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Functional Role of Serine Residues of Transmembrane Dopamin VII in Signal Transduction of CB2 Cannabinoid Receptor

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Abstract

Using site-directed mutagenesis technique, I have replaced serine 285 and serine 292 with the alanine, and assessed the binding of agonist and signaling such as the inhibition of adenylyl cyclase activity.

I have found that serine 292 has an important role in the signal transduction of cannabinoid agonists, HU-210 and CP55940, but not in that of amino-alkylindoles derivatives WIN55,212-2. All mutants express well in protein level determined by western blot using monoclonal antibody HA 11 as compared with the wild type receptor.

Interestingly, binding affinity of S285A and S292A mutants with classical cannabinoid agonist HU-243 was somewhat decreased. In signaling assay, the inhibition of adenylyl cyclase by HU-210, CP55940 and WIN55,212-2 is the same order in both wild type receptor and S285A mutant receptor. However, S292A have been shown that the inhibition curves of adenylyl cyclase activity moved to the right by HU-210 and CP55940, but those of adenylyl cyclase activity did not by aminoalkylindole WIN55,212-2, which is indicating that this residue is closely related to the binding site with HU-210 and CP55940. In addition, serine 292 might take more important role in CB2 receptor and G-protein signaling than serine 285.

Key Words : Cannabinoids, CB2, Serine, G protein, Adenylyl cyclase, Site-directed mutagenesis

Introduction

Two subtypes of cannabinoid (CB) receptors have been cloned so far, CB1 and CB2 (Matsuda et al., 1990; Munro et al., 1993). Both CB1 and CB2 are members of the seven transmembrane (TM) domain G protein-coupled receptor (GPCR) superfamily. The identity of amino acid sequences to CB1 and CB2 receptors is relatively low (44%): when compared in TM domain, it is increased by 63%. The CB2 receptor has been found to be expressed in immune cells, such as splenic macrophages, monocytes, B-cells, and natural killer cells, as well as in tonsil and bone marrow but not in brain (Munro et al., 1993; Galiègue et al., 1995). This distribution suggests that CB2 receptor has an role in the immune system and that CB2 recpetor is major target to develop drug, which devoid of psychoactive properties attributed to cannabinoids functioning via CB1 in the nervous system (Klein et al., 1998).

CB1 and CB2 cannabinoid receptors share a common characteristic in the signal transduction. For example, it has been reported that both types of cannabinoid receptors act via inhibitory G protein α subunit to inhibit certain types of adenylyl cyclase (AC) (Howlett et al., 2002; Rhee et al., 1998; Vogel et al., 1993) and activate the p42-44 mitogen-activated protein kinase activity (Bouaboula, 1996). In addition, most of cannabinoid agonist bind to CB1 and CB2 receptor with similarly affinity. However, there are some differences between them: $\triangle^9$-THC is known to be the CB1 agonist but it act as a neutral antagonist of CB2 receptor (Bayewitch et al., 1996). Aminoalkylindole derivative WIN55,212-2 is known to bind to CB2 more efficiently than to CB1 (Bouaboula et al., 1996), as is the same in signaling of AC inhibition (Rhee et al., 1998).

Site-directed mutagenesis of cloned cDNAs provides a good means of examining the specific functions of the proteins they encode (Savarese and Fraser, 1992; Baldwin JM., 1994). The selected-site for study have included residues that are highly conserved in GPCR superfamily or subset of receptors (Savarese and Fraser, 1992; Wess et al., 1993 Baldwin, 1994). On the other hand, relatively little is known about the structure of the CB2 cannabinoid receptor and the molecular interactions involved in the binding of ligand and signal transduction. There has recently been reported which amino acid take a role in the ligand binding and signaling in CB2 cannabinid receptor (Feng and Song, 2001; Rhee et al., 2000; Song et al., 1999; Tao et al., 1998). Rationale has been accepted that the binding site of classical cannabinoid and aminoalkylindole derivative is different. In addition, amino

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acids, known as having role in the signal transduction of CB2, is mainly located in the middle or extracellular TM helix. I have recently (Rhee et al., 2000; in preparation, 2002) reported that tryptophan in the TM segments of CB2 receptor take an important role in signaling as well as in the binding of ligand, suggesting that hydrophobic interaction or aromatic-aromatic stacking interaction between ligand and receptor exist. In addition, it is implied that binding pocket for cannabinoids in CB2 receptor might be consisted of several TM segments.

