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The Membrane Proximal Region of the Cannabinoid Receptor CB_1 N-Terminus Can Allosterically Modulate Ligand Affinity

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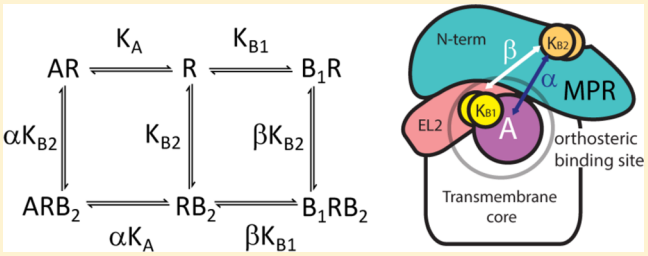
Supporting Information

ABSTRACT: The human cannabinoid receptor, CB_1, a G protein-coupled receptor (GPCR), contains a relatively long (~110 a.a.) amino terminus, whose function is still not defined. Here we explore a potential role for the CB_1 N-terminus in modulating ligand binding to the receptor. Although most of the CB_1 N-terminus is not necessary for ligand binding, previous studies have found that mutations introduced into its conserved membrane proximal region (MPR) do impair the receptors ability to bind ligand. Moreover, within the highly conserved MPR (~ residues 90−110) lie two cysteine residues that are invariant in all CB_1 receptors. We find these two cysteines (C98 and C107) form a disulfide in heterologously expressed human CB_1, and this C_{98}−C_{107} disulfide is much more accessible to reducing agents than the previously known disulfide in extracellular loop 2 (EL2). Interestingly, the presence of the C_{98}−C_{107} disulfide modulates ligand binding to the receptor in a way that can be quantitatively analyzed by an allosteric model. The C_{98}−C_{107} disulfide also alters the effects of allosteric ligands for CB_1, Org 27569 and PSNCBAM-1. Together, these results provide new insights into how the N-terminal MPR and EL2 act together to influence the high-affinity orthosteric ligand binding site in CB_1 and suggest that the CB_1 N-terminal MPR may be an area through which allosteric modulators can act.



The cannabinoid receptor, CB_1, is a G protein-coupled receptor (GPCR) found in high concentrations in the central nervous system.[1] CB_1 has been shown to mediate neurotransmitter release in presynaptic terminals,[2−4] by coupling with G_i or G_o proteins, which then inhibit adenylyl cyclase,[5,6] N- and P/Q-type calcium channels,[7] and activate A-type inwardly rectifying potassium channels.[8] The resulting modulation in amplitude and frequency of neurotransmission thus induced by CB_1 activation is thought to be one of the causes for the psychotropic effects known to accompany cannabis use.

From a biochemical and structural perspective, one intriguing question about CB_1 has been the purpose and role of its relatively long (~110 amino acid) N-terminus (Figure 1). The N-terminus has been shown to be involved in receptor biosynthesis and targeting, but its role in ligand binding and receptor activation is still not well-defined.

One would not expect the entire N-terminus would be required for ligand binding, since the hydrophobic cannabinoid ligands will partition into the membrane and thus can only interact with at most part of the extracellular N-terminus. Indeed, most of the N-terminus can in fact be deleted without abolishing ligand binding or G protein activation.[9−11]

However, some of the N-terminal region closest to the membrane, the so-called membrane proximal region (MPR) is apparently required for ligand binding. Kendall and colleagues have shown that dipeptide insertions into the MPR affects binding of the agonist CP 55940.[10] We have previously observed that a full deletion of the CB_1 N-terminus (up to residue 113) abolishes ligand binding, but retaining the MPR does not (Figure 2 and S1).

Interestingly, the CB_1 MPR is highly conserved among different species (see Figure 1) and contains two highly conserved cysteine residues (C98 and C107 in human CB_1), which are invariant across CB_1 N-termini from mammals, birds, fish, and amphibians (Figure 1B). Although previous studies (including our own) have found that these cysteine residues can be mutated to alanine or serine without abolishing agonist and antagonist binding or G protein activation,[12,13] no further investigations into whether these residues form a disulfide, nor what potential role such a disulfide might play, nor if these residues alter the effects of allosteric CB_1 ligands, have been reported.

Thus, in the present work, we set out to investigate if a potential disulfide in the CB_1 N-terminal MPR might affect or modulate ligand binding to CB_1. Our goal was to first determine if a C_{97}−C_{107} disulfide is in fact present in CB_1 receptors, and if so, if this disulfide could be used as a tool to assess if it (and the MPR to which it is attached) plays heretofore unappreciated roles in forming and stabilizing the orthosteric binding pocket for CB_1, thereby acting as built in allosteric modulators of the receptor.

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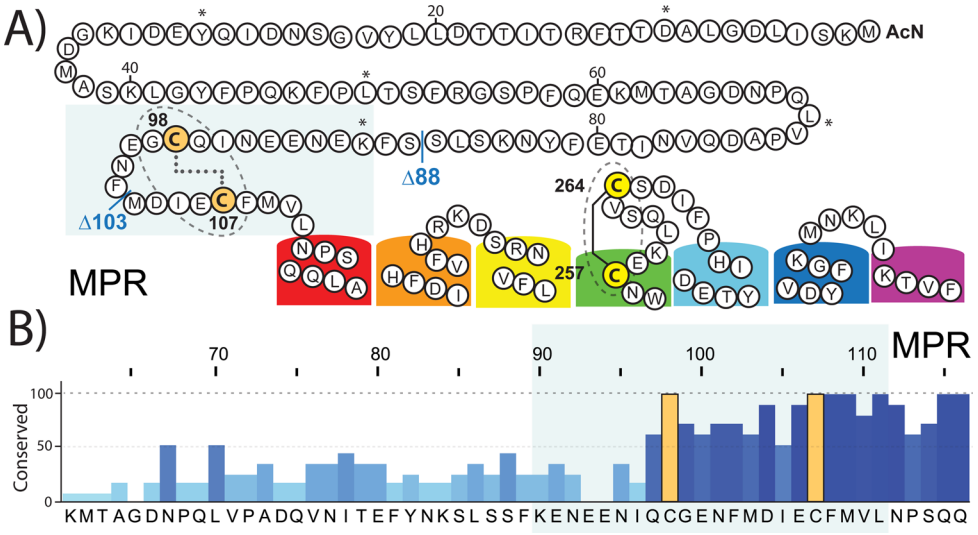

