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Functional roles of the tyrosine within the NP(X)$_n$Y motif and the cysteines in the C-terminal juxtamembrane region of the CB2 cannabinoid receptor

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Abstract In G protein-coupled receptors, a NP(X)$_n$Y motif in the seventh transmembrane domain and cysteine residues in the C-terminal juxtamembrane region are conserved. In the current study, the roles of Y299 within the NPVIY motif and C313 and C320 in the C-terminal juxtamembrane region of the human CB2 cannabinoid receptor were investigated by site-directed mutagenesis. Replacing Y299 with alanine resulted in a complete loss of ligand binding and a severe impairment of cannabinoid-induced inhibition of forskolin-stimulated cAMP accumulation. The C313A and C320A mutations markedly reduced functional coupling to adenylate cyclase, but had no effect on ligand binding and agonist-induced receptor desensitization. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cannabinoid receptor 2; Cannabinoid; Site-directed mutagenesis; Structure; Function

1. Introduction

To date, two types of cannabinoid receptors, CB1 and CB2, have been cloned [1,2]. CB2 exhibits 44% amino acid identity with CB1 [2]. CB1 is located in the central nervous system as well as in peripheral tissues [1,3,4]. CB2 is distributed primarily in peripheral tissues such as immune cells [2,3]. It is known that activation of both CB1 and CB2 cannabinoid receptors inhibits adenylate cyclase through coupling with pertussis toxin-sensitive G proteins [5,6]. However, currently very little information is available regarding the molecular mechanisms for the activation of these receptors. Studying the structure and function of CB2 has potential therapeutic implications. Since CB2 is not located in the central nervous system, ligands that selectively activate CB2 would be devoid of the psychoactive effects of marijuana.

Cannabinoid receptors belong to the G protein-coupled receptor (GPCR) superfamily [7–9]. These receptors contain seven putative transmembrane domains connected by three extracellular and three intracellular loops. In general, the extracellular loops and/or transmembrane regions are involved in ligand binding, whereas cytoplasmic regions contain sites for interactions with G proteins [10,11]. In many GPCRs, a NP(X)$_n$Y motif within the seventh transmembrane domain (TM7) near the cytoplasmic face of the plasma membrane is highly conserved. This sequence has been postulated to play important roles in receptor activation and regulation [12–15]. The NP(X)$_n$Y motif exists in TM7 of the CB2 receptor as NPVIY, with the Y being Y299 (Fig. 1). Furthermore, most GPCRs have one or two conserved cysteine(s) in the C-terminal juxtamembrane region. These cysteines are sites for palmitoylation, which has been suggested to play important roles for receptor–G protein coupling and receptor desensitization [15–18]. In the CB2 receptor, two cysteines, C313 and C320, are located in the C-terminal juxtamembrane region (Fig. 1).

The Y in the NP(X)$_n$Y motif and the cysteines in the C-terminal juxtamembrane region of GPCRs have been shown to play important roles in functional coupling and desensitization of these receptors [12–18]. Based on these previous findings, our hypothesis is that Y299 in the NPVIY motif and C313 and C320 in the C-terminal juxtamembrane region of the CB2 receptor may be crucial for the activation and desensitization of this receptor. To test this hypothesis, Y299, C313, and C320 of the CB2 receptor were mutated to alanine in this study. These mutant receptors were stably transfected into human embryonic kidney 293 (HEK293) cells and their ligand binding, signal transduction, and desensitization properties were compared with those of the wild-type CB2 receptor.

2. Materials and methods

2.1. Materials

Enzymes and reagents used for recombinant DNA experiments were purchased from Gibco-BRL (Gaithersburg, MD, USA) or Promega (Madison, WI, USA). Adenovirus-transformed HEK293 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin/streptomycin, L-glutamine, trypsin and Geneticin were purchased from Biowhittaker (Walkersville, MD, USA). Anandamide and WIN55212-2 were obtained from RBI (Batick, MA, USA). HU-210 and [^{3}H]HU-243 were obtained from Tocris (Ballwin, MO, USA). [^{125}I]Cyclic AMP used for radioimmunoassays was purchased from New England Nuclear (Boston, MA, USA).

