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Biochemical Pharmacology 65 (2003) 1623–1631
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Integrity of extracellular loop 1 of the human cannabinoid receptor 1 is critical for high-affinity binding of the ligand CP 55,940 but not SR 141716A

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Received 29 August 2002; accepted 15 November 2002

Abstract

Like other G-protein coupled receptors with hydrophobic ligands, the human cannabinoid receptor 1 (CB1) is thought to bind its ligands within the transmembrane region of the receptor. However, for some of these receptors the extracellular loops (ECs) have also been shown to play a role in ligand recognition and selectivity. We have taken a mutagenesis approach to examine the role of the amino terminus, EC1, and EC3 of CB1 in ligand binding. Eight mutant receptors, each with a dipeptide insertion, were constructed, expressed, and evaluated for binding to the cannabinoid ligands (−)-*cis*-3[2-hydroxy-4-(1′,1′-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP 55,940) and *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR 141716A). Mutants with insertions in the membrane distal region of the amino terminus or EC3 maintained affinity for both ligands. Those with insertions in the membrane proximal region of the amino terminus or EC1 exhibited a loss of affinity for CP 55,940 while retaining wild-type affinity for SR 141716A. Representative mutants were analyzed for agonist-induced inhibition of cyclic AMP accumulation, and the results indicated that G-protein coupling remained intact. Alanine substitution mutants were made to address whether it was the perturbation of the overall structure of the region or the displacement of particular side chains that was responsible for the loss of CP 55,940 binding. We conclude that a structurally intact EC1, but not the comparably short EC3, is essential for high-affinity CP 55,940 binding.

Keywords: Cannabinoid; Cannabinoid receptor; G-protein coupled receptor; Ligand binding; CB1; Extracellular loop

1. Introduction

The human CB1 binds Δ^9-THC, the psychoactive component of *Cannabis sativa* L., and other cannabimimetic compounds [1,2]. It is a G-protein coupled receptor that is primarily associated with neurons in the brain, spinal cord, and peripheral nervous system [3,4] and exerts its effects via coupling to G_i or G_o proteins to modulate adenylate cyclase [5–7], N- and P/Q-type calcium channels [8], and A-type and inwardly rectifying potassium channels [9]. Coupling to G_s proteins is also possible [10–12], but the functional significance of this association is not yet known. A subtype, CB2, is found mainly in peripheral tissues, including the marginal zone of the spleen, and in macrophages [13,14].

The pharmacological effects of Δ^9-THC include analgesia, inhibition of nausea, lowering of intraocular pressure, appetite stimulation, antiemetic activity, and bronchial dilation (for review, see Refs. [15–17]). Recognition that many of these effects are receptor-mediated [1] has considerably heightened research activity aimed at understanding the interactions of cannabinoids with their receptors.

Previous studies have suggested that the binding site(s) for CB1 ligands lies within the TM domain of the receptor. Mutational analysis of Lys^{192} in TM3 indicated that this residue is critical for binding to CP 55,940, HU 210, and

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Abbreviations: cAMP, cyclic AMP; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; EC, extracellular loop; FBS, fetal bovine serum; Δ^9-THC, Δ^9-tetrahydrocannabinol; TM, transmembrane; CP 55,940, (−)-*cis*-3[2-hydroxy-4-(1′,1′-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol [1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthyl)-methanone monomethanesulfonate.

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doi:10.1016/S0006-2952(03)00155-2

anandamide but not WIN 55,212-2 [18,19] and that other residues in TM3 play a role in the diminished affinity of CB1 for WIN 55,212-2 relative to CB2 [20]. In addition, replacement of Val^{282} in CB1 TM5 with Phe, as is found in the corresponding location in CB2, enhanced the affinity of the receptor for WIN 55,212-2 [21]. The investigators propose that WIN 55,212-2 binding is stabilized through aromatic stacking interactions with a cluster of aromatic residues contributed by TMs 3, 4, and 5 [21,22]. The importance of one aromatic residue (Trp^{172} in TM4 of CB2) was later confirmed experimentally [23]. Asp^{163} in TM2 of CB1 may also be a contact residue since mutation of this residue results in diminished WIN 55,212-2 binding [24]. Using chimeric receptors in which regions of the CB1 were replaced with regions of the CB2, Shire *et al*. [25] identified the segment encompassing TM4 and TM5 as critical for SR 141716A binding specificity. Molecular modeling analyses of ligand–receptor interactions in CB1 are consistent with the idea that the binding pocket is primarily hydrophobic and within the TM helix bundle [21,26,27], and that the different classes of ligands bind to different, though partly overlapping, sites within this region [28]. In a molecular modeling and mutagenesis study of CB2, Gouldson *et al*. [29] identified TMs 3, 4, and 5 as interacting with the CB2 specific antagonist SR 144528, analogous to the interactions of CB1 with SR 141716A.

