Absence of a conserved proline and presence of a conserved tyrosine in the CB2 cannabinoid receptor are crucial for its function

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Abstract A majority (84%) of G protein-coupled receptors have a proline (P5.50) in the middle of the fifth transmembrane domain. However, one of the unique structural features of cannabinoid receptors is the replacement of the conserved P5.50 by a leucine (L5.50). It has been shown that a conserved tyrosine (Y5.58), located at the cytoplasmic side of P5.50, is crucial for the signal transduction of several G protein-coupled receptors. We proposed that the replacement of P5.50 by L5.50 and the presence of the conserved Y5.58 in this context are important for the function of CB2. Mutating L5.50 to a proline abolished ligand binding, whereas mutating Y5.58 to an alanine resulted in a rightward shift of the competition binding curves. Both of these mutations led to a complete loss of the ability of cannabinoid agonists to inhibit forskolin-stimulated cAMP accumulation.
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Key words: Marijuana; Cannabinoid; G protein-coupled receptor; Mutagenesis; Transmembrane domain

1. Introduction

Marijuana (cannabis) is one of the oldest and most widely used drugs in the world. It has prominent effects on the central nervous system as well as peripheral systems [1]. In 1964, Δ^9-THC was recognized as the major psychoactive component of marijuana [2]. Based on their chemical structures, cannabinoid agonists can be classified into four main classes. They are classical cannabinoids [3], bicyclic cannabinoids [4], aminoalkylindoles [5] and fatty acid amines and esters [6,7].

To date, two types of cannabinoid receptors, CB1 [8] and CB2 [9], have been cloned. CB2 exhibits 44% amino acid identity with CB1 [9]. CB1 is located in the central nervous system as well as peripheral tissues [8,10,11]. CB2 is distributed primarily in peripheral tissues such as immune cells [9,11]. Many studies have demonstrated the modulatory effects of cannabinoids on the functions of immune system. It has been suggested that specific cannabinoid receptors may mediate the immunomodulatory effects of cannabinoids

[11,12]. It is known that activation of both CB1 and CB2 cannabinoid receptors inhibits adenylate cyclase through coupling with pertussis toxin sensitive G proteins [13,14].

Cannabinoid receptors belong to the rhodopsin family of G protein-coupled receptors [15–17]. These receptors contain seven putative transmembrane domains connected by three extracellular and three intracellular loops. The rhodopsin family of G protein-coupled receptors shares several highly conserved amino acid motifs in their transmembrane domains. Cannabinoid receptors have most, but not all of these highly conserved amino acid motifs.

One of the highly conserved features of G protein-coupled receptors is the presence of several highly conserved prolines in their transmembrane domains. The presence of proline in transmembrane helices plays potential structural and dynamic roles for transmembrane domain functions [18–20]. However, among the seven transmembrane domains of G protein-coupled receptors, proline is conserved only in selected transmembrane domains. Thus, it is possible that both proline-containing and proline-lacking transmembrane domains are important for the functions of G protein-coupled receptors. A proline residue (P5.50) is located in the middle of fifth transmembrane domains of majority (84%) of G protein-coupled receptors [17]. However, in cannabinoid receptors this proline is replaced by a leucine (Fig. 1). We hypothesized that this unique structural feature of cannabinoid receptors might be important for their functions. To test this hypothesis in CB2, we mutated this unique leucine to a proline, as highly conserved in most G protein-coupled receptors.

Previously, it has been demonstrated that a conserved tyrosine (Y5.58), located at the cytoplasmic side of the conserved proline (P5.50) in the fifth transmembrane domain, plays an important role in the signal transduction of several G protein-coupled receptors [21,22]. As mentioned above, one of the unique structural features of cannabinoid receptors is the absence of proline (P5.50) in the fifth transmembrane domain. However, the highly conserved tyrosine (Y5.58) does exist in the cannabinoid receptors (Fig. 1). It is unknown, whether in the absence of P5.50, this tyrosine still has a critical role in the signal transduction of CB2, as reported for other G protein-coupled receptors. To answer this question, we converted this tyrosine (Y5.58) in CB2 to an alanine.