2.2. Mutagenesis

A 1.8-kb full-length human CB2 cDNA was subcloned into pRC/CMV (Invitrogen, San Diego, CA, USA) to construct the expression plasmid pHCB2-RC/CMV [19]. The GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI, USA) was used to mutate the CB2 receptor. The following oligonucleotides were used: Y299A, 5′-AACCCTGTCATCGCTGCTCTACGGAG-3′; C313A, 5′-CTCTGCCCATCACGC-CCTGGCTCACTGG-3′; C320A, 5′-TCACTGGAAGAAGGCTGTGAGGGGCCTTG-3′. The presence of the mutation as well as the accuracy of the DNA sequences was confirmed by dideoxy sequencing.

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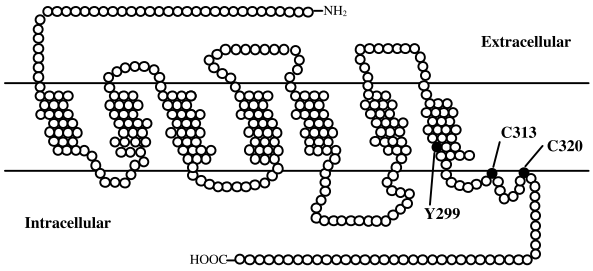

Fig. 1. Schematic depiction of the CB2 cannabinoid receptor membrane topology and the location of Y299, C313 and C320.

2.3. Cell transfection and culture

Expression plasmids containing wild-type and mutant CB2 receptors were transfected into HEK293 cells. Transfected cells were selected in culture medium containing 500 μg/ml Geneticin, and cell lines stably expressing wild-type and mutant cannabinoid receptors were established according to a previously established method [20]. Cells were grown as monolayers in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml Geneticin in a humidified atmosphere consisting of 5% CO_2 and 95% air, at 37°C.

2.4. Immunofluorescence staining

HEK293 cells stably expressing wild-type and mutant CB2 receptors were grown on cover glass. Cells were washed twice with 0.1 M phosphate-buffered saline (PBS), pH 7.4, fixed with 4% paraformaldehyde for 15 min, and washed twice with PBS for 5 min each time. The cells were incubated with PBS containing 5% normal goat serum (NGS) at room temperature for 1 h, and then incubated with the anti-CB2 antibody (Cayman, Ann Arbor, MI, USA) at room temperature for 2 h. After washing three times with PBS containing 5% NGS for 10 min each time, cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Zymed, San Francisco, CA, USA) at room temperature for 1 h. After washing four times with PBS, coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and viewed with an Olympus IX50 fluorescence microscope.

2.5. Ligand binding and cAMP accumulation assays

Ligand binding assays were performed as previously described [19,21]. Briefly, the cells were homogenized in membrane buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4) and cell membranes were obtained by centrifugation at $32\,000 \times g$ for 20 min. Membrane protein concentrations were determined by the use of a bicinchoninic acid protein reagent kit (Pierce, Rockford, IL, USA). Membranes were incubated with 0.2 nM [^{3}H]HU-243 and different concentrations of unlabeled ligands at 30°C for 60 min. Non-specific binding was determined in the presence of 0.1 μM unlabeled HU-210. Free and bound radioligands were separated by rapid filtration through polyethyleneimine-treated GF/B filters (Whatman International, Maidstone, UK). The filters were washed three times with 3 ml of cold 50 mM Tris–HCl, pH 7.4, and bound radioactivity was determined by liquid scintillation counting.

cAMP accumulation assays were performed using a previously published method [19,21]. Briefly, cannabinoid ligands in different concentrations were mixed with forskolin. Confluent cells were lifted and incubated with phosphodiesterase inhibitor RO20-1724 (Biomol, Plymouth Meeting, PA, USA) for 10 min. The stimulation was initiated by adding cells to the test tubes containing forskolin and cannabinoids, and incubated for 5 min at 37°C. The reaction was stopped with the addition of 0.1 N HCl, after which 50 μl was removed for cAMP radioimmunoassay, using a kit from DuPont-NEN (Wilmington, DE, USA).

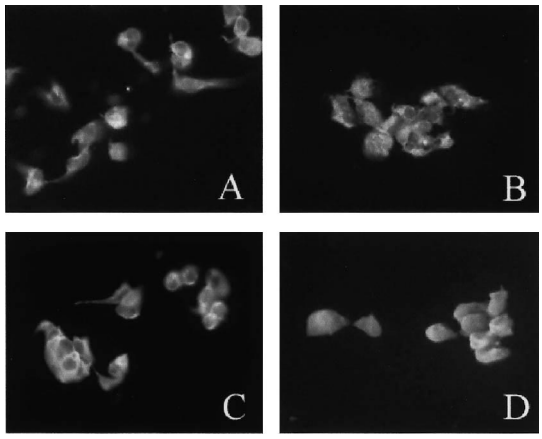

Fig. 2. Immunofluorescent staining of HEK293 cells stably transfected with the wild-type and mutant CB2 cannabinoid receptors. A: Wild-type CB2 receptor. B: Y299A mutant. C: C313A mutant. D: C320A mutant.