However, little is known about the initial stages of molecular recognition of ligands and the mechanism of association of ligand with the CB1 receptor prior to final docking in the binding pocket. Recent studies have suggested that for some receptors that bind small molecules, the ECs play a role in ligand recognition and differentiation even though the ligands may ultimately bind within the TM domain [30–32]. For example, residues in EC3 of the μ- and κ-opioid receptors are critical for the differential binding of the ligand DAMGO and may control the entry of the ligand to the TM binding pocket [33]. Particular residues in EC2 of the human A2a adenosine receptor [30] and in the human $P2Y_1$ receptor [34] have been shown to play a role in ligand binding. Moreover, for the latter receptor, computational studies suggest that disulfide bridges in the receptor poise EC2 over the opening of the TM cleft in such a way as to route entry of the ligand [34]. Aside from a requirement for the EC2 of CB1 for CP 55,940 binding [25], it is unclear what, if any, role the extracellular region of CB1 plays in ligand recognition.

We have used a mutagenesis approach to make a rapid assessment of the potential involvement of the amino terminus and EC1 and EC3 of CB1 in ligand binding. This involved using mutant receptors with dipeptide insertions to scan the sensitivity of these regions to binding the well-characterized ligands CP 55,940 and SR 141716A. Upon determining regions for which ligand binding was sensitive to disruption, we then focused on specific amino acid residues in these locations to determine whether the presence of particular side chains was critical. A clear pattern emerged indicating that the integrity of EC1 is important for interaction of CB1 with CP 55,940, while none of the regions tested here appear sensitive to SR 141716A binding.

2. Materials and methods

2.1. Materials

[^{3}H]CP 55,940 (180 Ci/mmol) was purchased from Perkin Elmer Life Sciences. [^{3}H]SR 141716A (19.3 Ci/mmol) was provided as part of the Chemical Synthesis Program of the National Institute of Drug Abuse. CP 55,940 was a gift from Pfizer, Inc. WIN 55,212-2 and SR 141716A were purchased from Research Biochemicals Inc. Protease inhibitor cocktail and fatty acid free BSA fraction V were purchased from the Sigma Chemical Co. Human CB1 cDNA was a gift from Dr. Marc Parmentier (University Libre de Bruxelles). Dulbecco's modified Eagle's medium (DMEM) and FBS were purchased from HyClone Laboratories, Inc. The calcium phosphate mammalian transfection kit was purchased from Invitrogen, Inc. cAMP levels were measured using the Biotrak Cyclic AMP [^{3}H] assay system from Amersham Biosciences.

2.2. Plasmid construction and mutagenesis

The human CB1 receptor cDNA was cloned into the pcDNA3 mammalian expression vector (Invitrogen Inc.) as previously described [18]. The resulting plasmid, pCNCB1, was used as a template for site-directed mutagenesis using the QuikChange system (Stratagene Inc.). Mutations coding for the dipeptide insertions were screened using the newly incorporated restriction enzyme sites (prolylglycine, encoded by CCCGGG, is recognized by *Sma*I, and glycylalanine, encoded by GGCGCC, is recognized by *Kas*I). Point mutations were also generated using the QuikChange system. All mutations were verified by DNA sequencing using the ABI Prism automated sequencing system (PE Applied Biosystems).

2.3. Transfection and membrane preparation of HEK-293T cells

Human embryonic kidney (HEK) 293T cells were grown in DMEM + 10% FBS with high glucose at 37° in a 5% CO_2, humidified incubator. Partially confluent culture plates were transiently transfected with pcDNA3 encoding the wild-type or mutant CB1 using calcium phosphate precipitation. The efficiency of transfection ranged between 50 and 95%, as indicated by β-galactosidase staining.

Membranes from these cells were prepared as described previously [10]. Briefly, cells were harvested 24 hr post-transfection, resuspended in PBS with 0.1% (v/v)

mammalian protease inhibitor cocktail, and subjected to nitrogen cavitation at 750 psi for 5 min with a Parr Cell Disruption Bomb. Fractionated cells were then centrifuged at 500 *g* for 10 min at 4° to remove cell debris and nuclei. The supernatant was subjected to ultracentrifugation at 100,000 *g* for 30 min at 4° to concentrate membrane vesicles. The resulting pellet was resuspended in TME buffer (25 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA) + 7% sucrose. Total protein concentration was determined by the Bradford method [35]. Membranes were diluted to 0.6 μg/μL of total protein and stored at −70° until used in the binding studies.

2.4. Ligand binding assays

The ligand binding assays were carried out as previously described [10]. Reactions were done in a 200-μL total volume in glass tubes treated with Regisil (Regis Technologies, Inc.). In [^{3}H]SR 141716A or [^{3}H]CP 55,940 saturation assays, approximately 30 μg of membrane protein was added to the reaction mixture and incubated at 30° for 90 min with various concentrations of tritiated ligand (0.4 to 30 nM) in TME + 0.1% fatty acid free BSA. In the competition binding assays, approximately 40 μg of membrane protein was incubated in the presence of 4 nM [^{3}H]SR 141716A with various concentrations of unlabeled CP 55,940 (between 10^{-10} and 10^{-6} M). For consistency, [^{3}H]SR 141716A was used as the radioligand for these assays since it bound well to most mutants, in contrast to CP 55,940 for which binding was highly dependent on the mutant receptor. The affinity for CP 55,940 binding to the wild-type receptor is comparable when determined by saturation binding or by competition against [^{3}H]SR 141716A [4.6 ± 0.6 nM and 8.3 (6.5 to 11), respectively]. Nonspecific binding was determined by the addition of 1 μM unlabeled ligand. The reaction was terminated by the addition of 250 μL of cold TME + 5% BSA. Free and bound ligand were separated by filtration through GF/C filter paper (Whatman Inc.) using a 24-manifold Brandel cell harvester (Brandel Inc.). Filters were washed with approximately 40 mL of cold TME buffer. Bound radioactivity was then determined by liquid scintillation counting. Specific binding was typically 60% of total binding. Data are given as the mean ± SEM of two independent experiments done in duplicate for saturation binding and as the mean with corresponding 95% confidence limits for competition experiments.

