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Structural Features of the Central Cannabinoid CB1 Receptor Involved in the Binding of the Specific CB1 Antagonist SR 141716A*

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The antagonist SR 141716A has a high specificity for the central CB1 cannabinoid receptor and negligeable affinity for the peripheral CB2 receptor, making it an excellent tool for probing receptor structure-activity relationships. From binding experiments with mutated CB1 and with chimeric CB1/CB2 receptors we have begun to identify the domains of CB1 implicated in the recognition of SR 141716A. Receptors were transiently expressed in COS-3 cells, and their binding characteristics were studied with SR 141716A and with CP 55,940, an agonist recognized equally well by the two receptors. The region delineated by the fourth and fifth transmembrane helices of CB1 proved to be crucial for high affinity binding of SR 141716A. The CB1 and CB2 second extracellular loops, e2, were exchanged, modifications that had no effect on SR 141716A binding in the CB1 variant but that eliminated CP 55,940 binding in both mutants. The replacement of the conserved cysteine residues in e2 of CB2 by serine also eliminated CP 55,940 binding, but replacement of those in CB1 resulted in the sequestration of the mutated receptors in the cell cytoplasm. The e2 domain thus plays some role in CP 55,940 binding but none in SR 141716A recognition, binding of the latter clearly implicating residues in the adjoining transmembrane helices.

The cellular effects elicited by Δ^9-tetrahydrocannabinol, the major psychoactive component of cannabis (1), are mediated through cell surface cannabinoid receptors. Complementary DNAs encoding rat (2) and human brain (3) receptors (CB1)[1] as well as a cDNA encoding a human peripheral (4) receptor (CB2) have been characterized. The predicted amino acid sequences show that these receptors have structures typical of the seven transmembrane domain G protein-coupled receptor superfamily (5). The human CB1 and CB2 share only 43% overall identity (64% similarity), rising to 51% identity (71% similarity) in the transmembrane bundle-loop domain; this rather large structural difference only partly correlates with agonist specificity. Although Δ^9-tetrahydrocannabinol and the classical synthetic agonists CP 55,940 and WIN 55212–2 were originally reported to be nondiscriminating (4), more recent investigations by us[2] and others (6, 7) have shown a significantly higher affinity of WIN 55212–2 for CB2 than for CB1. The endogenous brain and peripheral ligands, anandamide (8) and 2-arachidonyl glycerol (9), are essentially nondiscriminating (4, 6, 7, 9). In addition to their overall similarity in respect to agonist recognition, both CB1 (10, 11) and CB2 (12) mediate their action through the inhibition of adenylyl cyclase via a pertussis toxin-sensitive GTP-binding regulatory protein. CB1 has also been shown to be associated with the inhibition of N-type calcium channels in neuroblastoma-glioma cells (13) and Q-type calcium channels in AtT-20 cells (7), whereas a similar activity for CB2 could not be detected (7).

In order to determine the structural elements of receptors necessary for ligand recognition, it is useful to dispose of ligands that are species specific or receptor subtype specific. Considering the former possibility, it is unfortunate that the ligand binding properties of the rat CB1, whose sequence differs from human CB1 in only 13 amino acids, mainly in the amino-terminal region, are identical to those of human CB1 (14). The only other known CB1 sequence, the murine, differs from rat CB1 by only one amino acid in the amino-terminal region.[3] In contrast, the recently cloned murine CB2 is only 82% identical to human CB2 but has binding properties similar to those of the human receptor.[4] However, a highly specific CB1 ligand is already available. We have recently described SR 141716A (12), the first selective, potent antagonist of a cannabinoid receptor, which displays a 1000-fold higher specificity for CB1 than for CB2. This molecule therefore provides a powerful tool for studying *in vitro* and *in vivo* functions of the cannabinoid receptor as well as the structural features of the cannabinoid receptors important for ligand recognition.

Because of the paucity of biophysical data concerning G protein-coupled receptors, studies of ligand-receptor interactions have relied heavily on molecular biological techniques. Since the pioneering work by the Lefkowitz group (15) on the chimeric receptors that resulted from an exchange of structurally homologous domains between the α2- and β2-adrenergic receptors at the cDNA level, this strategy has proved to be an effective first approach for determining regions implicated in either ligand-receptor or receptor-effector interactions. Based on the results obtained, more precise details of the interactions follow the mutation of selected amino acids in the regions identified. This general approach has been followed successfully in studies of receptors for peptides and biogenic amines (see Refs. 16 and 17 for recent reviews).

In the present article we describe the expression of a series of CB1/CB2 chimeras and mutated wild-type receptors and their

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[1] The abbreviations used are: CB, cannabinoid receptor; e2, second extracellular loop region; TM, transmembrane region; PBS, phosphate-buffered; BSA, bovine serum albumin.

[2] M. Rinaldi-Carmona, B. Calandra, D. Shire, M. Bouboula, D. Oustric, F. Barth, P. Casellas, P. Ferrara, and G. Le Fur, submitted for publication.

[3] D. Shire, B. Calandra, M. Delpech, X. Dumont, M. Kaghad, G. Le Fur, D. Caput, and P. Ferrara, unpublished results.