2. Materials and methods

2.1. Materials
Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin/streptomycin, L-glutamine, trypsin and geneticin were obtained from

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Biowhittaker (Walkersville, MD, USA). Enzymes and reagents used for recombinant DNA experiments were purchased from Gibco-BRL (Gaithersburg, MD, USA), or Promega (Madison, WI, USA). Adenovirus-transformed 293 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Glass tubes used for diluting cannabinoid drugs and for ligand binding assays were silanized through exposure to dichlorodimethylsilane (Sigma Chemical Co., St. Louis, MO, USA) vapor while under vacuum for 3 h. Anandamide and WIN55212-2 were obtained from RBI (Batick, MA, USA). HU-210 was obtained from Tocris (Ballwin, MO, USA). [³H]WIN55212-2 and [¹²⁵I]cAMP were purchased from New England Nuclear (Boston, MA, USA).

2.2. Amino acid numbering system

The amino acid numbering system suggested by Ballesteros and Weinstein [23] was used. Each amino acid identifier starts with the transmembrane helix number, followed by the amino acid position relative to a reference amino acid in that helix. This reference amino acid is the most highly conserved residue across G protein-coupled receptors in that helix and is assigned a locant value of 0.50. This numbering system for the cannabinoid receptor has been previously described [24].

2.3. 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 [25]. The GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI, USA) was used to mutate the CB2 receptor. For CB2L5.50P, the mutagenic oligonucleotide 5′-TCGCCTTCCCCTTTTCCGG-3′ was used. For CB2Y5.58A, the mutagenic oligonucleotide 5′-ATCATCTACACCGCTGGGCATGT-TCTC-3′ was used. The presence of the mutation and the accuracy of the DNA sequences were confirmed by dideoxy sequencing.

2.4. Cell transfection and culture

Expression plasmids containing wild-type and mutant cannabinoid receptors transfected into human embryonic kidney 293 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 [26]. Cells were grown as monolayers in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere consisting of 5% CO_2 and 95% air, at 37°C.

2.5. Ligand binding

For binding assays, cannabinoid ligand dilutions were made in binding buffer containing 25 mg/ml bovine serum albumin and then added to assay tubes. [³H]WIN55212-2 (2 nM) was used as a labeled ligand for competition binding assays. Non-specific binding was determined in the presence of 1 μM unlabeled WIN55212-2. Binding assays were performed in 0.5 ml of binding buffer containing 5 mg/ml bovine serum albumin for 60 min at 30°C. Free and bound radioligands were separated by rapid filtration through polyethylenimine-treated GF/B filters (Whatman International, Maidstone, UK) that had been soaked in cold wash buffer (50 mM Tris–HCl, pH 7.4, and 1 mg/ml of bovine serum albumin). The filters were washed three times with 3 ml cold wash buffer. The bound [³H]WIN55212-2 was determined by liquid scintillation counting after overnight equilibration of the filter in 5 ml of scintillation cocktail (Hydrofluor, National Diagnostics, Manville, NJ, USA).

2.6. cAMP accumulation

To prevent the hydrolysis of cAMP, cells were incubated with 0.2 mM of Ro 20-1724, a phosphodiesterase inhibitor. Aliquot of cells (200 μl) were added to tubes containing forskolin and ±cannabinoids in a total volume of 250 μl and incubated for 10 min at 37°C. The reaction was terminated by the addition of 250 μl of 0.1 N HCl. 50 μl of the reaction solution was assayed for cAMP. Measurement of cAMP was performed by radioimmunoassay using [¹²⁵I]cAMP (Du-Pont-NEN, Wilmington, DE, USA). The results are expressed as percent inhibition of forskolin-stimulated cAMP accumulation.