2.5. Expression of cannabinoid receptors in CHO-K1 cells and cAMP determination

Chinese hamster ovary (CHO) K1 cells were used for cAMP assays due to their good adherence to culture plates over the course of the experiment. The ligand binding properties of CB1 in these cells, and the mutants examined, are the same as those with the HEK-293T cell line ([8] and our unpublished observations). CHO-K1 cells were grown in F-12 medium + 10% FBS. Cells were stably transfected with pcDNA3 encoding the wild-type or mutant CB1 using LipofectAMINE (Life Technologies, Inc.). Single cell clonal selection by limiting dilution was carried out in the presence of 0.5 mg/mL of G418 sulfate, and transfected cell lines were screened by reverse transcription–polymerase chain reaction (RT–PCR) as described previously [18].

The accumulation of cAMP was measured as previously described [18]. Briefly CHO-K1 cells expressing receptors were seeded in 24-well trays. After 48 hr, cells were treated with 1 μM forskolin to stimulate adenylyl cyclase and elevate cAMP levels. Cells were also treated with various amounts (between 10^{-10} and 10^{-5} M) of the cannabinoid receptor agonist WIN 55,212-2. The amount of cAMP was then determined using a [^{3}H]cAMP system.

2.6. Data analysis

Data obtained from the binding and cAMP determination assays were analyzed with the use of Prism (GraphPAD Software) as previously described [18]. The statistical differences between binding constants for wild-type and mutant receptors were determined using logged values with an unpaired *t*-test. Differences with values of $P < 0.05$ were considered significant.

3. Results

We have taken a mutagenesis approach to highlight regions in the extracellular domain of CB1 that are sensitive to ligand interactions. Eight mutant receptors were constructed in which a dipeptide insertion was placed in either the amino terminal tail, EC1, or EC3. Dipeptide insertions of prolylglycine (PG) or glycylalanine (GA) were chosen to cause sufficient disruption of the flanking segments so that sensitivity to ligand binding would be readily apparent. In addition, these insertions generated new restriction sites so that they could be quickly screened at the DNA level during construction. A schematic representation of the receptor and the positions of the dipeptide insertions are shown in Fig. 1A [36].

Each mutant receptor containing one dipeptide insertion was expressed by transient transfection of HEK-293T cells. All receptors were well expressed and yielded B_{max} values in the range of 0.4 to 3 pmol/mg protein. Variability in B_{max} values is attributed to a preparation-to-preparation variation in transfection efficiency, typically 50–95%. To assess the ligand binding properties of each mutant receptor, saturation and competition binding experiments were performed using the well-characterized ligands CP 55,940 and SR 141716A. CP 55,940, a non-classical cannabinoid agonist, is a bicyclic analog of Δ^9-THC lacking the pyran ring, and has a similarly high affinity for both CB1 and