Figure 1. The long N-terminus of CB_1 has a highly conserved membrane proximal region (MPR) containing two conserved cysteine residues. (A) Two-dimensional model of human cannabinoid (CB_1) receptor showing the extracellular region as well as the sites of cysteines and deletions studied in the present work. Disulfide pairs C_{98}–C_{107} and C_{257}–C_{264} are depicted as filled yellow or orange circles. The sites for truncation mutants Δ88 and Δ103 are also indicated. (B) The CB_1 N-terminal MPR is highly conserved, as indicated by alignment conservation plot of various sequences taken from a broad selection of species (sequences extracted from GPCR.org).[42] The multiple sequence alignment conservation was based on the AMAS program.[43] Cysteines (human C98 and C107) are colored in orange.

Table 1. Nomenclature Used for CB_1 Mutants

mutant[a]	N-terminal disulfide (C_{98}–C_{107})?	Cys residues (total number)	Description/comment
CB_1^{WT}	+	all 13 native Cys	"wild type" CB_1 background receptor.
$_{C98A,C107A}CB_1^{WT}$	–	11 cysteines	CB_1^{WT} without C_{98}–C_{107} N-term disulfide (C98, C107 mutated to alanine)
CB_1^{MC}	+	4 Cys: (C98, C107) (C257, C264)	"minimal cysteine" CB_1 background receptor. Has only N-term disulfide (C_{98}–C_{107}), and EL2 disulfide (C_{257}–C_{264}).
$_{C98A,C107A}CB_1^{MC}$	–	2 Cys: (C257, C264)	CB_1^{MC} without C_{98}–C_{107} N-term disulfide (C98, C107 mutated to alanine)
CB_1^{PUR}	+	4 Cys: (C98, C107) (C257, C264)	CB_1^{MC} with N- and C- terminal truncations (Δ88 and Δ417, respectively)
$_{C98A,C107A}CB_1^{PUR}$	–	2 Cys: (C257, C264)	CB_1^{PUR}, without C_{98}–C_{107} N-term disulfide (C98, C107 mutated to alanine). CB_1 mutant used for purification
CB^{RAT}	N.D.		wild-type CB receptors present in membranes prepared from rat brains

[a]All CB_1 mutants contain 1D4 antibody epitope (last nine amino acids of rhodopsin) on C-terminus.

EXPERIMENTAL PROCEDURES

Buffers. The definitions for buffers are PBSSC [0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4]; hypotonic buffer [5 mM Tris·HCl, 2 mM EDTA, PIC, pH 7.5]; TME [20 mM Tris·HCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4]; Rat A [320 mM sucrose, 2 mM Tris·EDTA, and 5 mM $MgCl_2$]; and Rat B: [50 mM Tris·HCl, pH 7, 2 mM EDTA and 5 mM $MgCl_2$].

Expression and Membrane Preparations of shCB_1 Genes in COS-1 Cells. The nomenclature used in the text for the various CB_1 mutants are as follows. Every mutant is referred to as CB_1. The "wild type" CB_1 receptor refers to a mutant synthetic human CB_1 "wild type "receptor which contains all native 13 cysteine residues and a 1D4 antibody epitope on the C-terminus, and this is referred to as $CB1^{WT}$. Proteins containing deletions or mutations are denoted as a subscript to the left of CB_1, and the cysteine "background" used for the mutant is denoted with a superscript on the right of CB_1. Thus, for example, $_{C98A}CB1^{WT}$ indicates that a C98A mutation was introduced into a "wild-type" CB_1 cysteine background. The various mutant DNA were expressed in COS-1 cells by transient transfection, and membranes were prepared as previously described.[13]

Labeling and SDS-PAGE Gel Shift Studies of CB_1 Mutants To Assess the Presence of a Disulfide Bond. Purification, labeling, and SDS-PAGE analysis of CB_1 mutants were performed as described previously.[11] In brief, CB_1 constructs were purified using a one-step immunoaffinity approach. While bound to the column, samples were either subjected to a 20-fold molar excess of bimane (or not) prior to elution and SDS-PAGE analysis in the absence or presence of reducing agent DTT. At least two separate gels were loaded to confirm the initially observed gel-shift.

Membrane Preparations of Rat Cannabinoid Receptors. Rat cortices were purchased from Pel Freeze Biologicals (Rogers, AR), and membrane preparations were performed as previously described.[14] Six grams of cortical tissue were homogenized in 45 mL of Rat A buffer, pelleted via centrifugation (1600*g* for 10 min), washed twice as above, and the combined supernatant fractions were then centrifuged at 39000*g* for 15 min. The pellet was resuspended in 90 mL of Rat B buffer, incubated at 37 °C for 10 min, and subsequently centrifuged at 11000*g* for 15 min whereupon the pellet was again resuspended in Rat B buffer, and incubated at 30 °C for 40 min. Final centrifugation occurred at 11000*g* for 15 min, pellets were homogenized to suspension in TME, aliquoted, snap frozen, and stored at −80 °C

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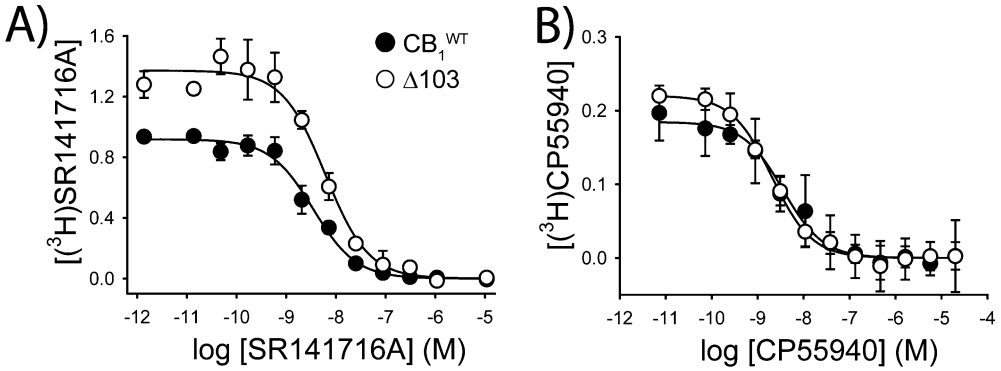

Figure 2. Most of the CB_1 N-terminus can be deleted without abolishing ligand binding. Competitive inhibition binding studies comparing CB_1^{WT} (●) and $_{\Delta 103}CB_1^{WT}$ (○) to binding tritiated (A) antagonist SR141716, and (B) agonist CP 55940. Binding was carried out using a Brandel 24-well filtration apparatus, and the data fit with a one-site binding model. Data represent one binding experiment performed in duplicate. See Experimental Procedures for more details.

until use. Protein concentration was determined using the modified DC protein assay kit (Bio-Rad).