2.7. Data analysis

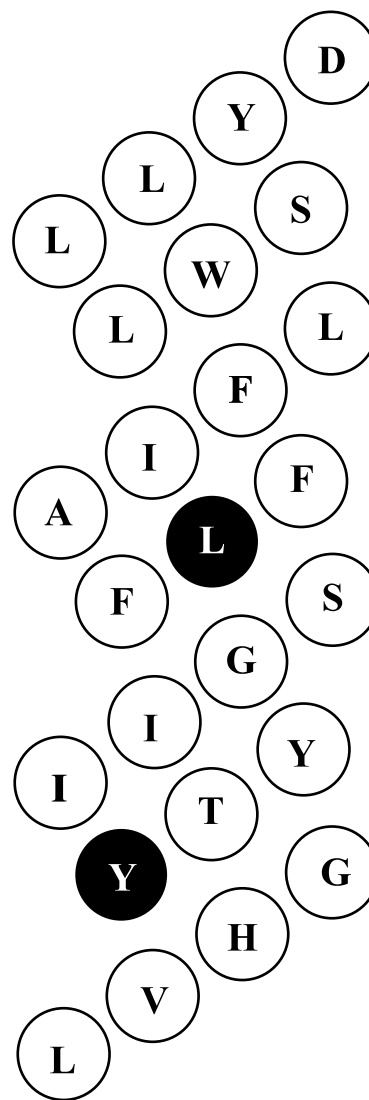Data from ligand binding and cAMP accumulation assays were

Fig. 1. Schematic view of the putative fifth transmembrane domain of the human CB2 cannabinoid receptor showing the amino acid sequence and positions of L5.50 and Y5.58. Letters in circles indicate the amino acid types. Two black circles indicate the positions where the mutations were made in this study.

analyzed, and curves were generated with use of the Prism program (GraphPad Software, San Diego, CA, USA). IC$_{50}$ and EC$_{50}$ values were determined through non-linear regression analysis performed with the Prism 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 [27]. The K_i values were calculated based on the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + L/K_d)$ [28].

3. Results

3.1. Comparison between wild-type and mutant CB2 receptors for ligand binding

Radioligand binding assays were performed to compare wild-type and mutant CB2 receptors. Specific high-affinity binding of cannabinoid ligand [³H]WIN55212-2 was observed with membranes prepared from 293 cells stably expressing wild-type CB2 receptor (Fig. 2). The K_d and B_{max} were determined to be 4.8 ± 0.4 nM and 2210.0 ± 373.5 fmol/mg protein, respec-
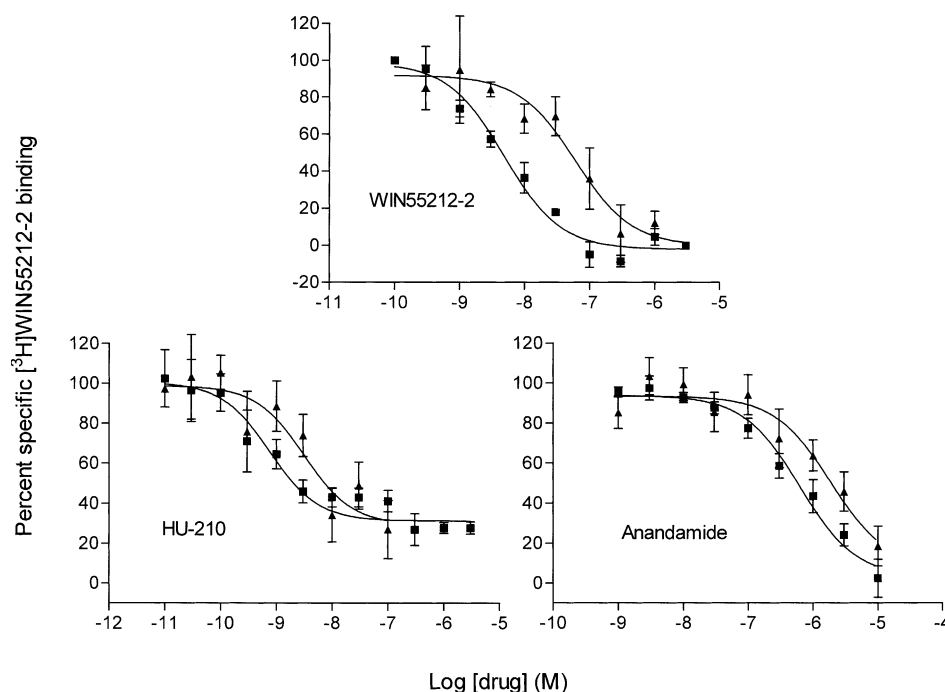
Fig. 2. Comparison between wild-type CB2 and CB2Y5.58A receptors for ligand binding. Competition binding assays were performed on membranes prepared from 293 cells stably expressing wild-type CB2 or CB2Y5.58A mutant receptor. [^{3}H]WIN55212-2 was used as a labeled ligand for competition binding assays. Membranes containing wild-type CB2 and CB2Y5.58A mutant receptors were incubated with various concentrations of ligands for 60 min at 30°C. Points, mean ± S.E.M. of three independent experiments performed in duplicate. Curves were generated with use of the Prism program. Squares represent wild-type CB2 and triangles represent CB2Y5.58A receptors.