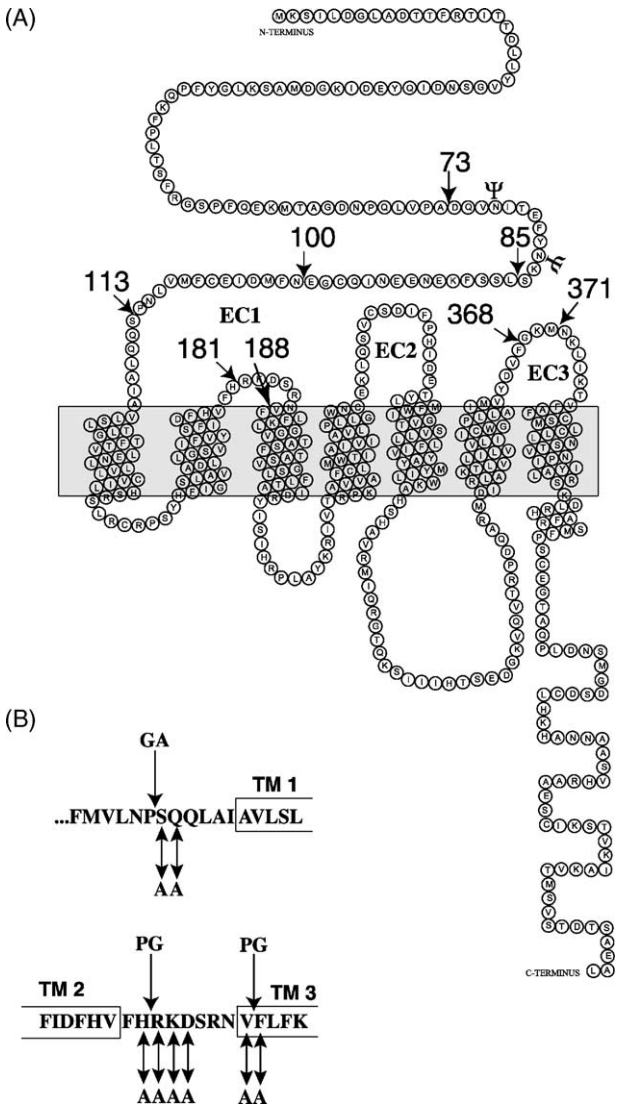

Fig. 1. (A) Helical net representation of the membrane topology of CB1. The shaded area indicates residues predicted to be within the membrane based on the model by Bramblett *et al.* [36]. The position of the dipeptide insertions is indicated by arrows. When these are referred to in the text, the number of the amino acid immediately amino terminal to the insertion is given to identify the location. Ψ indicates potential asparagine linked glycosylation sites. (B) Schematic representations of residues 108–124 containing the membrane proximal region of the amino terminus and residues 174–192 containing EC1. Boxed residues are within the flanking TM segments. Single-headed arrows above the sequence indicate the location of dipeptide insertions. Double-headed arrows below the sequence indicate the positions of the individual alanine substitutions.

CB2 receptor subtypes. SR 141716A, a 1,5-biarylpyrazole, is an inverse agonist, selective for the CB1 subtype.

The ligand binding properties of each of the mutant CB1 receptors carrying a dipeptide insertion are summarized in Table 1. Saturation and competition binding curves for the wild-type CB1 and each mutant receptor are shown in Fig. 2. Collectively, these data provide a profile for the involvement of different regions of the extracellular domain of CB1 in differential ligand binding. In general, the affinity for SR 141716A by the receptor was retained regardless of the position of the dipeptide insertion. In contrast, the affinity for CP 55,940 was variable and dependent upon position.

Disruption of the primary sequence by insertion early in the amino terminus through residue 100 had no effect on affinity for CP 55,940 [$K_i = 5.1$ (2.0 to 13) nM for 73PG; $K_i = 7.1$ (2.4 to 20) nM for 86PG; $K_i = 14$ (3.6 to 52) nM for 100PG] as compared to wild-type [$K_i = 8.3$ (6.5 to 11) nM]. However, when the insertion was introduced close to the predicted start of TM1, the affinity for CP 55,940 was lost altogether ($K_i > 1$ μM for 113GA).

A marked contrast was observed for the effects of the mutations in EC1 vs. EC3. Wild type-like affinity for [^{3}H]SR 141716A ($K_d = 4.7 \pm 0.48$ nM) was retained in all four cases ($K_d = 5.8 \pm 1.2$ nM for 181PG; $K_d = 1.8 \pm 0.44$ nM for 188PG; $K_d = 5.8 \pm 1.1$ nM for 368PG; $K_d = 3.0 \pm 1.0$ nM for 371PG). However, while wild-type-like affinity for CP 55,940 ($K_d = 4.6 \pm 0.6$ nM) was retained when EC3 was mutated ($K_d = 4.5 \pm 1.0$ nM for 368PG; $K_d = 3.1 \pm 0.79$ nM for 371PG), affinity was completely lost when insertions were made at positions 181 and 188 in EC1. No binding was detected in [^{3}H]CP 55,940 saturation binding experiments (data not shown). Furthermore, neither of these mutants exhibited any displacement of 4 nM [^{3}H]SR 141716A by CP 55,940 up to 1 μM.

The loss or reduction of affinity for CP 55,940, an agonist, and the retention of wild-type-like affinity for SR 141716A, an inverse agonist [37], observed for the mutants with insertions in EC1 raises the question of whether these insertion-mediated disruptions alter ligand interaction or whether they disrupt coupling to the G-protein via an indirect conformational change in the receptor. We found that in the representative mutant receptors examined G-protein coupling was maintained, as evidenced by agonist-induced modulation of the effector adenylyl cyclase. WIN 55,212-2 was chosen as the ligand for these experiments as it is an agonist like CP 55,940, yet it is believed to have different binding contacts with CB1. This is based on studies that suggest that CP 55,940 interacts with the receptor through hydrophobic contacts within the seven TM helical bundle core and through hydrogen bonding of its hydroxyl groups, whereas WIN 55,212-2 is believed to interact via an aromatic stacking network between TMs 3, 4, and 5 [22].