[4] D. Shire, B. Calandra, M. Rinaldi-Carmona, D. Oustric, B. Pessègue, N. Bonnin, D. Caput, and P. Ferrara, submitted for publication.

binding properties with the antagonist SR 141716A and the agonist CP 55,940, the latter proving useful for ensuring the integrity of the mutated receptors. In addition, several modified receptors failed to bind ligands, and we found that the expression of receptors fused to a c-*myc* epitope proved to be indispensable for ascertaining whether this failure was a result of nonexpression of the receptors, a consequence of poor insertion in the plasma membrane, or attributable to deleterious conformational perturbations.

EXPERIMENTAL PROCEDURES

Materials—SR 141716A, CP 55,940, and anandamide were synthesized at Sanofi Recherche (Montpellier, France). Drugs were dissolved either in ethanol (anandamide, Δ^9-tetrahydrocannabinol) or dimethyl sulfoxide (SR 141716A or CP 55,940), the amount of solvent in assays never exceeding 0.1% (v/v), an amount without effect on radioligand binding. Biofluor liquid scintillant and [^{3}H]CP 55,940 (111.9 Ci/mmol) were from New England Nuclear (Paris, France) and [^{3}H]SR 141716A (35–40 Ci/mmol) from Amersham Corp. (Les Ulis, France). The anti-c-*myc* mouse antibody 9E10 was a gift from B. Pau (CNRS, Montpellier). Human anti-mitochondrial serum was from Leinco Technology. A fluorescein-coupled anti-mouse antibody and a rhodamine-labeled anti-human antibody were from Silenus.

Site-directed Mutagenesis and Construction of Chimeric Receptors—The cDNA containing the coding region for CB1 was obtained by polymerase chain reaction from the human IM-9 cell line as described (18), and that for CB2 was obtained from the human premonocytic cell line U937. Site-directed mutagenesis was carried out using the Sculptor kit (Amersham Corp., Les Ulis, France). Constructions were made by overlap polymerase chain reaction extension (19). Receptor fusions were carried out at the junctions shown in Fig. 1, usually following or preceding an amino acid common to CB1 and CB2 at the extremity of a TM region. The nomenclature used for chimeric receptors is: CB parent receptor/replacement receptor (region replaced); for example CB1/2(6-Ct) is CB1 fused with the homologous TM6 to COOH-terminal CB2 sequence following Trp^{299} of CB1. The amino acids preceding and following the points of fusion are as follows: CB1/2(1-Ct), CB1(Ala^{118})/CB2(Val^{36}); CB1/2(2-Ct), CB1(His^{154})/CB2(Phe^{72}); CB1/2(3-Ct), CB1(Asn^{187})/CB2(Val^{155}); CB1/2(4-Ct), CB1(Lys^{232})/Cb2(Ala^{150}); CB1/2(5-Ct), CB1(Thr^{274})/CB2(Tyr^{190}); CB1/2(6-Ct), CB1(Trp^{299})/CB2(Lys^{215}); CB1/2(7-Ct), CB1(Val^{364})/CB2(His^{267}); CB1/2(Ct), CB1(Ser^{401})/CB2(Gly^{304}); CB2/1(5-Ct), CB2(Asp^{189})/CB2(Tyr^{275}). "Sandwich" fusions were: CB1/2(4–5)/1, CB1(Lys^{232})/CB2(Ala^{150}-Trp^{214})/CB1(Lys^{300}); CB2/1(4–5)/2, CB2(Arg^{149})/CB1(Ala^{233}-Trp^{299})/CB2(Lys^{215}). Other modifications are described under "Results." To express receptors having an NH_2-terminal c-*myc* epitope, a double-stranded oligonucleotide encoding the epitope together with a Kozac consensus sequence was inserted into the *Hin*dIII site, thereby leading to the expression of receptors carrying the supplementary 13-amino acid NH_2-terminal sequence (MEQKLISEEDLKL) in front of the second residue of the receptors. The DNA sequences of all constructs were confirmed by dideoxy sequencing (21). All the constructions were inserted into p658, an expression vector derived from p7055 (20) by replacing the IL-2 coding sequence with a polylinker flanked by *Hin*dIII and *Eco*RI sites. The vectors were transfected into COS-3 cells by a modified DEAE-dextran method (22).

Immunofluorescence—Transiently transfected COS-3 cells (1.6×10^5 cells) were incubated for 2 days in pairs of slide flasks (Nunc, Roskilde, Denmark). The cells were washed with phosphate-buffered saline (PBS), on one of the pairs of slide flasks the cells were fixed by methanol treatment for 6 s at −20 °C and then followed by further washing with PBS, 1% bovine serum albumin, and sodium azide. Fixed and unfixed cells were treated for 60 min at 4 °C with PBS solution containing mouse anti-*myc* antibody (1/500) and human anti-mitochondrial antibody (1/1000). After washing with the same solution at 4 °C, the slides were incubated with the labeled anti-antibodies (each at 1/100). After further washing the slide bearing the fixed cells was dried with filter paper, and the cells on the second slide were methanol-fixed and dried. The doubly labeled cells were examined using a Leitz Dialux 20 phase contrast microscope.