2.6. Desensitization experiments

To study receptor desensitization, cells were pretreated in DMEM containing 0.2% bovine serum albumin and 3 nM HU-210 at 37°C for 1 h. Subsequently, cAMP accumulation assays were performed as described in Section 2.5.

2.7. Data analysis

Data from ligand binding and cAMP accumulation assays were analyzed, and curves were generated with use of the Prizm program (GraphPad Software, San Diego, CA, USA). IC_{50} and EC_{50} values were determined through non-linear regression analysis performed with the Prizm program. K_d and B_{max} values were estimated from competition binding experiments with the following equations: $K_d = IC_{50} - L$ and $B_{max} = (B_0 IC_{50})/L$, where L is the concentration of free radioligand, and B_0 is specifically bound radioligand [22]. The K_i values were calculated based on the Cheng–Prusoff equation: $K_i = IC_{50}/(1+L/K_d)$ [23].

3. Results

3.1. Expression of wild-type and mutant CB2 receptors

Immunofluorescent microscopy study was performed to examine the expression of wild-type and mutant CB2 receptors. Using the anti-CB2 antibody, which is directed against the extracellular N-terminal of CB2 receptor, positive immunofluorescence staining signals were observed in non-permeabilized HEK293 cells stably transfected with Y299A, C313A, and C320A, as well as wild-type CB2 receptors (Fig. 2). The results indicate that these receptors were properly targeted into the plasma membranes.

Table 1
The ligand binding parameters of the wild-type and mutant CB2 receptors

		Wild-type	C313A	C320A
	B_{max}, fmol/mg protein	2846.9 ± 837.9	2782.4 ± 458.9	2502.2 ± 415.7
HU-210	K_d/K_i, nM	0.47 ± 0.06	0.39 ± 0.11	0.23 ± 0.06
WIN55212–2	K_d/K_i, nM	3.4 ± 1.0	3.1 ± 0.4	6.5 ± 0.7
Anandamide	K_d/K_i, nM	314.5 ± 64.8	448.4 ± 48.5	234.1 ± 31.9

Competition assays were performed on membranes prepared from cells expressing wild-type, C313A, and C320A mutant CB2 receptors using [^{3}H]HU-243 as radioligand. Values are shown as mean ± S.E.M. of at least three independent experiments, each performed in duplicate.

3.2. Ligand binding

Fig. 3 and Table 1 show the ligand binding curves and parameters of wild-type and mutant CB2 receptors. The wild-type CB2 receptor displayed high affinity binding of [^{3}H]HU-243 with a K_d of 0.47 ± 0.06 nM and a B_{max} of 2846.9 ± 837.9 fmol/mg of membrane protein. While no specific binding was detected with the Y299A mutant receptor, specific and high affinity binding of [^{3}H]HU-243 was found with C313A and C320A mutant receptors. For the wild-type CB2 receptor, the rank order of potency for three structurally distinct cannabinoid ligands to compete for the binding of [^{3}H]HU-243 was HU-210 > WIN55212–2 > anandamide. For the C313A and C320A mutant receptors, HU-210, WIN55212-2, and anandamide had ligand binding affinities similar to those for the wild-type CB2 receptor, and the rank order of potency for the three ligands to compete for the binding of [^{3}H]HU-243 was the same as that for wild-type CB2 receptor. Furthermore, the B_{max} values of neither C313A nor C320A mutant receptors are significantly different from that of the wild-type CB2 receptor ($P > 0.05$).