Our results are shown in Table 2 and Fig. 3. All three mutants tested (181PG, 188PG, and 371PG) behaved like the wild-type receptor with respect to the EC_{50} and maximal inhibition for WIN 55,212-2-induced inhibition of forskolin-stimulated cAMP accumulation. A trend emerged in which mutants that did bind SR 141617A but not CP 55,940 were still coupled to the G-protein and were able to be activated by WIN 55,212-2, leading to inhibition of forskolin-stimulated cAMP accumulation. The results also suggest that, unlike CP 55,940, perturbation of EC1 does not affect binding of WIN 55,212-2. Proper coupling

Table 1
Effects of insertion mutations in CB1 on ligand binding affinities[a]

Receptor	Location	[^{3}H]SR 141716A		CP 55,940
		K_d[b] (nM)	B_{max}[b] (fmol/mg)	K_i[c] (nM)
WT		4.7 ± 0.48	1400 ± 56	8.3 (6.5–11) (4.6 ± 0.6[d])
73PG	N-terminus	2.4 ± 0.66	1400 ± 130	5.1 (2.0–13)
86PG	N-terminus	3.3 ± 1.2	1300 ± 180	7.1 (2.4–20)
100PG	N-terminus	2.3 ± 0.61	590 ± 50	14 (3.6–52)
113GA	N-terminus	4.7 ± 2.0	1500 ± 260	>1 μM
181PG	EC1	5.8 ± 1.2	1900 ± 190	>1 μM
188PG	EC1	1.8 ± 0.44	2800 ± 210	>1 μM
368PG	EC3	5.8 ± 1.1	340 ± 21	4.5 ± 1.0[d]
371PG	EC3	3.0 ± 1.0	910 ± 110	3.1 ± 0.79[d]

[a] Ligand binding assays using HEK-293T cells were as described in Section 2. Values were determined from at least two experiments performed in duplicate. WT = wild-type.
[b] K_d and B_{max} values for [^{3}H]SR 141716A were determined by saturation binding and are expressed as means ± SEM.
[c] K_i values for CP 55,940 were determined by competition binding against 4 nM [^{3}H]SR 141716A and are expressed as means with corresponding 95% confidence limits in parentheses.
[d] Indicates affinity determined as K_d by saturation binding with [^{3}H]CP 55,940, and are expressed as means ± SEM.

Table 2
WIN 55,212-2-induced inhibition of forskolin-stimulated cAMP accumulation[a]

Receptor	Location	EC_{50} (nM)	Maximum inhibition (%)
WT		67 (41–110)	100
181PG	EC1	50 (27–93)	100
188PG	EC1	45 (28–72)	100
371PG	EC3	71 (28–180)	92

[a] cAMP accumulation was determined as described in Section 2. The EC_{50} values are presented as the means of two independent experiments performed in duplicate with corresponding 95% confidence limits in parentheses. WT = wild-type.

further suggests that the dipeptide insertions have produced only localized perturbations and that overall folding of the receptor is comparable to the wild type.

To probe whether the effects of these mutations were due to the displacement of a critical amino acid side chain(s) or to a structural perturbation of the loop, additional mutations consisting of individual alanine substitutions within the amino terminus and EC1 were constructed (Fig. 1B). Alanine was chosen for the substitutions since it does not offer a reactive side chain and is not likely to introduce obtrusive structural alterations although some perturbation can be expected. The substitutions made included residues immediately adjacent to the locations of the insertions in the dipeptide mutants. Affinity for SR 141716A was determined by saturation binding and affinity for CP 55,940 by displacement of [^{3}H]SR 141716A. As shown by the saturation binding curves in Fig. 4, the receptors with alanine substitutions in EC1 (H181A, R182A, K183A, D184A, V188A, and F189A) retained affinity for SR 141716A in each case (Table 3). The six substitution mutants showed a range of CP 55,940 binding [K_i = 25 (13 to 46) nM, K_i = 18 (13 to 26) nM, K_i = 23 (15 to 36) nM, K_i = 220 (100 to 460) nM, K_i = 83 (65 to 110) nM, and K_i = 520 (240 to 1100) nM, respectively] weaker than that of the wild-type receptor but less of a loss

Table 3
Effects of alanine substitutions in CB1 on ligand binding affinities[a]

Receptor	Location	[^{3}H]SR 141716A		CP 55,940
		K_d[b] (nM)	B_{max}[b] (fmol/mg)	K_i[c] (nM)
WT		4.7 ± 0.48	1400 ± 56	8.3 (6.5–11)
S114A	N-terminus	3.5 ± 0.27	2300 ± 65	26 (14–47)*
Q115A	N-terminus	3.9 ± 0.27	1800 ± 45	80 (41–160)**
H181A	EC1	2.8 ± 0.35	360 ± 33	25 (13–46)*
R182A	EC1	5.0 ± 0.43	1300 ± 45	18 (13–26)*
K183A	EC1	2.5 ± 0.37	1000 ± 51	23 (15–36)*
D184A	EC1	5.3 ± 1.2	340 ± 29	220 (100–460)**
V188A	EC1	2.9 ± 0.44	710 ± 32	83 (65–110)**
F189A	EC1	6.4 ± 1.3	970 ± 74	520 (240–1100)**

[a] Ligand binding assays were as described in Section 2. Values were determined from at least two experiments performed in duplicate. WT = wild-type.
[b] K_d and B_{max} values for [^{3}H]SR 141716A were determined by saturation binding and are expressed as means ± SEM.
[c] K_i values for CP 55,940 were determined by competition binding against 4 nM [^{3}H]SR 141716A and are expressed as means with corresponding 95% confidence limits in parentheses.
*,** Significantly different from wild-type: $^*P < 0.05$, and $^{**}P < 0.01$.