Western Blot Analysis—Western blot analysis of total cell proteins was carried out. Transiently transfected COS-3 cells (1.6×10^5 cells) were incubated for 2 days in 10-cm wells and then scraped into 100 μl of PBS. After centrifugation for 5 min at 1000 rpm, the pellet was resuspended in 30 μl of 125 mM Tris pH 7.4, 4% SDS, and 20% glycerol containing bromphenol blue. An equal volume of 8% dithiothreitol was added, the mixture was heated for 5 min at 100 °C and sonicated, and 10 μl samples separated on 10% SDS-polyacrylamide gel electrophoresis for 18 h at 70 V. The proteins were transferred to Immobilon P membranes (Millipore S.A., St. Quentin-Yvelines, France), which were saturated for 30 min with PBS with 5% BSA at 37 °C before washing three times with PBS and 0.1% BSA. The membranes were incubated for 18 h at 4 °C with anti-c-*myc* antibody (1/1000) in PBS with 0.1% BSA and then washed three times for 30 min with PBS and 0.1% BSA. Positive bands were revealed after incubating for 2 h at 20 °C with an anti-mouse Auroprobe (1/100) (Amersham Corp.) in PBS, 0.1% BSA, and 5% (v/v) gelatine, followed by washing two times for 1 min with distilled water and then adding silver enhancer from the Amersham kit.

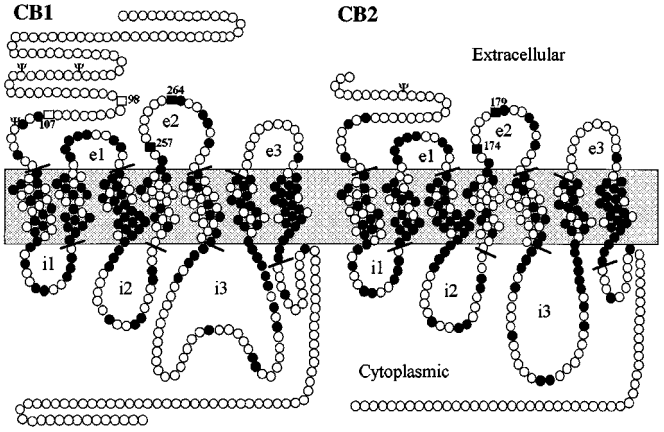

FIG. 1. **Schematic representation of the human CB1 and CB2 receptors.** *Black circles* represent amino acids common to the two receptors, and *white circles* different amino acids. The mutated cysteines described in the present work are shown as *open squares* (CB1-specific) or as *solid squares* (common to CB1 and CB2). Potential Asn-linked sugar residues are shown as ψ. The *bars* represent the sites of fusion to create the chimeras.

Binding on Subcellular Fractions—COS-3 cells transiently expressing cannabinoid receptors were treated 56 h after transfection. Cells were washed twice with PBS, scraped into 50 mM Tris-HCl, pH 7.7, crushed in a Polytron for 1 min at 7000 rpm, and then separated into two halves. One half was centrifuged for 60 min at 108,000 × *g*, and the pellet was resuspended in PBS (fraction 1). The second half was centrifuged for 15 min at 2000 × *g*, the pellet was suspended in PBS (fraction 2), and the supernatant was centrifuged at 108,000 × *g*, the pellet being taken up in PBS (fraction 3). Protein content was measured by the Bradford method (23). The fractions were stored at −80 °C before binding assays (12, 44).

RESULTS

Binding Characteristics of Wild-type and Chimeric Receptors—Using polymerase chain reaction, chimeric cDNA constructions were obtained in which CB1-encoding domains were systematically replaced by the corresponding CB2 regions, junctions being made at the putative intra/extramembrane interfaces, in all but one case next to an amino acid common to the two receptors (Fig. 1). Membranes from transfected COS-3 cells transiently expressing the chimeras were used for binding experiments. Membranes from COS-3 cells transfected with empty vector do not bind cannabinoids (data not shown). The synthetic agonist CP 55,940 has been shown to have similar binding affinities for both CB1 and CB2 (4) and was therefore expected to be useful as a universal ligand for all chimeric constructs. In a first series of saturation binding experiments, the dissociation constants for [^{3}H]CP 55,940 with each of the expressed receptors was measured (Table I). Where binding occurred, all the values were in the subnanomolar range, indicating insertion of these receptors in the membrane apparently with little structural perturbation. From the B_{max} values it can be seen that comparable numbers of each of the receptors were to be found in the plasma membranes. The antagonist SR 141716A displaced the radioligand in a competitive manner, revealing a clear relationship between the binding affinity of

TABLE I
Equilibrium dissociation constants of CP 55,940 and SR 141716A for chimeric cannabinoid receptors

Chimeric receptors were constructed as described under "Experimental Procedures," and binding studies were performed on membranes isolated from transiently transfected COS-3 cells. The K_d values for CP 55,940 were obtained from saturation experiments with the tritiated ligand. The B_{max} values are in italics.