DTT Treatment of CB_1 Containing Membranes. Membrane preparations containing receptor protein were diluted to 4.4 mg/mL of total protein. Importantly, samples were passed through a 24-gauge needle five times and allowed to sit on ice for 1 h prior to treatments. Treatments consisted of diluting membrane preparations to 2.2 mg/mL in various concentrations of DTT (0−300 mM) and allowing the treated samples to nutate at room temperature for 20 min. Treated membranes were then immediately used for equilibrium binding studies.

Radioactive Ligand Binding Studies. The ligand binding experiments were carried out as previously described.[11,13] Data were globally fit and error estimates for the parameters were derived from least-squares fits. All radioactive binding experiments were performed at least twice in duplicate, unless otherwise indicated. Additionally, an allosteric two-site model (eq 1), described previously,[15] was used to fit our data:

$$\mathrm{Fb} = \frac{[\mathrm{A}]\left(1 + \frac{\alpha[\mathrm{B}]}{K_{\mathrm{B2}}}\right)}{[\mathrm{A}]\left(1 + \frac{\alpha[\mathrm{B}]}{K_{\mathrm{B2}}}\right) + K_{\mathrm{A}}\left(1 + \frac{[\mathrm{B}]}{K_{\mathrm{B1}}} + \frac{[\mathrm{B}]}{K_{\mathrm{B2}}}\left(1 + \frac{\beta[\mathrm{B}]}{K_{\mathrm{B1}}}\right)\right)} \quad (1)$$

where Fb denotes the fraction bound, R denotes the receptor, A denotes the orthosteric ligand, and B denotes the allosteric ligand. The K_A, K_{B1}, and K_{B2} are the dissociation constants where the subscripts B1 and B2 represent the two sites that the allosteric ligand can interact with, orthosteric site and allosteric site, respectively. The cooperativity factors, α and β, denote the allosteric interaction between A and B or between the two molecules of B. Cooperativity values greater than 1 denote positive cooperativity (increased affinity), whereas values less than one denote negative cooperativity (decreased affinity), and values equal to one are neutral.

RESULTS

Biochemical Evidence of N-Terminal Disulfide in CB_1. The only previously confirmed disulfide bond in CB_1 is in extracellular loop 2 (EL2), between C257 and C264.[a] For example, as shown in Figure 3A, a purified CB_1 mutant containing only the two cysteine residues at 257 and 264 (termed $_{C98A,C107A}CB_1^{PUR}$) is unreactive to thiol-reactive fluorophores, unless first treated with reducing agents (see also ref 11) providing biochemical evidence that these two cysteines in extracellular loop two (EL2)

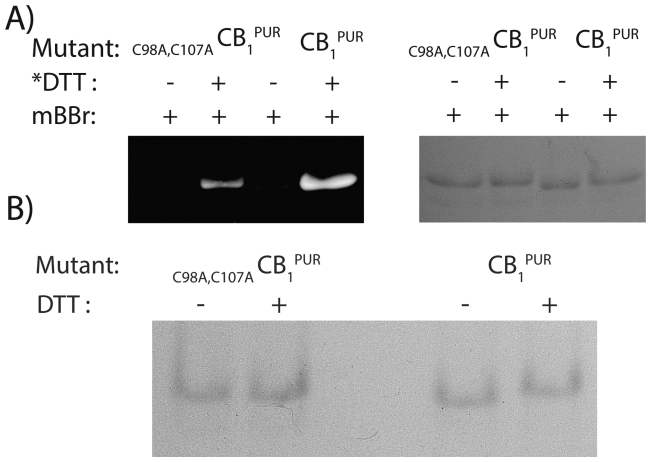

Figure 3. Biochemical evidence for a disulfide between C98 and C107 in the CB_1 N-terminus is indicated by their lack of reactivity toward thiol-specific probes, and their presence causing a faster running species on a nonreduced SDS-PAGE. (A) The purified, minimal-cysteine construct mutants $_{C98A,C107A}CB_1^{PUR}$ (containing C257 and C264) and CB_1^{PUR} (containing C98, C107, C257, and C264) do not react to thiol-reactive fluorophore bimane as indicated by the lack of detectable in-gel bimane fluorescence in the absence of DTT (left), even though equivalent amounts of protein are present (right, Coomassie staining of the same gel). However, reducing both samples with DTT (+DTT) prior to bimane labeling, results in label incorporation. (B) Evidence for a disulfide is also observed when the mobility for the purified CB_1 receptors $_{C98A,C107A}CB_1^{PUR}$ and CB_1^{PUR} are compared in the presence or absence of DTT. In nonreducing conditions, CB_1^{PUR} runs faster than $_{C98A,C107A}CB_1^{PUR}$, but this behavior is abolished upon DTT treatment.

are in a disulfide bond consistent with previous mutagenesis studies.[12,13]

We became interested in the possibility that C98 and C107 in the CB_1 N-terminus also form a disulfide based on observations made during experiments aimed at identifying a nonreactive CB_1 "background" mutant for fluorescence studies.[11] We noticed that a purified CB_1 mutant containing the N-terminal cysteines, C98 and C107, in addition to the two EL2 cysteines C257 and C264 (called CB_1^{PUR}) was also nonreactive to fluorescent labeling (Figure 3B), as was our nonreactive background $_{C98A,C107A}CB_1^{PUR}$. Like $_{C98A,C107A}CB_1^{PUR}$, CB_1^{PUR} also became susceptible to labeling with the thiol-reactive fluorescent probe bimane if treated with DTT prior to labeling, suggesting that C98 and C107 also form a reducible disulfide bond.