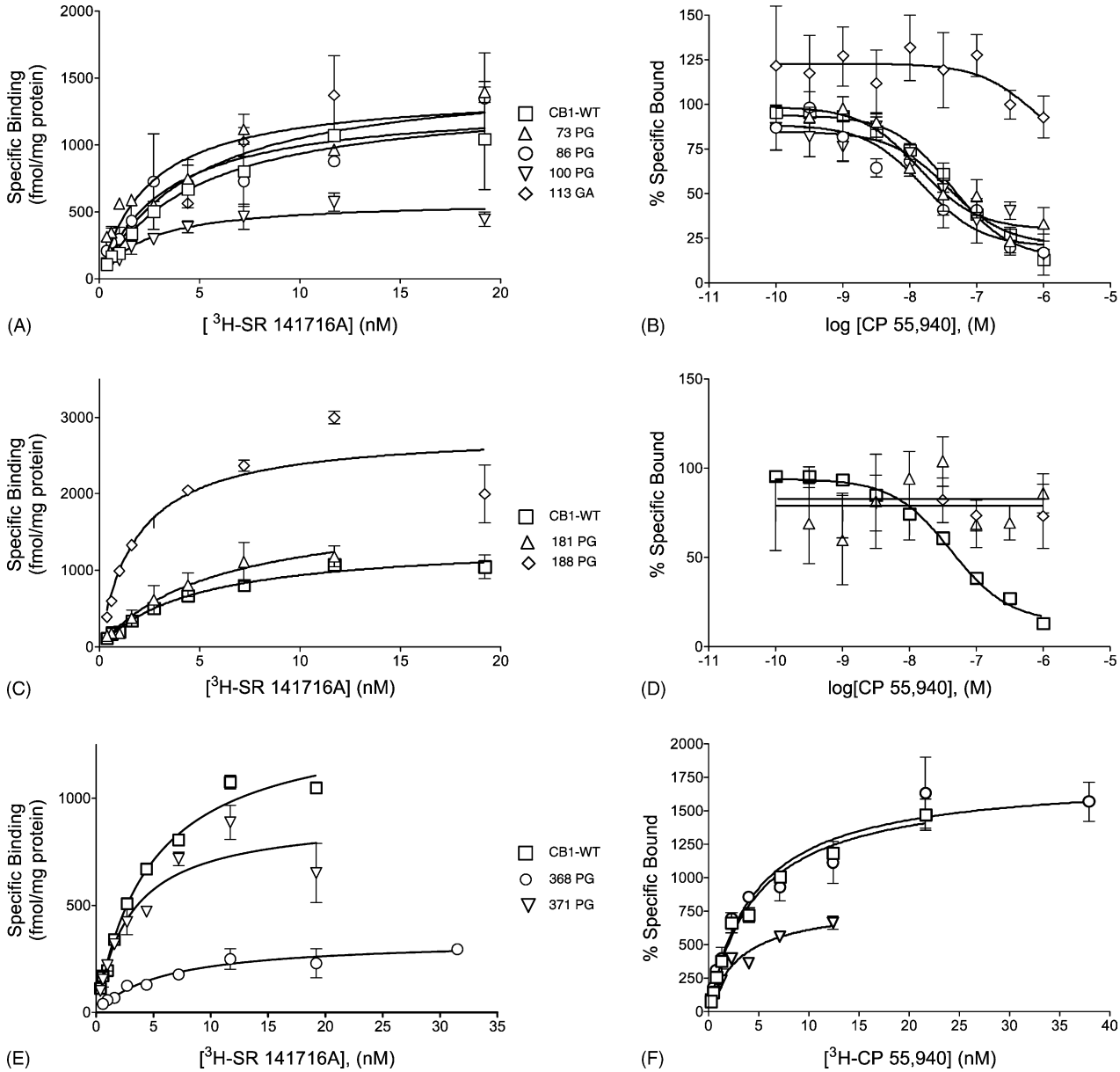

Fig. 2. Ligand binding analysis of insertion mutants. Individual mutants are identified by the symbol key centered horizontally between each set of panels. (A and B) Mutants within the amino terminus. (C and D) Mutants with insertions within EC1. (E and F) Mutants with insertions within EC3. Panels A, C, and E show [^{3}H]SR 141716A saturation binding. Panels B and D show CP 55,940 competition binding against 4 nM [^{3}H]SR 141716A. Panel F shows [^{3}H]CP 55,940 saturation binding. Results with the wild-type receptor are also shown for comparison. The results (means ± SEM) are from two independent experiments performed in duplicate.

in affinity than observed with the corresponding insertion mutant. The overall pattern suggests that several positions are sensitive to substitution rather than any one residue providing a critical functional group for ligand contact. This is consistent with the substitutions, and more so the dipeptide insertions, perturbing the overall structure of this short loop region in a manner that ultimately impacts CP 55,940 binding.

Two alanine substitution mutations were made near position 113 (Fig. 1B). S114A and Q115A in the amino terminus exhibited measurable affinity for CP 55,940 [K_i = 26 (14 to 47) nM and K_i = 80 (41 to 160) nM, respectively], though weaker than the wild type (Table 3). Affinity was lost completely by the dipeptide insertion at this location. The data suggest that, like EC1, the overall structure of this portion of the amino terminus is essential for binding CP 55,940 but not SR 1417161A.