	CP55,940	SR141716A
	Kd nM (mean ± SEM), n=2-4 *Bmax pmole/mg protein*	IC50 nM (mean ± SEM), n=1-4
WT CB1	0.4 ± 0.09 *18.7 ± 2.1*	6.4 ± 2.2
CB1/2(Ct)	0.28 ± 0.1 *9.98 ± 1.1*	3.4 ± 0.8
CB1/2(7-Ct)	0.6 ± 0.2 *6.2 ± 0.9*	23.4 ± 7.7
CB1/2(6-Ct)	no binding	no binding
CB1/2(5-Ct)	0.21 ± 0.05 *12.7 ± 1.0*	43.7 ± 25
CB1/2(4-Ct)	0.1 ± 0.01 *4.9 ± 0.17*	734 ± 134
CB1/2(3-Ct)	no binding	no binding
CB1/2(2-Ct)	no binding	no binding
CB1/2(1-Ct)	0.26 ± 0.14 *4.4 ± 0.64*	643
WT CB2	0.2 ± 0.07 *18.8 ± 2.3*	>1000
CB2/1(5-Ct)	0.14 ± 0.04 *4.3 ± 1.36*	49.2 ± 31.9

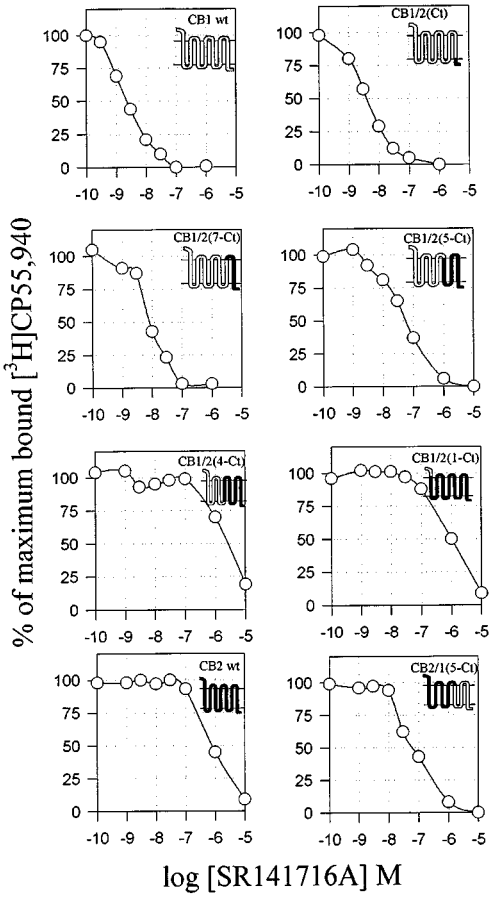

FIG. 2. **Competition binding profiles for the antagonist SR 141716A with the wild-type CB1 and CB2 receptors and chimeric receptors composed of CB1 and CB2 regions.** Data are expressed as the percentage of maximum bound radioligand and are representative of the number of experiments given in Table I. The binding assay was carried out on isolated membranes as described under "Experimental Procedures." Schematic representations of the receptors are shown next to each curve, *open regions* being those from CB1 and *solid regions* from CB2.

the antagonist and receptor structure (Fig. 2). The IC_{50} values for SR 141716A with the wild-type CB1 and CB2 receptors were 6.4 and >1000 nM, respectively.

Replacement of the COOH-terminal tail of CB1 by that of CB2 in the CB1/2(Ct) chimera resulted in a 2-fold increase in antagonist affinity (Table I). The subsequent replacement of the CB1 TM7 region by that of CB2, giving CB1/2(7-Ct), resulted in a 4-fold loss in SR 141716A affinity compared with the wild-type receptor. This loss in affinity suggests some slight perturbation in receptor structure rather than a direct effect on the ligand binding site. No ligand binding was obtained with the chimeric receptor CB1/2(6-Ct). This chimera was reconstructed with a c-*myc* epitope inserted between Met^1 and Lys^2, and its characteristics compared with those of epitope-tagged wild-type CB1 and CB2. The epitope proved to have relatively little effect on CP 55,940 binding (K_d 0.63 ± 0.29 nM, B_{max} 2.5 ± 0.6 pmol/mg of protein for CB1; K_d 0.24 ± 0.05 nM, B_{max} 12.7 ± 1.0 pmol/mg of protein for CB2) compared with that of the wild-type receptors (see Table I), in line with previous findings with amino-terminally tagged receptors (24, 25). Western immunoblot analysis of the tagged receptors using a labeled anti-*myc* antibody confirmed the expression of CB1 (*lane 5*, Fig. 3*a*) and CB2 (*lanes 1* and *2*, Fig. 3*b*) as well as of CB1/2(6-Ct) (*lane 4*, Fig. 3*a*).