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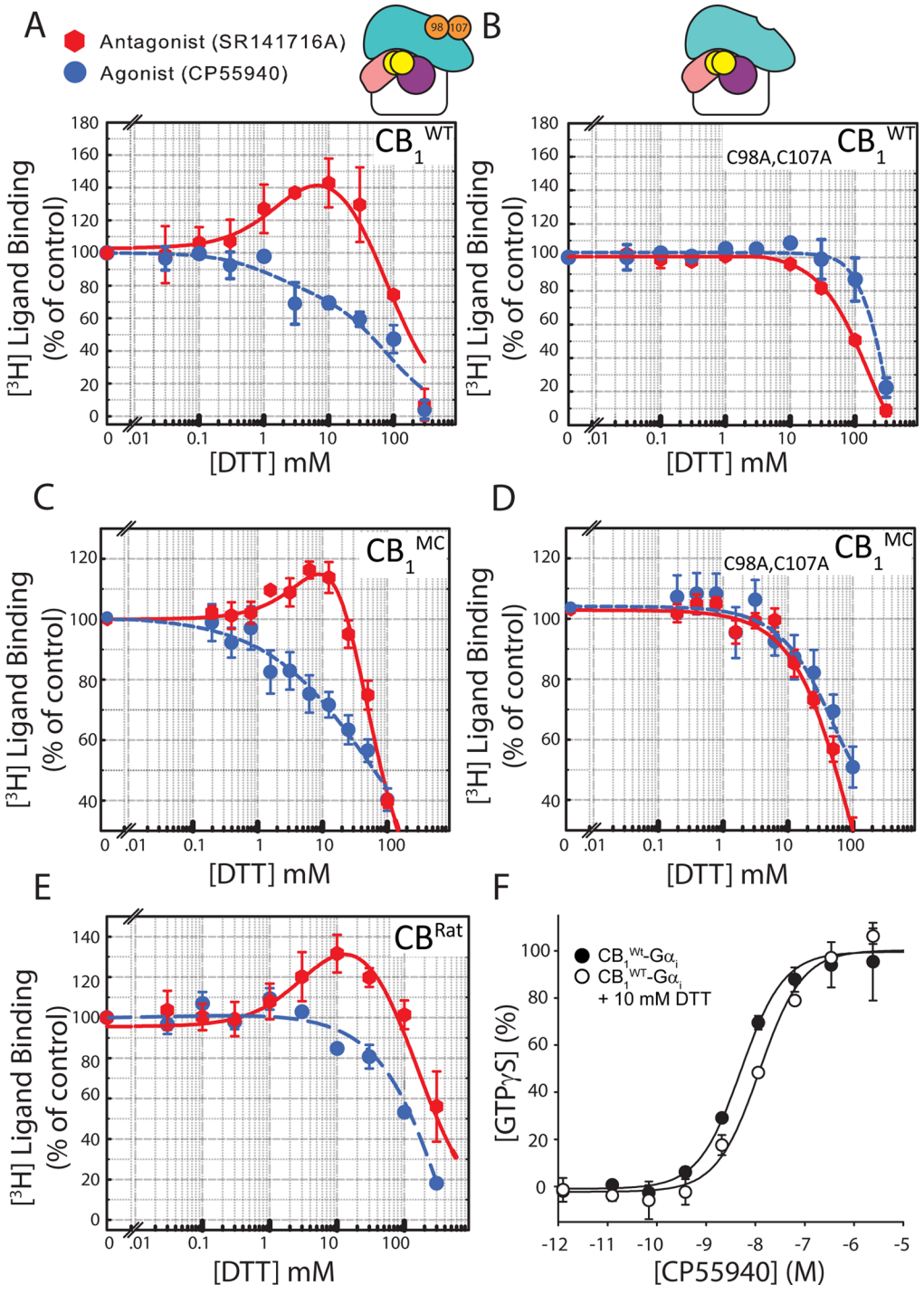

Figure 4. Increasing concentrations of the reducing agent DTT causes different allosteric modulation of agonist (CP55940, blue circles) and antagonist (SR14171A, red hexagons) ligand binding to CB_1. (A) Effect of DTT on ligand binding to "wild-type" (CB_1^{WT}) receptors. (B) Effect of DTT on ligand binding to a receptor lacking N-terminal cysteines ($_{C98A,C107A}CB_1^{WT}$). (C) Binding to CB_1 mutant containing only C98, C107, C257, and C264 (CB_1^{MC}). (D) Binding to CB_1 mutant containing only C257 and C264 ($_{C98A,C107A}CB_1^{MC}$). (E) Binding to wild-type cannabinoid receptors present in membranes prepared from rat cortices (CB^{RAT}). (F) Agonist-stimulated GTPγS binding to CB_1^{WT}-Gαi in the (●) absence (EC_{50} = 5.1 ± 1 nM) or (○) presence of a 10 mM DTT pretreatment (EC_{50} = 12 ± 2 nM). Experiments were performed at least twice in duplicate, and data are presented as the mean ± SEM. Data are normalized to specific fraction bound for respective radioligands determined in the absence or presence of saturating concentration of respective cold ligand. For more details see Experimental Procedures.

Upon closer examination, we also noticed a shift in the Coomassie stain of CB_1^{PUR} under nonreducing SDS-PAGE conditions (Figure 3A). This was confirmed by comparing the mobility of $_{C98A,C107A}CB_1^{PUR}$ versus CB_1^{PUR} on SDS-PAGE, both with and without reducing agent (Figure 3B). Reduction by DTT causes the purified CB_1^{PUR} receptor to run more slowly, comparable to its $_{C98A,C107A}CB_1^{PUR}$ counterpart (Figure 3B), providing further evidence that an N-terminal disulfide exists that imparts structure and/or stability to the CB_1 receptor.