4. Discussion

In this report, we describe a mutagenesis strategy for identifying regions of the extracellular domain of CB1 critical for high-affinity binding of CP 55,940 to the

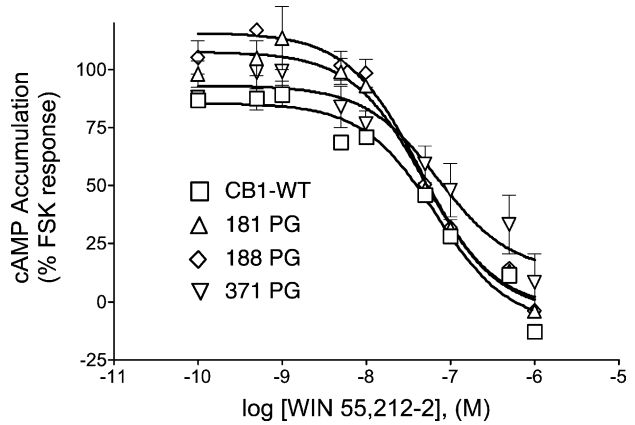

Fig. 3. Agonist-induced inhibition of cAMP accumulation in CHO cells expressing either wild-type or mutant CB1 receptors. The percentage change of cAMP levels was determined following treatment with 1 μM forskolin and various concentrations of WIN 55,212-2. Results (means ± SEM) are from two independent experiments performed in duplicate. The EC_{50} values are given in Table 2.

receptor. This approach was designed to provide a rapid overview of the relative sensitivity of CP 55,940 binding to perturbation of different subsegments of the receptor. A clear pattern emerges with respect to the relative importance of EC1 and EC3. Namely, mutations within EC1 are found to affect binding to CP 55,940, whereas those in EC3 do not. Binding to SR 141716A was unaffected by mutations in either loop. EC2 was determined to be important in a study where CB1/CB2 chimeric receptors, involving the exchange of EC2, were constructed, and CP 55,940 binding was eliminated while SR 141716A binding was retained by the CB1 variant [25].

Although the amino terminus of CB1 is particularly long (about 120 residues) and might be expected to influence ligand recognition, our data suggest that, except for the membrane proximal region, the amino terminus can be perturbed by dipeptide insertions with no effect on binding of the ligands tested here. This is consistent with studies of CB1A, a truncated isoform of CB1, which indicated that it displays CB1-like affinity for CP 55,940 [38].

The hydrophobic nature of ligands for CB1 has motivated studies aimed at identifying the binding site(s) within the transmembrane domain of the receptor [26]. Our data indicate that, in addition to residues within the membrane-associated portion of the receptor, extracellular regions of the receptor contact, if only transiently, the non-classical cannabinoid CP 55,940. A possible role for the extracellular loops in the molecular recognition of nonpeptidic ligands by other G-protein coupled receptors has been suggested recently. For the human $P2Y_1$ receptor, a mechanism for ligand binding has been proposed, which involves initial interactions of the extracellular loops with