The question then arose as to whether or not the latter protein was present in the plasma membrane. We carried out microscopical immunofluorescence examination of transfected COS cells after incubating the cells with fluorescein-labeled anti-*myc* antibodies both before and after fixation on slide flasks. At the same time, the cells were incubated with a rhodamine-labeled anti-mitochondrial antibody as a control of cell integrity. *Panels A* and *B* of Fig. 4 show the detection on intact cells of tagged CB1 and CB2, respectively. A uniform pattern of distribution of the receptors over the entire plasma membrane surface can be observed. The *insets* in Fig. 4 (*A* and *B*) show that mitochondria were not detectable, indicating that the cells were intact. In contrast, Fig. 4*C* shows the immunofluorescence observed with CB1/2(6-Ct). The *inset* (Fig. 4*C*) shows that no immunofluorescence was observed in intact cells, whereas it can be seen that the c-*myc* epitope could be easily detected in fixed cells, indicating sequestration of the chimera in the interior of the cells. Fig. 4*D* shows the result of incubation of the same, fixed cells with the antimitochondrial antibody. However, if CB1/2(6-Ct) was present in the interior of the cell, was it nevertheless capable of recognizing the universal ligand CP 55,940? Whole cell fractions were compared with the membrane fractions and the nonmembrane fractions for binding experiments, wild-type c-*myc*-CB1 being used as a control. Specific binding of tritiated CP 55,940 was 73, 74, and 68% for the three fractions of wild-type CB1, respectively. In contrast, no specific binding to CB1/2(6-Ct) was observed, radioactivity being indistinguishable from background levels for each fraction.

The subsequent replacement of the CB1 TM5 region in CB1/2(6-Ct) by that of CB2 restored CP 55,940 binding and compe-

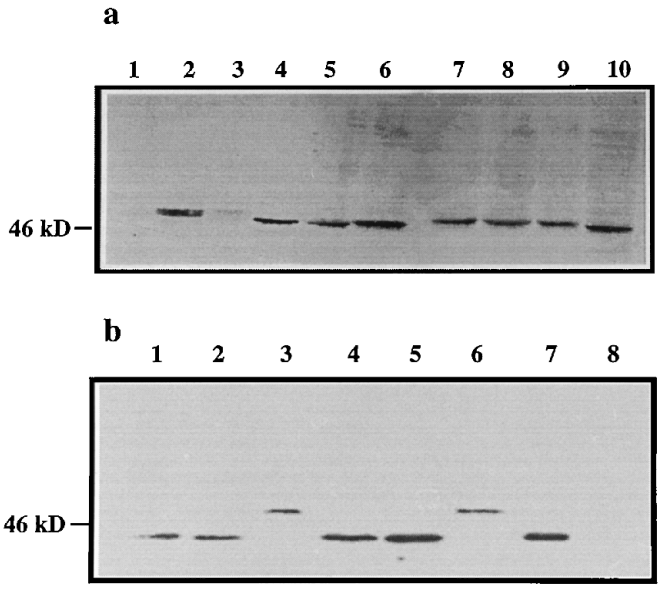

FIG. 3. **Western immunoblot analysis of cannabinoid receptor mutants and chimeras.** Proteins were prepared as described under "Experimental Procedures." *a*, *lane 1*, nontransfected COS-3 cells; *lane 2*, CB1/2(5-Ct); *lane 3*, CB1/2(5-Ct) c-*myc*(Ct); *lane 4*, CB1/2(6-Ct); *lane 5*, CB1; *lane 6*, CB1(C98, 107S); *lane 7*, CB1(C257S); *lane 8*, CB1(C264S); *lane 9*, CB1(C257, 264S); *lane 10*, CB1(CPRPe2). *b*, *lanes 1* and *2*, CB2; *lane 3*, CB1/2(e2); *lane 4*, CB2(C179S); *lane 5*, CB2/1(e2); *lane 6*, CB1/2(CPRPe2); *lane 7*, CB2(C174S); *lane 8*, mock-transfected COS.