I hypothesized that ser-292 and ser-285 in the 7th TM segment could interact with hydroxyl group of cannabinoids (e.g., HU-210 and CP55940) but not with WIN55,212-2, devoid of hydroxyl group. In addition, ser-292 is highly conserved in almost all rhodopsin-like GPCR superfamily (attword et al., 1991; Probst et al., 1992). To test this hypothesis, I investigated the functional interaction between the CB2 receptor and various agoist after the mutation of Ser-292 to Ala and of Ser-285 to Ala. I show here that Ser-292 take an important role in the binding of cannabinoids (e.g., HU-210 and CP55940) and the resulting activation of CB2 receptor, but not in aminoalkylindole derivative (e.g., WIN55,212-2).

Materials and Methods

Materials

[^{3}H-2]adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Phosphodiesterase inhibitors, 1-methyl-3-isobutylxanthine (IBMX) and RO-20-1724, were from Calbiochem (La Jolla, CA). Forskolin (FS), cAMP, and fatty acid-free bovine serum albumin (FAF-BSA) were from Sigma (St. Louis, MO). The cannabinoid agonists, HU243, HU-210, CP55940 and WIN55,212-2, were kindly obtained from Dr. R. Mechoulam (Jerusalem, Israel). Tissue culture reagents were from Life Technologies (Gaithersburg, MD).

Plasmids

β-gal cDNA in pXMD1 vector, as well as the AC-V plasmid, were described previously (Rhee et al., 1998).

Construction and HA-tagging into human CB2 plasmids

The plasmid MC36F1, containing the human peripheral cannabinoid receptor cDNA, was cloned into the COS cell expression vector CDM8. The following oligonucleotide primers (P1 and P2) were synthesized and used to amplify a 1100 bp fragment containing the cannabinoid coding sequence:

P1: 5′-GCGGATCCGAGGAATGCTGGGTG-3′ sense primer

P2: 5′-GCGCGGCCGCTCAGCAATCAGAGAG-3′ antisense primer

P1 is homologous to the cDNA sequence at the CB2 start site and was engineered to contain a unique BamH I site (underlined) for subcloning into pcDNA 3 with HA following Bgl II digestion. The P2 sequence was designed to allow for the amplification of a unique Not I site (underlined) for ligation into the multiple cloning site of pcDNA 3 following Not I digestion. The PCR reaction was carried out using a Mastercycler 5330 Plus (Eppendorf) that was programmed for 25 cycles in the following manner: 1-min denaturation at 92℃, 1-min annealing step at 45℃, and 1-min extension at 72℃. The cloning vector pcDNA 3 was digested with Bgl II and Not I, and the PCR product, 1100 bp of CB2 cannabinoid receptor, was digested at the unique enzyme sites of BamH I and Not I. These digested vectors and PCR product were electrophoresed with a DNA mini gel, cleaned, extracted with phenol/chloroform, and ligated. The sequence of CB2 cannabinoid receptor was confirmed by sequencing.

Preparation of point mutations in CB2

Mutations in CB2 were prepared using the PCR-overlap extension method as previously described (Ho et al., 1989; Rhee et al., 2000). In brief, two general primers were designed for PCR that cover the region in CB2 where the mutations were planned. The 5′ general primer 5′-TAATACGACTCACTATAGGG-3′ and the 3′ general primer 5′-TTGACCTGGTCACTGAGCGTAGT-3′ were used in conjunction with internal sense and matching anti-sense primers that contained the desired mutation. Three PCR reactions were run, the first two providing the 5′ and 3′ ends of the mutagenized fragment, and the third consecutive reaction joining the separate fragments to provide a clonable DNA product to place back into CB2. Wild type CB2 and the PCR products were cut with the restriction enzymes BamH I and BstE II (unique sites in CB2 that surround the area of interest), and the mutagenized fragment was subsequently cloned into CB2. The sequence of the CB2 cannabinoid receptor was confirmed in the Sequencing Unit of the Weizmann Institute of Science.

Transient cell transfection.