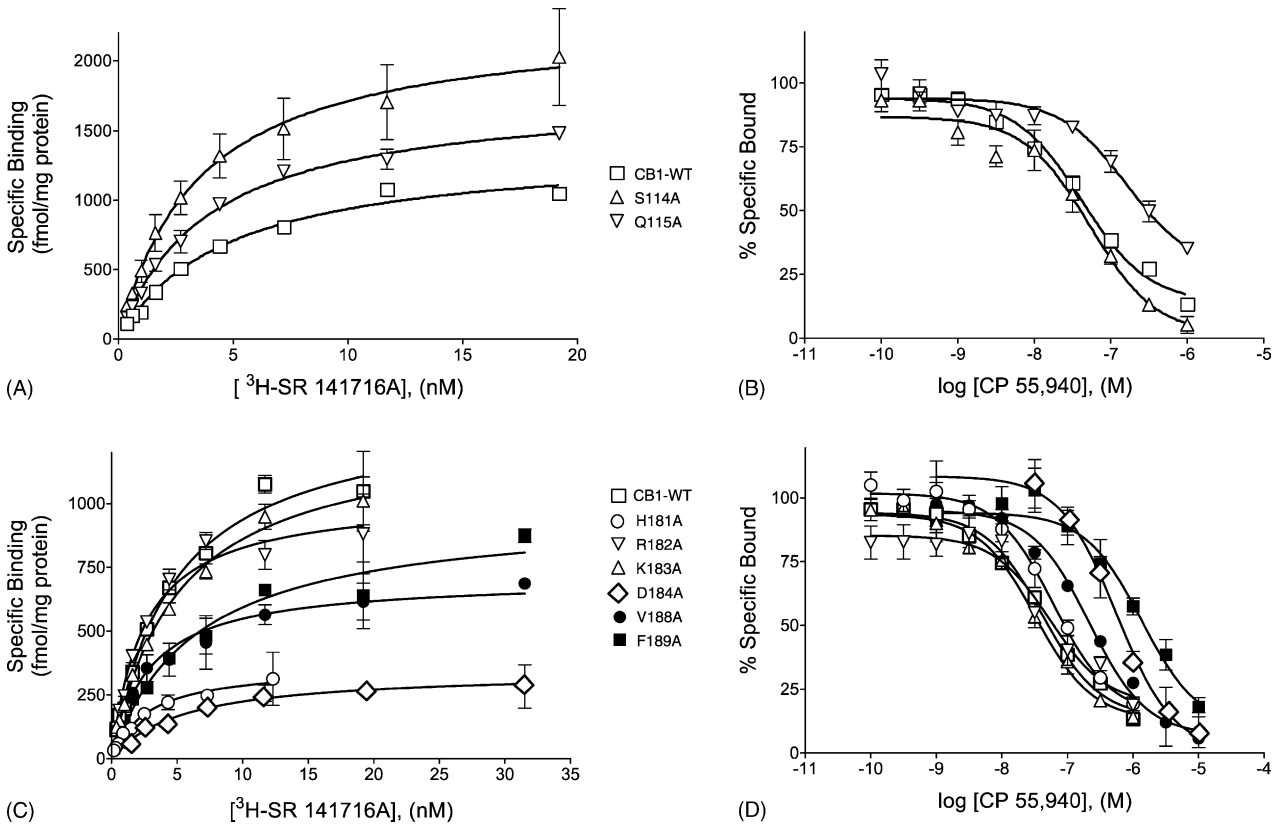

Fig. 4. Binding analysis of alanine substitution CB1 mutants. Individual mutants are identified by the symbol key centered horizontally between each set of panels. Panels A and C show [^{3}H]SR 141716A saturation binding. Panels B and D show CP 55,940 competition binding against 4 nM [^{3}H]SR 141716A. Wild-type CB1 is shown for comparison. Results (means ± SEM) are from two independent experiments performed in duplicate.

the ligand in an energetically favorable manner [34]. The extracellular loops then facilitate access and routing to the principal binding site within the TM domain. This is not unlike models for recognition of peptidic hormones. Moroder *et al*. [39] proposed that following preadsorption to a target cell membrane, the ligand undergoes reorientation and conformational adjustments. The loops may help direct the proper positioning of the ligand by constraining it in a particular way [40] so that subsequent diffusion and docking in the binding site are rapid. Our data suggest that recognition of CP 55,940 by CB1 may involve a similar mechanism.

Since little is currently known about the involvement of the extracellular domain in CB1–ligand interactions, global mapping of important segments by classical site-directed mutagenesis would have been an exhaustive process. As a first step, it would have been difficult to predict the nature and location of single-site substitutions, which would be most informative. The alternate approach of generating chimeric receptors with different subtypes can often be useful. However, in previous studies it was found that problems in plasma membrane expression precluded the analysis of many chimeras involving extracellular and/or transmembrane regions of the cannabinoid receptors [25]. In contrast, our approach has provided an overall picture of the regions within the extracellular domain sensitive to ligand binding. These results can then be used as a directive for more detailed analyses that will be necessary in order to delineate the specific role of a given subsegment in ligand binding. While dipeptide insertion mutagenesis does not involve the loss of any residues in the native sequence, it does result in the displacement of flanking residues by two. The result could alter significantly the position of key functional groups and could disrupt secondary structural elements. Our results suggest that for insertions within the loops, perturbations are not propagated to the intracellular loops because G-protein coupling is still retained. Furthermore, the effect on ligand binding seems to be primarily due to structural alterations within the loop as opposed to displacement of key contact groups because binding was altered less by the corresponding alanine substitutions. If the replacement with alanine had resulted in the loss of a critical functional element, a complete loss in CP 55,940 binding like that observed with the insertions would be expected. In addition to shifting the position of residues on either side of the insertion, the addition of proline and/or glycine would be expected to produce a more irregular structure and could introduce a kink in a helical segment [41]. The NMR structure of EC1 from the human parathyroid hormone receptor shows that it consists of an eight residue surface embedded α-helix [42]. The EC1 of the cannabinoid receptor is similarly very short, and if it is in a similar arrangement, this could explain the severe effect of a helix breaking dipeptide insertion compared to the less severe effects of single-site substitutions. Furthermore, this underscores the relative importance of EC1 vs. EC3 in ligand binding; EC3 is also short but the dipeptide insertions did not impact binding to either SR 141716A or CP 55,940.

There is considerable structural diversity across the five classes of cannabinoid compounds. The effect on ligand binding of a mutation that perturbs the structure of a localized region of CB1 may vary among compound classes. This study centered on the effects of mutations in the extracellular portion of CB1 with respect to the affinity for CP 55,940 and SR 141716A. Future studies involving screening with compounds from the classical, aminoalklyindole and endogenous classes will be valuable in determining to what extent the structure of the extracellular region is critical for binding other compounds as well. Understanding the involvement of the extracellular domain in ligand interactions will be important in the design of new therapeutic agents. In addition to using this information to mimic the binding mechanism of known ligands, it is conceivable that new agonists could be developed in which the loops are used as a binding scaffold to torque the receptor into the activated state and antagonists could be developed that bind the loops, thus blocking entry of ligands to the transmembrane binding pocket.

Acknowledgments

The authors thank Dr. Chen-ni Chin, Dr. Alexander Miller, and Sharyn Rusch for helpful discussions and critically reading this manuscript.

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