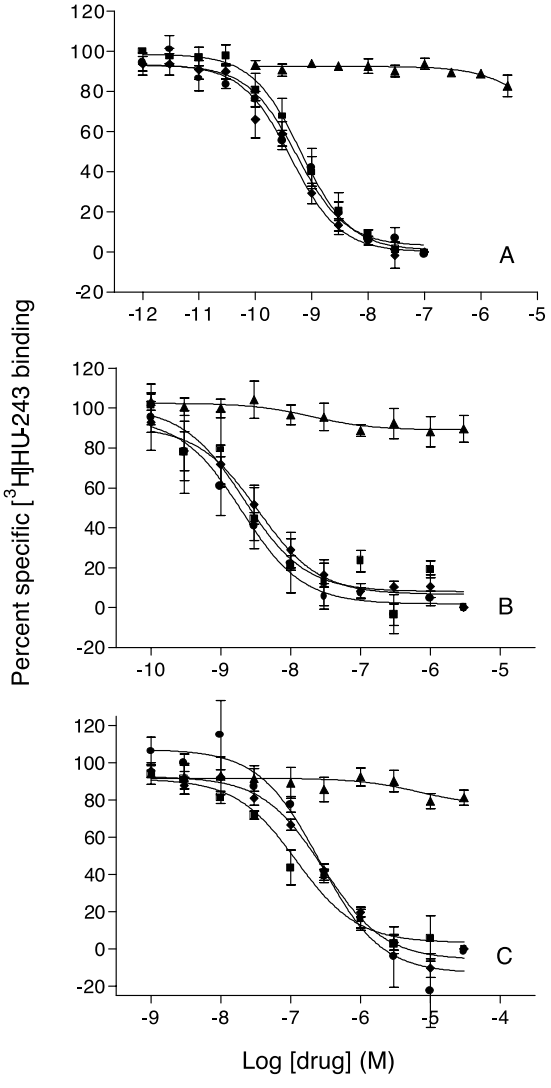

Fig. 3. Comparison of the wild-type and mutant CB2 receptors for cannabinoid ligand binding. Three cannabinoid ligands, HU-210 (A), WIN55212-2 (B), and anandamide (C), were used for ligand binding experiments using membranes prepared from HEK293 cells stably expressing the wild-type (■), Y299A (▲), C313A (●) and C320A (♦) mutant CB2 receptors. [^{3}H]HU-243 specific binding in the absence of unlabeled ligand was defined as 100%. Data shown represent the mean ± S.E.M. of at least three independent experiments performed in duplicate. Curves were generated as described in Section 2.

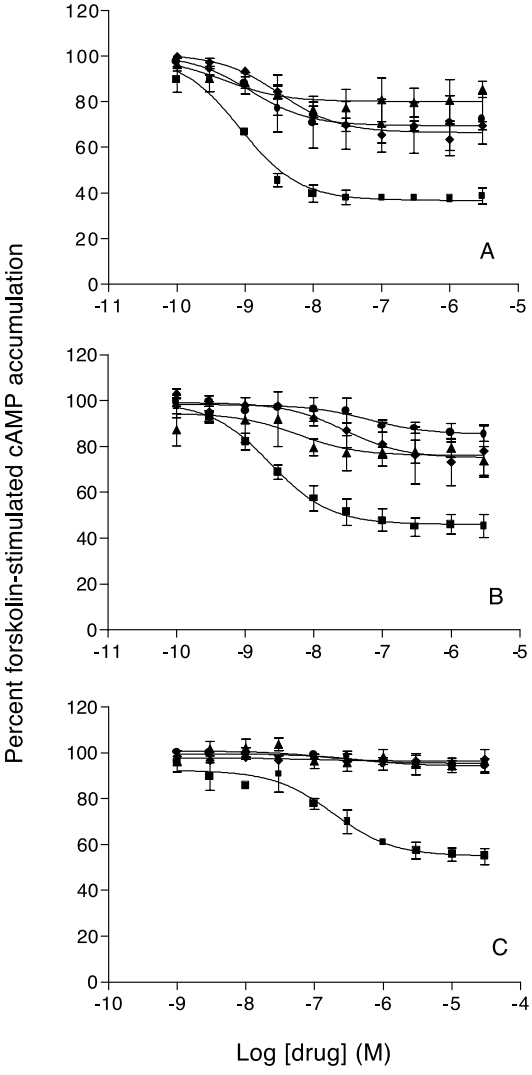

Fig. 4. Comparison of the wild-type and mutant CB2 receptors for agonist-induced inhibition of forskolin-stimulated cAMP accumulation. Three cannabinoid ligands, HU210 (A), WIN55212-2 (B), and anandamide (C), were used for cAMP accumulation assay using HEK293 cells stably expressing wild-type (■), Y299A (▲), C313A (●) and C320A (♦) mutant CB2 receptors. The forskolin-stimulated cAMP accumulation in the absence of cannabinoid ligands (6.83 ± 0.24 pmol/10^5 cells) was defined as 100%. Data shown represent the mean ± S.E.M. of at least three independent experiments performed in triplicate. Curves were generated as described in Section 2.

