible alternative hydrogen bonding partners are available on transmembrane domain 3 of the cannabinoid CB_1 receptor, namely, the conserved residues Thr^{114} (gn323) and Thr^{118} (gn327). Although we have no data that support their implication in SR 141716A binding to the CB_1 receptor, molecular modelling shows that hydrogen bond interactions with both residues could be accomplished by turning the molecule through 180° from the position occupied by SR 144528. This orientation would maintain the aromatic interaction with Trp^{172} and Trp^{194} and also the hydrophobic interactions of the pyrazole ring with hydrophobic residues in transmembrane domains 4 and 5.

It is clear from the present study and others carried out elsewhere (Abood et al., 1998; Song and Bonner, 1996; Tao et al., 1999), that the binding sites of ligands of different chemical structure on the cannabinoid receptors, if overlapping, are not identical. It is hoped that by establishing the exact architecture of agonists and antagonists docked into the cannabinoid receptors, we can develop ligands of higher specificity and potency for each subtype.

References

Abood, M.E., Tao, Q., McAllister, S.D., Hurst, D.P., Norris, J., Reggio, P.H., 1998. A critical role for a tyrosine residue in the cannabinoid receptors for recognition of several compounds excluding the indoles. In: 1998 Symposium on the Cannabinoids, Burlington, Vermont, International Cannabinoid Research Society 2-2.

Bouaboula, M., Perrachon, S., Milligan, L., Canat, X., Rinaldi-Carmona, M., Portier, M., Barth, F., Calandra, B., Pecceu, F., Lupker, J., Maffrand, J.P., Le Fur, G., Casellas, P., 1997. A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1 — evidence for a new model of receptor/ligand interactions. J. Biol. Chem. 272, 22330–22339.

Calandra, B., Portier, M., Kerneis, A., Delpech, M., Carillon, C., Le Fur, G., Ferrara, P., Shire, D., 1999. Dual intracellular signaling pathways mediated by the human cannabinoid CB1 receptor. Eur. J. Pharmacol. 374, 445–455.

Ciucci, A., Palma, C., Riitano, D., Manzini, S., Werge, T.M., 1997. Gly^{166} In the NK1 receptor regulates tachykinin selectivity and receptor conformation. FEBS Lett. 416, 335–338.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., Casellas, P., 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur. J. Biochem. 232, 54–61.

Gérard, C.M., Mollereau, C., Vassart, G., Parmentier, M., 1991. Molecular cloning of a human cannabinoid receptor which is also expressed in testis. Biochem. J. 279, 129–134.

Gouldson, P.R., Snell, C.R., Reynolds, C.A., 1997. A new approach to docking in the β_2-adrenergic receptor that exploits the domain structure of G-protein-coupled receptors. J. Med. Chem. 40, 3871–3886.

Krebs, A., Villa, C., Edwards, P.C., Schertler, G.F.X., 1998. Characterisation of an improved two-dimensional p22121 crystal from bovine rhodopsin. J. Mol. Biol. 282, 991–1003.

Labbé-Jullié, C., Barroso, S., Nicolas-Etève, D., Reversat, J.L., Botto, J.M., Mazella, J., Bernassau, J.M., Kitabgi, P., 1998. Mutagenesis and modeling of the neurotensin receptor NTR1 — identification of residues that are critical for binding SR 48692, a nonpeptide neurotensin antagonist. J. Biol. Chem. 273, 16351–16357.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., Pertwee, R.G., Griffin, G., Beyewitch, M., Barg, J., Vogel, Z., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50, 83–90.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61–65.

Oliveira, L., Paiva, A.M., Vriend, G., 1993. A common motif in G-protein-coupled seven transmembrane helix receptors. J. Comput.-Aided Mol. Des. 7, 649–658.

Pertwee, R.G., 1997. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol. Ther. 74, 129–180.

Quiza, M., Zaki, P.A., 1999. Investigation of the extracellular accessibility of the connecting loop between membrane domains I and II of the bradykinin B2 receptor. J. Biol. Chem. 274, 14773–14778.

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, J.-C., Le Fur, G., 1994. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. 350, 240–244.

Rinaldi-Carmona, M., Calandra, B., Shire, D., Bouaboula, M., Oustric, D., Barth, F., Casellas, P., Ferrara, P., Le Fur, G., 1996. Characterization of two cloned human CB1 cannabinoid receptor isoforms. J. Pharmacol. Exp. Ther. 278, 871–878.

Rinaldi-Carmona, M., Barth, F., Millan, J., Derocq, J.M., Casellas, P., Congy, C., Oustric, D., Sarran, M., Bouaboula, M., Calandra, B., Portier, M., Shire, D., Brelière, J.C., Le Fur, G., 1998. SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. J. Pharmacol. Exp. Ther. 284, 644–650.

Shire, D., Calandra, B., Delpech, M., Dumont, X., Kaghad, M., Le Fur, G., Caput, D., Ferrara, P., 1996a. Structural features of the central cannabinoid CB1 receptor involved in the binding of the specific CB1 antagonist SR 141716A. J. Biol. Chem. 271, 6941–6946.

Shire, D., Calandra, B., Rinaldi-Carmona, M., Oustric, D., Pessegue, B., Bonnin-Cabanne, O., Le Fur, G., Caput, D., Ferrara, P., 1996b. Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. Biochim. Biophys. Acta Gene Struct. Expression 1307, 132–136.

Shire, D., Calandra, B., Bouaboula, M., Barth, F., Rinaldi-Carmona, M., Casellas, P., Ferrara, P., 1999. Cannabinoid receptor interactions with the antagonists SR 141716A and SR 144528. Life Sci. 65, 627–635.

Showalter, V.M., Compton, D.R., Martin, B.R., Abood, M.E., 1996. Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. J. Pharmacol. Exp. Ther. 278, 989–999.

Song, Z.H., Bonner, T.I., 1996. A lysine residue of the cannabinoid receptor is critical for receptor recognition by several agonists but not WIN 55212-2. Mol. Pharmacol. 49, 891–896.

Song, Z.H., Slowey, C.A., Hurst, D.P., Reggio, P.H., 1999. The difference between the CB_1 and CB_2 cannabinoid receptors at position 5.46 is crucial for the selectivity of WIN 55212-2 for CB_2. Mol. Pharmacol. 56, 834–840.

Strader, C.D., Fong, T.M., Tota, M.R., Underwood, D., Dixon, R.A.F., 1994. Structure and function of G protein-coupled receptors. Annu. Rev. Biochem. 63, 101–132.

Tao, Q., McAllister, S.D., Andreassi, J., Nowell, K.W., Cabral, G.A., Hurst, D.P., Bachtel, K., Ekman, M.C., Reggio, P.H., Abood, M.E., 1999. Role of a conserved lysine residue in the peripheral cannabinoid receptor (CB2): evidence for subtype specificity. Mol. Pharmacol. 55, 605–613.

Vriend, G., 1990. WHATIF: a molecular modelling and drug design program. J. Mol. Graphics 8, 52–56.

Zeng, F.Y., Wess, J., 1999. Identification and molecular characterization of m3 muscarinic receptor dimers. J. Biol. Chem. 274, 19487–19497.

Zeng, F.Y., Soldner, A., Schoneberg, T., Wess, J., 1999. Conserved extracellular cysteine pair in the M-3 muscarinic acetylcholine receptor is essential for proper receptor cell surface localization but not for G protein coupling. J. Neurochem. 72, 2404–2414.