Pharmacological Evidence that N-Terminal Cysteines Are in a Disulfide Bond. We reasoned that any effect of an N-terminal disulfide bond on ligand binding must be more subtle

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than previously appreciated, since almost all of the N-terminus can be deleted while ligand binding ability is retained. On the basis of previous work by Karnik et al.,[16] we decided to explore this phenomenon by systematically monitoring binding of either agonist or antagonist as a function of DTT, using transiently expressed receptors in COS cells (Figure 4).

These results show that DTT causes a decrease in agonist binding, with a surprising concomitant increase in antagonist binding to CB_1^{WT} (Figure 4A). We next tested for this effect in a CB_1 mutant in which two cysteines were mutated to alanine (C98A and C107A) in an CB_1^{WT} background (referred to as $_{C98A,C107A}CB_1^{WT}$). As seen in Figure 4B, the $_{C98A,C107A}CB_1^{WT}$ mutant does not show the DTT-dependent increase and decrease in antagonist and agonist binding, only a decrease in both at very high DTT concentrations. Furthermore, the individual Cys to Ala mutations ($_{C98A}CB_1^{WT}$ and $_{C107A}CB_1^{WT}$) also behave like the double Cys to Ala mutant in that they do not show the DTT-dependent increase and decrease in ligand binding at the lower DTT concentrations (Figure S2, Supporting Information). Thus, we conclude the DTT effect described above is due to reduction of the $C_{98}-C_{107}$ disulfide bond in the N-terminus.

We reasoned that reduction of the disulfide bond between C257 and C264 in EL2 explains why the higher DTT concentrations abolish binding of both agonist and antagonist, as this disulfide is known to be required for functional CB_1 ligand binding.[12,13] We confirmed this by testing the effect of DTT on our previously characterized $_{C98A,C107A}CB_1^{MC}$ mutant[13] and found it behaves like $_{C98A,C107A}CB_1^{WT}$ (Figure 4D). These results are consistent with the interpretation that the $C_{257}-C_{264}$ disulfide bond in EL2 confers stability to the CB_1 receptor orthosteric site.

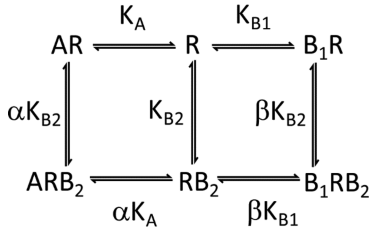
The necessity for higher concentrations of DTT also indicates $C_{257}-C_{264}$ disulfide in EL2 is less accessible and/or is more readily reversible than the $C_{98}-C_{107}$ disulfide in the N-terminus. Other reducing agents (TCEP and GSH) showed a similar behavior, requiring higher concentrations to reduce the EL2 disulfide (Figure S3, Supporting Information), again supporting the presumably inaccessible location of the $C_{98}-C_{107}$ disulfide bond. Furthermore, when the N-terminal cysteine pair is reintroduced into $_{C98A,C107A}CB_1^{MC}$ (a mutant we call CB_1^{MC}) the DTT-dependent change in ligand binding is restored (Figure 4C).

Importantly, the DTT effect is also observed in experiments carried out on rat cerebral cortex membranes (CB^{RAT}, Figure 4E), which contain native cannabinoid receptors. This important control confirms that the DTT effect on ligand binding is not just an artifact caused by the use of a transient expression system (COS cells) to express the mutant human CB_1 receptors.

Finally, we tested the possible functional effect of the N-terminal $C_{98}-C_{107}$ disulfide bond using a CB_1-G protein fusion system. These assays measured the ability of a CB_1 agonist to stimulate radioactive $GTP\gamma S^{[35]}$ binding to a CB_1^{WT} receptor with a $G_{\alpha i}$ subunit fused to its C-terminal tail.[13] The results, shown in Figure 4F, indicate that addition of DTT under conditions that should selectively reduce the N-terminal $C_{98}-C_{107}$ disulfide, but not the EL2 $C_{257}-C_{264}$ disulfide, causes a small but reproducible shift in the EC_{50} value of agonist required for G protein activation (the conditions were the same as the maximum inflection point on Figure 4A, 10 mM DTT treatment for 20 min prior to the assay).

Analysis Suggests the N-Terminal $C_{98}-C_{107}$ Disulfide Allosterically Regulates the CB_1 Orthosteric Binding Site. The behavior seen in Figure 4 is highly indicative of an allosteric effect, where reducing the allosteric N-terminal disulfide imparts positive cooperativity to the orthosteric (antagonist) ligand binding site, and negative cooperativity to agonist binding. We tested this using an allosteric two-site model and find excellent agreement of the fits to our experimental data ($R^2 = 0.95 \pm 0.01$). The model, represented in a cartoon in Figure 6A and schematically in Scheme 1, presumes that the DTT-dependent

Scheme 1. Allosteric Two Site Model



enhancement/decrease of binding at the N-terminal disulfide is the "other site" (i.e., allosteric) and that EL2 disulfide is part of the orthosteric site, as it is known that the EL2 disulfide is required for orthosteric ligand binding.[12,13] Note that in this two-site model, DTT is not binding to these sites in a traditional sense, but rather, is modifying/interacting with these two sites in a manner that can be interpreted (for the case of this model) as "binding".

Although not perfect, this two-site model provides a unique way to conceptualize and quantify our data, and the fits indicate both positive or negative cooperativity (α) is imparted on the orthosteric ligand by modulation of the "allosteric effect" disulfide in the N-terminus (Table 2). Moreover, the mean

Table 2. Mean Fit Parameters[a]

$K_{B1}^{C257-C264}$	95 ± 20 mM	$\alpha_{SR141716A}$	2.3 ± 0.5
$K_{B2}^{C98-C107}$	4.4 ± 1.3 mM	$\alpha_{CP55940}$	0.69 ± 0.08

[a]An allosteric two site model (eq 1) was used to fit the data in Figure 4. Mean parameter values ± SEM are listed above where $K_{B1}^{C257-C264}$ and $K_{B2}^{C98-C107}$ are the dissociation constants for the orthosteric and allosteric effector disulfide respectively. The α value is the cooperativity factor between A and B for CP55940 or SR141716A (denoted as subscript). The mean coefficient of determination for all fits is 0.95 ± 0.01. For more details see Supplemental Table 1, Supporting Information and Experimental procedures.

dissociation constant indicates a high "affinity" of DTT toward for N-terminal cysteines. In contrast, the EL2 disulfide shows a lower "affinity" for DTT (Table 2), consistent with a model where the N-terminal cysteine residues are more accessible than the EL2 disulfide.