tively. The CB2L5.50P mutation completely abolished ligand binding (data not shown). In contrast, specific high-affinity binding of [^{3}H]WIN55212-2 was observed with CB2Y5.58A mutant receptor (Fig. 2). The K_d and B_{max} values were determined to be 37.2 ± 7.9 nM and 2256.0 ± 505.7 fmol/mg protein, respectively. HU-210 and anandamide, two cannabinoid ligands with distinct chemical structures, were tested for their ability to compete for specific [^{3}H]WIN55212-2 binding (Fig. 2). The CB2Y5.58A mutation resulted in a rightward shift of the competition curves, as compared with the wild-type CB2. For HU-210, the K_i values for wild-type CB2 and CB2Y5.58A were 0.62 ± 0.14 and 3.0 ± 1.1 nM, respectively. For anandamide, the K_i values for wild-type CB2 and CB2Y5.58A were 687.5 ± 176.3 and 2936.0 ± 137.5 nM, respectively.

3.2. Comparison between wild-type and mutant CB2 receptors for inhibition of forskolin-stimulated cAMP accumulation

cAMP accumulation assays were conducted to compare wild-type and mutant CB2 receptors (Figs. 3 and 4). In 293 cells stably expressing wild-type CB2, three cannabinoid ligands inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent manner. The EC_{50} values for WIN55212-2, HU-210, and anandamide were 0.71 ± 0.14, 0.28 ± 0.07, and 93.3 ± 25.8 nM, respectively. In contrast, in 293 cells stably transfected with CB2L.50P and CB2Y5.58A receptors, these three cannabinoid agonists completely lost their ability to inhibit forskolin-stimulated cAMP accumulation.

4. Discussion

About 84% of G protein-coupled receptors have a con-

served proline (P5.50) at the fifth transmembrane domain [17]. However, in cannabinoid receptors this conserved proline is missing, instead, a leucine is located at the corresponding position [24]. In this study, the CB2L5.50P mutation completely eliminated ligand binding and agonist-induced inhibition of cAMP accumulation, indicating that the replacement of conserved proline by leucine at position 5.50 of CB2 is critical for the functions of this receptor.

Presently, it is unknown why the CB2L5.50P mutation caused a complete loss of function of the CB2 receptor. Proline is considered the classical helix-breaker [18–20], thus the introduction of CB2L5.50P mutation into the fifth transmembrane domain of CB2 would create a kink in the helical backbone. Most G protein-coupled receptors have a highly conserved proline at the fourth, fifth, sixth, and seventh transmembrane domains. On the other hand, there is no conserved proline in the first, second, and third transmembrane domains. It is likely that the presence of both 'angled', proline-containing, and 'straight', proline-lacking helices is important for the relative movements of helical bundles that are necessary for receptor activation. Cannabinoid receptors have the conserved proline at the fourth, sixth, and seventh transmembrane domains, but the conserved proline at the fifth transmembrane domain is missing. The current study on CB2 indicates that the 'straight', proline-lacking fifth transmembrane domain is crucial for the function of this receptor.