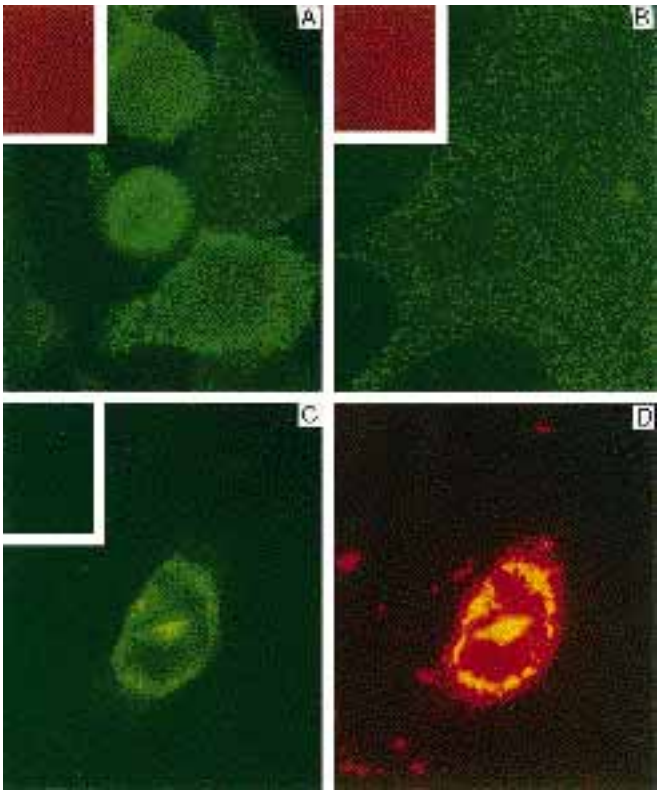

FIG. 4. **Immunofluorescence of cannabinoid receptors transiently expressed in COS-3 cells.** Fluorescein-coupled anti-mouse antibodies used to detect anti-epitope c-*myc* antibodies on c-*myc*-receptor fusions and rhodamine-coupled anti-mitochondrial antibodies were used as described under "Experimental Procedures." *A* and *B* show wild-type CB1 and CB2, respectively, on unfixed cells, and the *insets* show the results with the anti-mitochondrial antibody. *C* shows CB1/2(6-Ct) on fixed cells, with an *inset* showing the result on unfixed cells. *D* shows the same cells as in *C* with the rhodamine-coupled anti-mitochondrial antibody.

tition by SR 141716A for the binding site. Compared with CB1/2(7-Ct), the addition of a further two CB2 transmembrane regions only resulted in a 2-fold drop in affinity for SR 141716A, which represented a relatively small loss. In contrast, a more spectacular loss in affinity for SR 141716A was observed with CB1/2(4-Ct), the replacement of TM4 and e2 of CB1 by that of CB2 resulting in an 18-fold drop in the IC_{50} value, thereby practically attaining that of wild-type CB2. No further significant changes in binding affinity for SR 141716A occurred on incorporating further amino-terminal regions of CB2 into the chimeras. It must be noted that two other chimeras in the series, CB1/2(3-Ct) and CB1/2(2-Ct), failed to bind CP 55,940.

Results from the direct binding of [^{3}H]SR 141716A to the wild-type and chimeric receptors correlated with the competition studies, because this ligand recognized only wild-type CB1, CB1/2(Ct), and CB1/2(7-Ct) The binding affinities of the ligand with each of these receptors were 0.38, 0.41, and 1.0 nM, respectively, in line with the IC_{50} values (Table I). The binding of SR 141716A with CB1/2(5-Ct) was too poor to allow its direct binding affinity to be measured with any degree of accuracy.

We also constructed the chimeric receptor reciprocal to CB1/2(5-Ct), namely CB2/1(5-Ct), containing the CB2 sequence up to and including TM4 and e2, the remaining sequence being that of CB1 (Table I). This had a binding affinity for SR 141716A similar to that of CB1/2(5-Ct), suggesting that structural elements in the TM4 and TM5 regions and/or in the e2 loop of CB1 might be implicated in the binding of the antagonist. To investigate this further, we constructed sandwich chimeras in which the TM4-e2-TM5 regions of the two receptors were interchanged (Fig. 5). [^{3}H]CP 55,940 bound with high affinity to the CB1/2(4–5)/1 sandwich, whereas as predictable from the preceding experiments, SR 141716A completely failed to compete for its binding site. In addition, tritiated SR 141716A failed to bind to this receptor, providing a further indication that particular amino acids in the TM4-e2-TM5 might indeed be implicated in binding this ligand. Neither CP 55,940 nor SR 141716A bound to the CB2/1(4–5)/2 sandwich.

Investigations into the Role of e2 Loop Residues in Ligand Binding—Having found that SR 141716A apparently recognized residues in the TM4-e2-TM5 region, we first focused on the role of the e2 loop. Replacement of the entire e2 loop of CB2 between the conserved G(W/Y)L residues (Fig. 5) by the CB1 e2 loop (CB2/1(e2)) resulted in a total loss of ligand binding. Immunoblot analysis (*lane 5*, Fig. 3*b*) confirmed, however, that the protein was indeed expressed, and furthermore immunofluorescence analysis showed the receptors to be present in the plasma membrane. In contrast, the CB1 receptor containing the CB2 e2 loop (CB1/2(e2), Fig. 5) was expressed (*lane 3*, Fig. 3*b*) and recognized tritiated SR 141716A with the same affinity as wild-type CB1 (K_d 0.44 ± 0.2 nM, B_{max} 3.5 ± 0.7 pmol/mg protein). This receptor, however, completely failed to bind CP 55,940. We then restricted the exchange in the e2 loop to the gap region between the conserved Cys residues (Fig. 5*b*), replacing EKLQSV in CB1 by CPRP (CB1(CPRPe2)). This mutation and foreshortening of the CB1 e2 region resulted in the sequestration of the receptor and thereby loss of binding, but positive immunoblots confirmed receptor expression (*lane 10*, Fig. 3*a*; *lane 6*, Fig. 3*b*).