It is important to note that the dissociation values derived from our fits likely depend on the experimental conditions (time, temperature, concentration, etc.), and we did not explore the effect of these possible variables. Also, it might formally be possible to fit our data with a dimer/oligomer model, but since we find no evidence that significant amounts of our CB_1 mutants form dimers through a disulfide by SDS-PAGE analysis (see Figure S4, Supporting Information) we have not done so. The implications of conformational coupling between the N-terminal disulfide and the orthosteric site are discussed in greater detail below.

The N-Terminal $C_{98}-C_{107}$ Disulfide Bond Affects the Actions of CB_1 Allosteric Ligands Org 27569 and PSNCBAM-1. The fact that the N-terminal MPR can act to

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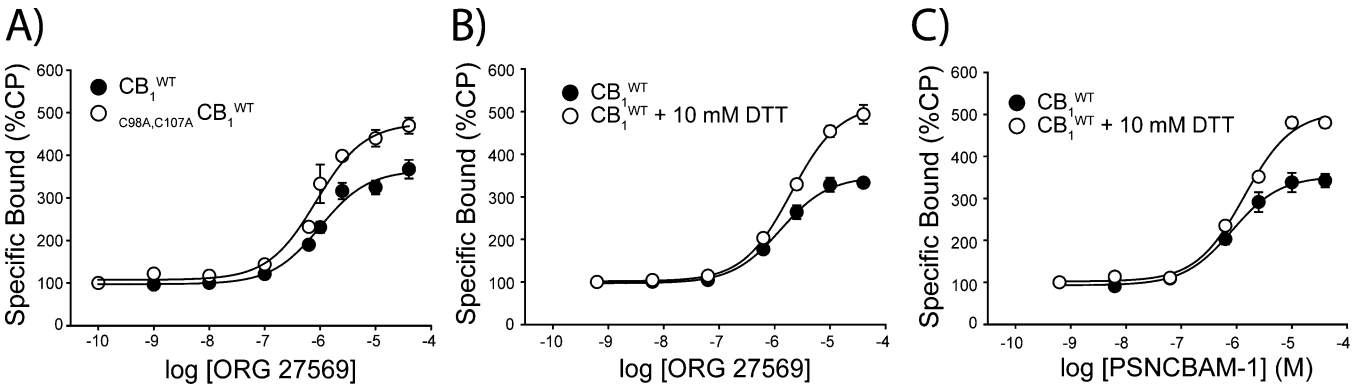

Figure 5. The CB_1 N-terminal disulfide alters the cooperativity of the allosteric ligand Org 27569 and PSCNBAM-1. Equilibrium binding of agonist [^{3}H]CP55940 was determined in the presence of various concentrations of allosteric modulator. (A) The effect of Org 27569 on agonist binding of CB_1^{WT} (●) compared with a CB_1 mutant lacking the N-terminal disulfide (A, $_{C98A,C107A}CB_1^{WT}$, ○). (B) Comparison of the effect of Org 27569 on CB_1^{WT} treated with 10 mM DTT for 20 min prior to the assay (B, 10 mM DTT ○). (C) Comparison of the effect of PSCNBAM-1 on CB_1^{WT} treated with 10 mM DTT for 20 min prior to the assay (C, 10 mM DTT ○). The data show that removing the CB_1 N-terminal disulfide enhances the effects of the allosteric ligands Org 27569 and PSCNBAM-1. When the allosteric ternary complex is fit to the above data, the resulting allosteric cooperativity factors with respect to Org 27569 are 4.9 ± 0.4 for CB_1^{WT} and 7.8 ± 0.5 or 6.8 ± 0.3 for $_{C98A,C107A}CB_1^{WT}$ or CB_1^{WT} treated with 10 mM DTT, respectively. With respect to PSCNBAM-1, the cooperativity factors are 4.2 ± 0.2 for CB_1^{WT} and 5.9 ± 0.3 for CB_1^{WT} treated with 10 mM DTT. Each radioactive binding shown above is representative of at least two independent experiments performed in triplicate and is reported as the mean ± SEM. The cooperativity factors were determined by fitting the combined respective data sets, and errors were determined from least-squares fitting. See Experimental Procedures for more details.

allosterically modulate ligand binding to CB_1 (described above) is reminiscent of the way some small molecule allosteric ligands can affect GPCRs. Several such allosteric ligands exist for CB_1, but their ability to modulate receptor binding and function has not been tested in relation to the N-terminal C_{98}–C_{107} disulfide bond in CB_1 we describe here. Thus, we tested how a known CB_1 specific allosteric ligand Org 27569[11,17] is affected by the presence or absence of the N-terminal disulfide. We find the positive cooperativity of Org 27569 (α = 4.9 ± 0.4) for agonist binding is significantly enhanced (Figure 5) when the N-terminal C_{98}–C_{107} disulfide is removed by either mutation or incubation of the sample with 10 mM DTT (combined mean value α = 7.2 ± 0.6 for the two conditions [$t(8) = -3.3$, $p < 0.02$]). Interestingly, the effects of the allosteric antagonist, Org 27569 are additive with the allosteric effects of the N-terminal disulfide. For instance, the sum of cooperativity factors (4.9 ± 0.4 for "wt CB_1" (Figure 5) and 2.3 ± 0.5 for antagonist binding (Figure 4, Table 2) is equal to the higher cooperativity value observed for Org 27569 when the N-terminal disulfide is absent (7.2 ± 0.6 vs 7.2 ± 0.6). We also tested the effect of DTT reduction on another allosteric ligand for CB_1, PSNCBAM-1. As shown in Figure 5C, addition of DTT at a concentration shown to reduce the N-terminal C_{98}–C_{107} disulfide bond resulted in an increase in agonist binding.

DISCUSSION

Our data indicate the membrane proximal region (MPR) of the CB_1 N-terminus can affect the ligand binding properties of the receptor in a previously unappreciated way and yield insights into where known allosteric ligands may exert their action on CB_1. Specifically, we find direct evidence for an intramolecular disulfide in the CB_1 N-terminal MPR (Figure 3B) and find that reduction of this disulfide results in altered ligand binding in an unexpected way when ligand binding is monitored as a function of increasing DTT (Figure 4).