3.3. Coupling to adenylate cyclase

To compare the signaling properties of C313A and C320A mutant receptors with that of wild-type CB2 receptor, the ability of cannabinoid agonists to inhibit forskolin-stimulated cAMP accumulation was determined in HEK293 cells stably transfected with these receptors. As shown in Fig. 4, in cells expressing wild-type CB2 receptor, three cannabinoid agonists inhibited forskolin-stimulated cAMP accumulation in a con-

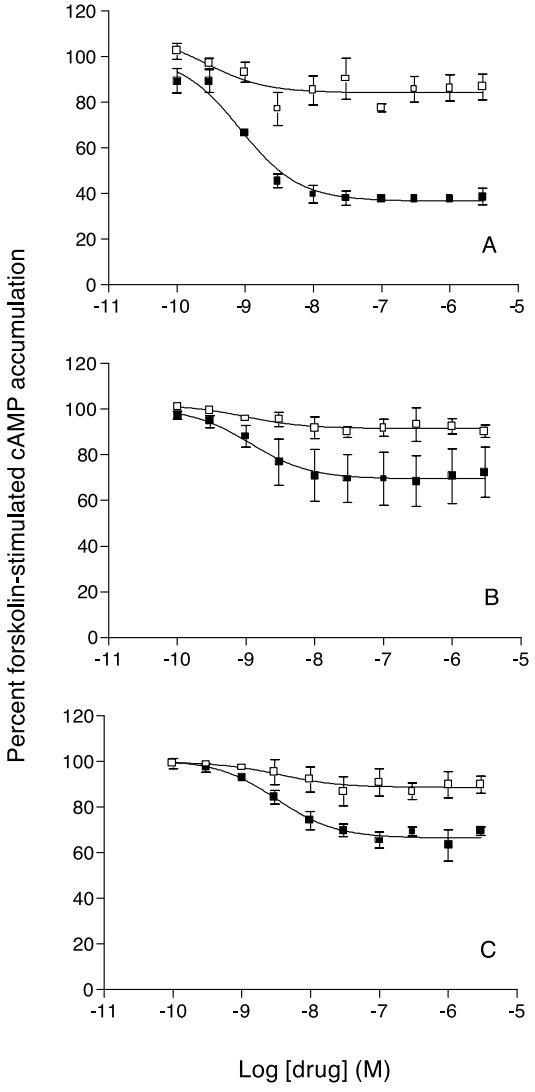

Fig. 5. Agonist-induced desensitization of the wild-type (A), C313A (B), and C320A (C) mutant CB2 receptors. Cells were pretreated with 3 nM HU-210 (□) or vehicle (■) for 1 h. The forskolin-stimulated cAMP accumulation in the absence of cannabinoid ligands was defined as 100%. Data shown represent the mean ± S.E.M. of at least three independent experiments performed in triplicate. Curves were generated as described in Section 2.

centration-dependent manner. The rank order of potency was HU-210 > WIN55212-2 > anandamide. The maximum inhibition of forskolin-stimulated cAMP accumulation was 63.3 ± 2.1% by HU-210, 54.1 ± 4.4% by WIN55212-2, and 30.2 ± 4.8% by anandamide. At the highest concentrations used on transfected cells, none of these cannabinoid agonists inhibited cAMP accumulation in untransfected cells (data not shown). Thus, all of the inhibition of cAMP accumulation shown is receptor-mediated.

In HEK293 cells stably transfected with Y299A, C313A, and C320A mutant receptors, the ability of HU-210 and WIN55212-2 to inhibit forskolin-stimulated cAMP accumulation was severely impaired, and the effect of anandamide was completely abolished (Fig. 4).