We next turned our attention to the conserved extracellular Cys residues, because some evidence exists for the implication of Cys in ligand binding or disulfide bridge formation (26, 27). The mutation to serine of either of the two cysteines in e2 of CB1, C257S, and C264S, resulted in a complete loss of binding of ligands to isolated COS-3 membranes. Here again, the lack of binding was entirely attributable to the absence of the mu-

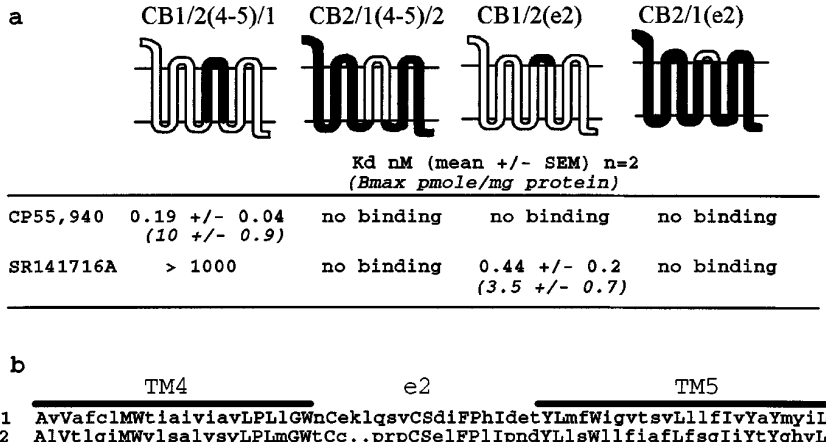

FIG. 5. **Modifications to the TM4-e2-TM5 region of CB1 and CB2.** *a*, schematic representation of sandwich constructs. The *open regions* are those from CB1, and the *solid regions* from CB2. *b*, alignment of the TM4-e2-TM5 regions of human CB1 and CB2. The *solid bars* represent the putative transmembrane regions, TM4 and TM5. Common amino acids are in *bold capital letters*. The *dots* in hCB2 represent a gap. The entire amino acid sequences shown were exchanged in the CB1/2(4–5)/1 and CB2/1(4–5)/2 sandwich chimeras; those between GW and YL in the CB1/2(e2) and CB2/1(e2) chimeras and CPRP replaced EKLQSV between the conserved C and CS in CB1(CPRPe2).

tated receptors at the cell surface, because although immunoblotting confirmed their expression (*lanes 7* and *8*, Fig. 3*a*), immunofluorescence was detectable only in permeabilized cells (not shown). Not surprisingly, a similar loss in binding was observed for the double CB1 mutant C257,264S, although here again immunoblot analysis confirmed its expression (*lane 9*, Fig. 3*a*), and immunofluorescence confirmed its sequestration in the interior of the cells (not shown). It was recently shown by the Khorana group (28) that replacement of cysteine by alanine resulted in a mutant rhodopsin receptor that had a greater resemblance to the native form than that resulting from a serine replacement (29). However, the CB1 mutants C257A and C264A, although expressed, failed to bind CP 55,940. We investigated the possibility that replacement of the e2 Cys residues had resulted in the elimination of a crucial putative disulfide bridge formed with one of the only two other extracellularly located Cys, situated in the NH_2-terminal extremity. The double mutant C98,107S was well expressed (*lane 6*, Fig. 3*a*) and bound both CP 55,940 (K_d 0.2 ± 0.05 nM, B_{max} 5.3 ± 0.6 pmol/mg protein) and SR 141716A (K_d 0.3 ± 0.08 and B_{max} 9.3 ± 1.0 pmol/mg protein), showing that these residues are not implicated in ligand binding and that a disulfide bridge between the NH_2-terminal region and an e2 Cys is either not present in CB1 or is not crucial for ligand binding.

Human CB2 has three cysteines in e2, two of which, Cys^{174} and Cys^{179}, correspond to the positions of those in CB1 (Figs. 1 and 5*b*). Each of these was independently mutated to serine in an amino-terminal c-*myc*-CB2 fusion receptor. As observed with the corresponding CB1 mutants, although western immunoblot confirmed a normal expression of the mutated receptors (*lanes 4* and *7*, Fig. 3*b*), ligand binding was completely eliminated. However, in contrast to the CB1 mutants, immunofluorescence on intact cells was positive (not shown), indicating a profound modification of the CP 55,940 binding site. We have not mutated either Cys^4 or Cys^{180}.

DISCUSSION

SR 141716A is a highly selective antagonist for the central cannabinoid receptor CB1 (12, 30). This ligand is the only one to date that can readily differentiate between CB1 and CB2, which share only 51% identity in their central, transmembrane loop regions. As a first step in the identification of the amino acid residues in CB1 implicated in SR 141716A recognition, we have undertaken the construction of a series of chimeric receptors with the aim of determining the regions of CB1 essential for the binding of the antagonist. The large drop in affinity for SR 141716A on replacing the TM4-e2 amino acids of CB1/2(5-Ct) by the corresponding CB2 residues was particularly striking, strongly suggesting that this region of CB1 contains structural features important for the high affinity binding of the antagonist. However, the same binding affinity was found for the mirror image receptor CB2/1(5-Ct), which pointed at the same time to the presence of residues important for SR 141716A binding in TM5.