In the current study, Y5.58 was selected for mutational analysis because it is highly conserved in most G protein-coupled receptors, it is very close to the third intracellular loop, and it has been shown to be a critical amino acid for receptor activation in other G protein-coupled receptors. We wanted to test whether in the absence of P5.50, this tyrosine
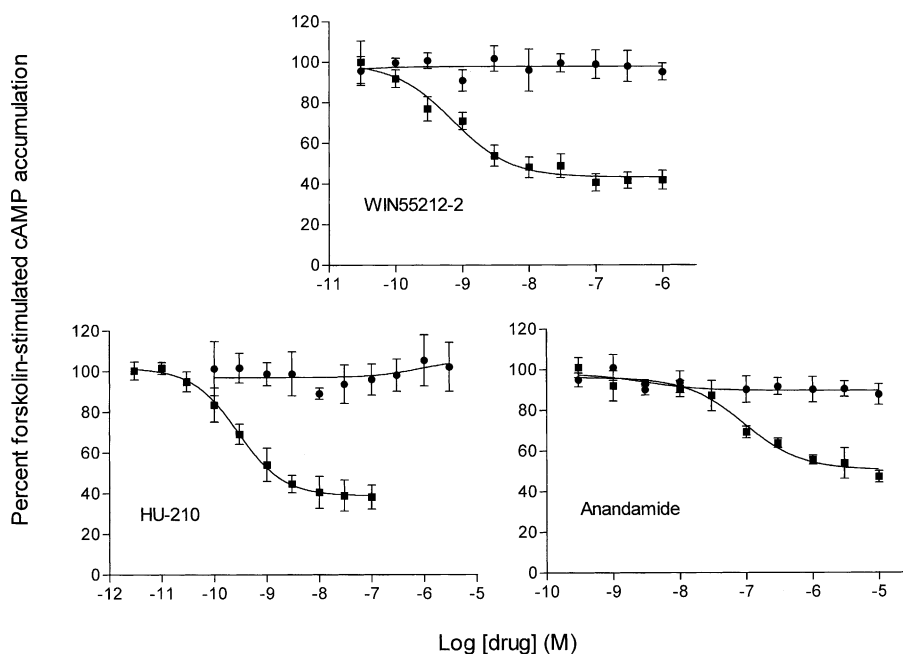
Fig. 3. Comparison between wild-type CB2 and CB2L5.50P receptors for agonist-induced inhibition of cAMP accumulation. cAMP accumulation assays were performed on 293 cells stably expressing wild-type CB2 or CB2L5.50P mutant receptor. Measurement of cAMP was carried out by radioimmunoassay using [^{125}I]cAMP. Cells expressing wild-type CB2 and CB2L5.50P mutant receptors were incubated with various concentrations of ligands for 10 min at 37°C. The results are expressed as percent forskolin-stimulated cAMP accumulation. Points, mean ± S.E.M. of three independent experiments performed in triplicate. Curves were generated with use of the Prism program. Squares represent wild-type CB2 and circles represent CB2L5.50P receptors.
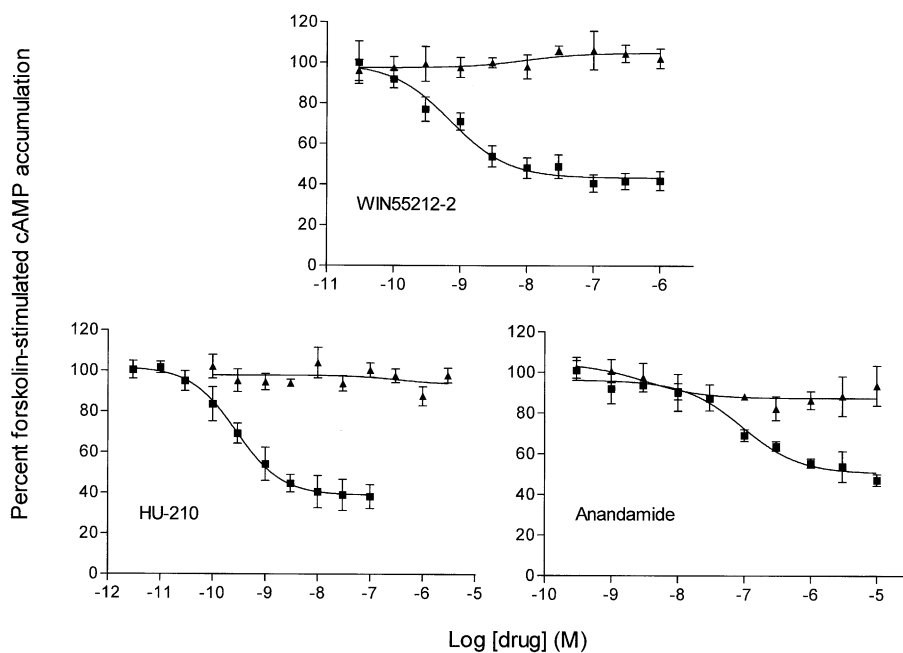