At lower DTT concentrations, reducing the N-terminal C_{98}–C_{107} disulfide can impart either positive or negative cooperativity (α) on the orthosteric ligand binding site, depending on whether

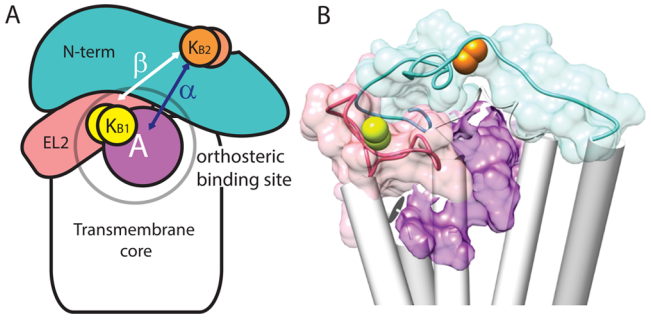

Figure 6. Proposed model showing how interaction with the CB_1 N-terminus effects orthosteric ligand biding pocket. (A) Cartoon model depicting the allosteric two-site model for regulation of CB_1 by the N-terminus. The model shows the orthosteric ligand, A (purple circle), and the orthosteric binding pocket (gray circle), which also includes the critical C_{257}–C_{264} disulfide in EL2 (pink). The N-terminal region that allosterically modulates binding is also shown (turquoise), as well as the N-terminal C_{98}–C_{107} disulfide (orange circles). The cooperativity factors governing interactions between K_{B2} and the orthosteric site (α) or between the two disulfides (β) are represented by blue and white arrows, respectively. (B) A homology model of CB_1 developed using coordinates from the structure of GPCR S1P1 (Protein Data Bank (PDB): 3V2Y). The model shows the three domains that are conformationally coupled in the model: (1) The N-terminal domain (turquoise, the C_{98}–C_{107} disulfide in orange), (2) loop EL2 (pink, the C_{257}–C_{264} disulfide in yellow), and (3) the orthosteric ligand binding cavity (purple). Transmembrane helices 6 and 7 have been omitted for clarity.

the ligand is an antagonist or agonist. High DTT concentrations caused a clear attenuation of ligand binding, presumably due to reduction of the EL2 disulfide (Figure 4). These results are consistent with previous mutation studies that suggest a disulfide between C257/C264 in EL2 is critical for the stability of the orthosteric ligand binding site.[12,13] Below, we discuss the implications resulting from analyzing this data using an allosteric two-site model.

Effect of DTT on Ligand Binding Can Be Described Using an Allosteric Model. Our findings suggest that the CB_1

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N-terminal cysteines allosterically regulates the binding characteristic for the orthosteric site on CB_1, since reducing the N-terminal disulfide with DTT changes the affinity for agonist and antagonists in a reciprocal manner. This reciprocity is illustrated by the cooperativity factors; for instance, the inverse of the mean positive cooperatively value for antagonist binding (α_{SR}^{-1} = 0.50 ± 0.09) is close to the mean negative cooperative for agonist binding (α_{CP} = 0.69 ± 0.08). It is also reasonable to assume the converse effects may exist, where agonist binding is enhanced by the presence of an N-terminal disulfide, and antagonist specific binding is reduced by the presence of an N-terminal disulfide.

The N-Terminal Disulfide in CB_1 Is More Solvent Accessible than the EL2 Disulfide. On the basis of the K_{B1} and K_{B2} values determined from the model (Table 2), we find the EL2 disulfide (K_{B1}) has an approximately 20-fold higher dissociation constant than the N-terminal disulfide (K_{B2}). Our data suggest that the N-terminal disulfide is much more accessible to reducing agents than the EL2 disulfide, consistent with our results using other reducing agents (see also Figure S3, Supporting Information).

The fact that high DTT concentrations are required to reduce the EL2 disulfide is reminiscent of rhodopsin, where the highly conserved disulfide between EL2 and TM3 is completely buried and inaccessible to reducing agents (in the absence of denaturants).[18]

Possible Structural Roles of an N-Terminal Disulfide in CB_1. What insights do these data and analysis provide about the role of the CB_1 N-terminus? One insight from our data is that the CB_1 N-terminal MPR couples with the orthoseteric ligand binding site. On the basis of this observation, we propose that this part of the N-terminus forms a "lid" over the orthosteric binding site (as depicted in our model in Figure 6B). This idea is intriguing since almost all known GPCR structures to date show solvent accessible binding pockets, except for rhodopsin and sphingosine 1 phosphate receptor (S1P1), two GPCRs that, like CB_1, bind hydrophobic ligands. Rhodopsin and S1P1 both show significant occlusion of the orthosteric binding pocket and limited solvent accessibility presumably due to the hydrophobic nature of their endogenous ligands. Since cannabinoid ligands are also hydrophobic, it is reasonable to speculate that CB_1 also possesses an occluded binding pocket.

In summary, our results suggest the highly conserved N-terminal MPR of CB_1 may play several roles. One role is to interact with and allosterically regulate the orthosteric binding site of CB_1, leading to a subtle change in conformational and functional states of the receptor. This may explain the highly conserved nature of this region. Another role for the CB_1 N-terminus, stabilized by the C_{98}–C_{107} disulfide, could also be to act as a domain over the binding pocket in CB_1. Thus, we propose there is domain coupling at least between the CB_1 N-terminal MPR and the ligand binding pocket of CB_1, where reduction of the exposed N-terminal disulfides can perturb the N-terminal domain structure and thus the entire conformational landscape of the extracellular domain.

Interestingly, the N-terminus of rhodopsin plays a similar role for that receptor. It forms a stable domain, and when constrained to the receptor by engineered disulfides, produces a receptor with enhanced thermostability.[19] Other GPCRs have also shown a role of extracellular disulfides, including some chemokine receptors[20,21] and the angiotensin II receptor.[16] Interestingly, metabotropic glutamate and calcium sensing receptors have been reported to have intermolecular disulfides between receptors,[22,23] but we have not found evidence for this in CB_1 (Figure S4, Supporting Information).