3.4. Receptor desensitization

To study agonist-induced desensitization of adenylate cyclase responses, HEK293 cells expressing wild-type and C313A and C320A mutant CB2 receptors were pretreated with 3 nM HU-210 for 1 h before the cAMP accumulation assay. As shown in Fig. 5, for wild-type CB2 receptor, HU-210 pretreatment markedly decreased the ability of HU-210 to inhibit forskolin-stimulated cAMP accumulation, with a 76% (from 63.3 ± 2.1% to 15.2 ± 5.0%) decrease of maximum inhibition by HU-210. For C313A and C320A mutants, the maximum inhibition by HU-210 was decreased by 72% (from 30.7 ± 11.5% to 8.5 ± 4.1%) and 76% (from 48.7 ± 15.0% to 11.5 ± 4.8%), respectively, due to HU-210 pretreatment. Therefore, in cells expressing C313A and C320A mutant receptors, the maximum inhibition by HU-210 was decreased by a similar extent as the wild-type CB2 receptor due to HU-210 pretreatment-induced receptor desensitization.

4. Discussion

4.1. Roles of Y299 in ligand binding and coupling to adenylate cyclase

In this study we hypothesized that conserved Y299 in the TM7 NPVIY motif of the CB2 receptor might be important for ligand binding and functional coupling. To test our hypothesis, the Y299A mutation was made and examined. This mutation produced a receptor that was correctly targeted into the plasma membrane. However, this mutation led to a complete loss of ligand binding and a severe impairment of functional coupling to adenylate cyclase. These data indicate a critical role of Y299 in ligand binding and functional coupling of the CB2 receptor. Since the location of Y299 is very close to the cytoplasmic face of the plasma membrane, this amino acid is unlikely to be involved directly in ligand binding. Therefore, the loss of ligand binding caused by Y299A mutation is most likely due to indirect, conformational changes. Our results on Y299A mutation are in agreement with those of the studies on β_2-adrenoceptor [13] and human somatostatin receptor type 5 [15]. These studies also demonstrated a severe loss of ligand binding when the tyrosine residue in the $NP(X)_nY$ motif was mutated to alanine. However, our data are different from the studies with human angiotensin II type 1 receptor [12] and α_{1B}-adrenergic receptor [14]. In these studies, the tyrosine residue in the $NP(X)_nY$ motif has been shown to be important for functional coupling, but not for ligand binding. Taken together, this suggests that the roles of tyrosine in the $NP(X)_nY$ motif are receptor-specific.

4.2. Roles of C313 and C320 in ligand binding, coupling to adenylate cyclase, and receptor desensitization

Experiments were performed in this study to test our hypothesis that two cysteine residues in the C-terminal juxtamembrane region of the CB2 receptor may be important for its function. Our data showed that mutating C313 and C320 to alanine in the CB2 receptor gave correctly expressed receptor protein and had no effect on ligand binding. These results indicate that these two cysteines of the CB2 receptor are not crucial for receptor expression or ligand binding. However, in HEK293 cells expressing C313A and C320A mutant receptors, agonist-induced inhibition of forskolin-stimulated cAMP accumulation was severely impaired. These data demonstrate that these two cysteine residues in the C-terminal juxtamembrane region of the CB2 receptor have critical roles in negatively coupling to adenylate cyclase. In addition, our

desensitization experiments showed that the C313A and C320A mutations had no effects on agonist-induced receptor desensitization.

Our observation on the ligand binding properties of C313A and C320A mutant receptors is in agreement with those of several other GPCRs, which also indicated that the conserved cysteines in the C-terminal juxtamembrane region are not crucial for ligand binding [15,18]. Regarding the roles of these cysteines in functional coupling, our data are consistent with those obtained with human β_2-adrenergic receptor [16], which also showed a critical role of these cysteines for functional coupling. However, our results differ from several other GPCRs, such as, human somatostatin receptor type 5 [15] and α_{2A}-adrenergic receptor [17], in which the conserved cysteines are not crucial for functional coupling. Furthermore, we demonstrated that the C313 and C320 are not essential for agonist-induced receptor desensitization in the CB2 receptor. This finding is in contrast to the previous reports on human somatostatin receptor type 5 [15] and glucose-dependent insulinotropic peptide receptor [18], which have shown that the conserved cysteine residues are crucial for receptor desensitization. It remains to be studied why the conserved C-terminal juxtamembrane region cysteines play different roles among different GPCRs.

In summary, we have shown that the Y299 in the TM7 NPVIY motif of the CB2 receptor is critical for ligand binding and functionally coupling to adenylate cyclase. We have also demonstrated that C313 and C320 at the C-terminal juxtamembrane region of the CB2 receptor is critical for coupling to adenylate cyclase, but not for ligand binding and receptor desensitization.

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