Fig. 4. Comparison between wild-type CB2 and the CB2Y5.58A receptors for agonist-induced inhibition of cAMP accumulation. cAMP accumulation assays were performed on 293 cells stably expressing wild-type CB2 or CB2Y5.58A mutant receptor. Measurement of cAMP was carried out by radioimmunoassay using [^{125}I]cAMP. Cells expressing wild-type CB2 and CB2Y5.58A mutant receptors were incubated with various concentrations of ligands for 10 min at 37°C. The results are expressed as percent forskolin-stimulated cAMP accumulation. Points, mean ± S.E.M. of three independent experiments performed in triplicate. Curves were generated with use of the Prism program. Squares represent wild-type CB2 and triangles represent CB2Y5.58A receptors.

was still crucial for the activation of CB2. The CB2Y5.58A mutation caused a reduction in agonist binding affinities in the current study. Since Y5.58 is located at the bottom of the fifth transmembrane domain, the decrease in agonist binding affinities in the CB2Y5.58A mutant receptor is most likely due to indirect, conformational changes on the ligand-binding pocket, rather than direct effects on the ligand binding sites.

CB2Y5.58A mutation completely abolished cannabinoid-induced inhibition of cAMP accumulation in cells stably expressing CB2. These data are consistent with previous reports on the critical roles of this tyrosine in the activation of angiotensin II and neurokinin-1 receptors [21,22]. Currently, the exact roles for this highly conserved tyrosine in the activation of G protein-coupled receptors are not clear. A recent study by Howlett et al. [29] suggested that N-terminal portion of the third cytoplasmic loop of CB1 are very important for interacting with G proteins. Y5.58 is located at the bottom of the fifth transmembrane domain, a position adjacent to the N-terminal of the third cytoplasmic loop. Modeling studies have suggested that this amino acid is in molecular proximity to regions that are important in receptor activation, including the DRY motif located at the end of the third transmembrane helix [22]. Therefore, this conserved tyrosine may play an important role in propagation of the signal from the ligand binding domain to the third cytoplasmic loop of these receptors. Replacement of the tyrosine with an alanine may impair the proper propagation of the agonist-induced conformational changes of the receptor.

In summary, the present study provides experimental evidences to support the conclusion that a 'straight', proline-lacking fifth transmembrane, and a highly conserved tyrosine residue located at the bottom of the fifth transmembrane domain, play critical roles in the functions of CB2.

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