The importance of the TM4 to TM5 region was confirmed by the finding that the CB1 sandwich containing this particular region of CB2 bound CP 55,940 normally but showed no affinity for SR 141716A. This chimeric receptor was of particular interest because it provided a starting point for mutational studies aimed at recovering the high affinity binding site for the antagonist and hence identifying critical amino acids. It is clear that despite the considerable difference in primary structure between CB1 and CB2, the overall architecture of the wild-type and chimeric receptors must be well retained in those receptors binding CP 55,940. A three-dimensional theoretical model of CB1 has recently been proposed (31), based on a seven-transmembrane helix bundle arrangement in rhodopsin (32), derived from a low resolution electron cryomicroscopic analysis of this G protein-coupled receptor (33). Using this model for ligand-receptor docking studies, Bramblett and Reggio (45) have hypothesized a three-point interaction site for CP 55,940 and other agonists with CB1 with residues in TM3, TM5, and TM6. This conforms with the most widely accepted model for agonist binding in the G protein-coupled receptor family (reviewed in Ref. 16). The postulated residues are Lys^{192} in TM3, Tyr^{275} in TM5, with Val^{350} and Ile^{354} in TM6, all residues found in equivalent positions in CB2. If this hypothesis is correct, because the same high affinity binding of CP 55,940 has been found for the receptors studied here, these noncontiguous amino acids are most probably identically oriented in all the chimeric receptors as well as in wild-type CB1 and CB2. SR 141716A, a pyrazole derivative, is structurally dissimilar to CP 55,940 and the other cannabinoids and therefore probably binds to quite different amino acids in CB1. Nevertheless, some of the amino acids involved in SR 141716A binding to CB1 but perhaps not all may also be those conserved in CB2. Because the transmembrane bundles are correctly orientated for CP 55,940 binding in all of the receptors, except those modified in e2 (see below), the evidence points away from the fact that the specificity for SR 141716A for CB1 may be merely attributable to slightly different orientations of amino acids conserved in

both CB1 and CB2. But as a caveat it must be noted that the species selectivity for substance P antagonists was indeed attributable to relatively minor local conformational perturbations between species (34).

The TM4-e2-TM5 region is the internal domain that contains the lowest identity (35%) between CB1 and CB2, and because the e2 loops of CB1 and CB2 differ both in size and primary sequence, we first investigated their possible participation in ligand binding. In general, although extracellular loop residues are principally involved in peptide ligand recognition (see Ref. 16 for a recent review), they can contribute to antagonist binding, as found for the substance P (35, 36), κ opioid (37), and adenosine (38) receptors. Unfortunately, our investigation was severely hampered by the fact that several of the modifications made to this part of the CB receptors resulted in sequestration of the proteins in the interior of the transfected cells, which also occurred with three of the CB1/CB2 chimeric constructs. This particular problem is often encountered with mutated G protein-coupled receptors and chimeric constructs (39–42); for example, confocal microscopic examination of a nontranslocated, mutated, tagged β2-adrenergic receptor showed it to be trapped in the endoplasmic reticulum (40). Its sequestration was assumed to be a result of steric incompatibility, leading to misfolding, because compensatory mutation subsequently rescued the receptor and allowed it to be successfully translocated (40). Cellular extracts of the CB1/2(6-Ct) chimeric receptor failed to bind CP 55,940, whereas in the same experiment, cellular extracts of wild-type CB1 correctly bound the agonist. Because the chimeric receptor, although present, does not bind the ligand, this would indicate that the chimera contains incompatible structural features that impede correct folding in the interior of the cell and would suggest, furthermore, that only a correctly folded receptor is able to translocate. It is well documented that the degradation of misfolded, endoplasmic reticulum-bound proteins can be very slow (43), and in support of this report is our observation that our Western immunoblot analyses showed little evidence of degradation of these trapped proteins.

We were particularly interested in the cysteine residues in e2, because they are a characterizing feature of the cannabinoid receptors and have been suggested to be implicated in receptor tertiary structure or directly in ligand binding (26, 27). Unfortunately, among the proteins that failed to reach the plasma membrane were the CB1 variants in which the e2 cysteines had been mutated singly or doubly. On the other hand, the two Cys situated in the amino-terminal of CB1 do not appear to play an important role in its function, because their replacement had no effect on ligand binding. Unlike the CB1 mutants, the $Cys^{174} \rightarrow$ Ser and $Cys^{179} \rightarrow$ Ser mutants of CB2 were successfully translocated to the plasma membrane. However, they failed to bind CP 55,940. The other e2 modifications that resulted in successful receptor translocation were those in which the entire loops were interchanged. The effect of these changes on ligand binding was contrasting. CB2 containing the e2 of CB1 failed to bind either CP 55,940 (or, predictably, SR 141716A), although immunofluorescence analysis showed the receptor to be present at the cell surface. Similarly, in CB1 containing the e2 of CB2, the CP 55,940 binding site was again lost, but SR 141716A bound as well as to the wild-type receptor. Several possibilities arise from these results: i) the Cys may directly participate in binding CP 55,940; ii) the Cys may play an essential role in correctly orientating the CP 55,940 binding site; iii) modifications to the e2 region may disorient the neighboring Tyr^{275}, if indeed this residue is implicated in CP 55,940 binding (45); iv) e2 residues in CB1 are unimportant for the binding of SR 141716A; and v) amino acid side chains interacting with SR 141716A have to be sought within both of the transmembrane helices adjoining e2. Extensive mutagenesis studies will be necessary before we can identify specific SR 141716A-recognizing amino acids in this particular part of the CB1 receptor.

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