Possible Role for the CB_1 N-Terminal MPR in Binding Allosteric Ligands and/or Enabling Them to Exert Their Influence. As with several other GPCRs, CB_1 has been shown to undergo regulation by allosteric exogenous ligands, several of which been described.[17,24−29] Our results in Figure 5 indicate interplay between the agonist CP 55940, the allosteric ligands Org 27569 (and PSNCBAM-1), and the CB_1 MPR and N-terminal disulfide. One possible mechanism through which this occurs could be that allosteric ligands bind near this region and affect the orthosteric ligand binding/dissociation pathway, thereby preventing orthosteric ligand dissociation. Consistent with this, the allosteric compounds described by Price et al. have been shown to slow the dissociation of CP 55940 from the orthosteric binding site.[17] Recent computational studies on the muscarinic acetylcholine receptor 3 suggest an orthosteric ligand with slower dissociation rates toward M3 can bind to a second site along the binding pathway.[30] Thus, it is tempting to speculate that small-molecule allosteric CB_1 allosteric ligands may work in a similar fashion. However, given the lipophilic nature of cannabinoid ligands, this site would presumably be in the egress pathway that connects the orthosteric binding site to the membrane and not directly to extracellular parts of the N-terminus.

Interestingly, the lipid binding GPCR S1P1 structure hints at a lipid access channel between TM1 and TM7.[31] Reggio and coworkers have proposed a similar channel may exist between helices in cannabinoid receptors.[32−34] If such a channel exists in CB_1, it may represent an area where the N-terminal MPR of CB_1 induces its allosteric effect. In support of this model, we find that the absence of the N-terminal disulfide in the MPR enhances the cooperativity between the allosteric ligand Org 27569 and the agonist CP 55940 (Figure 5). However, since the cooperativity for these interactions appears to be additive, it is likely that these two allosteric events occur through somewhat different mechanisms.

Possible Biological Roles for CB_1 N-Terminal Disulfide. Finally, although we have no direct evidence for this yet, it is tempting to speculate that the N-terminal disulfide in CB_1 could act as a redox sensor. Redox-dependent structural switches have been observed in other proteins, such as OxyR, INAD, and angiotensinogen.[35] A redox sensor in CB_1 could be neuroprotective, since traumatic brain injury results in the release of endocannabinoids in addition to reactive oxygen intermediates.[36,37] Taken together, this redox-switch could potentially enhance CB_1 receptor ligand occupancy and, in part, modulate the neuroprotective properties of CB_1 activation. Altering the effect would likely be subtle (as suggested by our functional data in Figure 5F), but it could change the "set point" at which the receptor responds to endogenous signals.

We also note that the antioxidant tripeptide glutathione (GSH) can be released from neurons in a depolarization-dependent fashion [38] and GSH has been shown to play a role in modulation of excitatory neurotransmission[39] similar to endocannabinoids.[40] Thus, GSH release may be a way to locally regulate presynaptic CB_1 receptor "set points". Moreover, impaired GSH homeostasis or increase in reactive oxygen species is associated with diseases[41] that coincide with CB_1 receptor-associated diseases, including Parkinson's and Alzheimer's.[1] The importance of the potential disulfide bond-mediated redox sensitivity in normal and diseased states has implications in the development of antioxidant-based therapeutic approaches and warrants further exploration.

In summary, we find clear evidence for the presence of an extracellular disulfide bond between C98 and C107 in the N-terminus of CB_1, evidence that the MPR of the CB_1 N-terminus

acts to allosterically modulate agonist and antagonist binding, and evidence that this disulfide can affect the behavior of known CB_1 allosteric ligands. Together these data suggest the mechanism of action for some CB_1 allosteric ligands may lie in the MPR of the CB_1 N-terminus. Future studies are underway to test this theory and further investigate the mechanism of this allosteric effect.

■ ASSOCIATED CONTENT

Supporting Information

G protein activity of Δ103 truncation mutant, immunoblot analysis of CB_1 receptor mutants used in membrane radioligand binding assays, the effect of DTT on individual Cys to Ala mutants ($_{C98A}CB_1^{WT}$ and $_{C107A}CB_1^{WT}$), and the effect of other reducing agents on CB_1 mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

GPCR, G protein-coupled receptor; CB_1, cannabinoid type-1 receptor; CP55940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; SR141716A, 5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1*H*-pyrazole-3-carboxylic acid piperidin-1-ylamide hydrochloride; PDT-bimane, 2,3,6-trimethyl-5-[(2-pyridinyldithio)methyl]-1*H*,7H-pyra-zolo[1,2-a]pyrazole-1,7-dione; shCB1, synthetic human CB1 receptor; DTT, dithiothreitol; CB_1^{WT}, synthetic human "wild type " CB_1 receptor with all 13 cysteines retained and a 1D4 antibody epitope on the C-terminus; $_{\Delta 103}CB_1^{WT}$, CB_1^{WT} with N-terminal truncation at site 103; $_{C98A,C107A}CB_1^{WT}$, CB_1^{WT} receptor containing C98A and C107A mutations; CB_1^{MC}, "minimal cysteine" CB_1 background receptor, has only N-terminal disulfide (C_{98}−C_{107}), and EL2 disulfide (C_{257}−C_{264}); $_{C98A,C107A}CB_1^{MC}$, CB_1^{MC} receptor without C_{98}−C_{107} N-term disulfide (C98, C107 mutated to alanine); CB_1^{PUR}, CB_1^{MC} receptor with N- and C-terminal truncations (Δ88 and Δ417, respectively); $_{C98A,C107A}$ CB_1^{PUR}, CB_1^{PUR} receptor lacking the C_{98}−C_{107} N-term disulfide (C98, C107 mutated to alanine), a CB_1 mutant used for purification; CB^{RAT}, wild-type CB receptors present in membranes prepared from rat brains; TCEP, (tris(2-carboxyethyl)phosphine; EL2, extracellular loop 2; TM, transmembrane helix; PIC, protease inhibitor cocktail

■ ADDITIONAL NOTE

[a]Interestingly, this disulfide appears to take the place of the canonical disulfide bridge connecting EL2 to transmembrane helix 3 present in most Class A GPCRs.

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