Twenty-four hr before transfection, a confluent 10-cm plate of COS-7 cells in Dulbeccos modified Eagles medium (DMEM) supplemented with 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37℃, was trypsinized and split into five 10-cm plates. The cells were transfected, using the DEAE-dextran chloroquine method (Keown et al., 1990), with wild type human cannabinoid receptor cDNA (2 μg/plate) or mutant cDNAs (4 μg/plate), as well as either AC-V cDNAs (2 μg/plate) or pXMD1-gal (for mock DNA transfection), where indicated, for AC assay. Fourty-eight h later, the cells were trypsinized and re-cultured in 24-well plates, and after an additional 24 h, the cells were assayed for AC activity as described below. For binding assay after 72 h of transfection, COS cells were washed with PBS 2 times, scraped, centrifuged at 3,000 rpm for 10 min, and stored at -70°C before use. Transfection efficiencies were normally in the range of 40-80%, as determined by staining for β-galactosidase activity (Lim

and Chae, 1989).

AC activity

The assay was performed in triplicate as described previously (Salomon et al., 1991). In brief, cells cultured in 24 well plates were incubated for 2 hr with 0.25 ml/well fresh growth medium containing 5 μCi/ml [2-^{3}H]adenine. This medium was replaced with DMEM containing 20 mM HEPES (pH 7.4), 1 mg/ml FAF-BSA, and the phosphodiesterase inhibitors RO-20-1724 (0.5 mM) and IBMX (0.5 mM). Cannabinoids diluted in 10 mg/ml FAF-BSA were then added. AC activity was stimulated in the presence or absence of cannabinoids by the addition of FS. After 10 min at 37℃, the medium was removed and the reaction terminated by adding to the cell layer 1 ml of 2.5% perchloric acid containing 0.1 mM unlabeled cAMP. Aliquots of 0.9 ml of the acidic extract were neutralized with 100 μl of 3.8 M KOH and 0.16 M K_2CO_3 and applied to a two-step column separation procedure (Salomon, 1991). The [^{3}H]cAMP was eluted into scintillation vials and counted. Background levels (cAMP accumulation in the absence of stimulator) were subtracted from all values.

Competition binding assay with [^{3}H]HU-243

This assay was performed as described previously (Rhee et al., 1997). In brief, the assay is performed in 1.5 ml Eppendorf tubes in a final volume of 1 ml of 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 2.5 mM EDTA, pH 7.4, and 1 mg/ml FAF-BSA. The protein concentration of cell homogenate (determined by the Bradford method) was 10-20 μg per assay. The reaction was started by adding 300 pM of [^{3}H]HU 243 to each tube. The binding mixture was incubated at 30 ℃ for 90 min with gentle shaking and centrifuged at 14,000 rpm for 10 min. The bottoms of the 1.5 ml tubes were then cut, and counted for radioactivity. Non-specific binding determined in the presence of 1 μM HU-210 was subtracted.

SDS-PAGE and western immunoblotting

COS-7 cells transfected with human HA-tagged CB2 cDNA were harvested with cold PBS and spun down at 3000 rpm (at 4℃ for 5 min), and cell pellets were mixed with 100 μl of Laemmli sample buffer, sonicated, and frozen at -20℃ before use. Dithiothreitol (0.1 M final) was added and the samples incubated for 5 min at 100℃ prior to loading onto 1.5-mm thick 10% polyacrylamide gel. Following electrophoresis, proteins were transferred overnight at room temperature onto nitrocellulose membrane at 100 mA using a Bio-Rad Blot cell (Bio-Rad Laboratories). The blot was blocked in PBS containing 5% fat-free milk and 0.5% Tween-20 followed by 1.5 hr incubation with HA 11 monoclonal antibody diluted 1:1,000 in 5% fat-free milk and 0.5% Tween-20. Blots were washed three times with PBS containing 0.3% Tween-20 and secondary antibodies (horseradish peroxidase (HRP)-coupled rat anti-mouse; Jackson Immuno-research Laboratories, Inc.) diluted 1:20,000 in 5% fat-free milk plus 0.5% Tween-20, incubated with the blot for 1 hr and the blot extensively washed with PBS containing 0.3% Tween-20. Peroxidase activity was observed by the ECL chemiluminescence technique (Amersham).

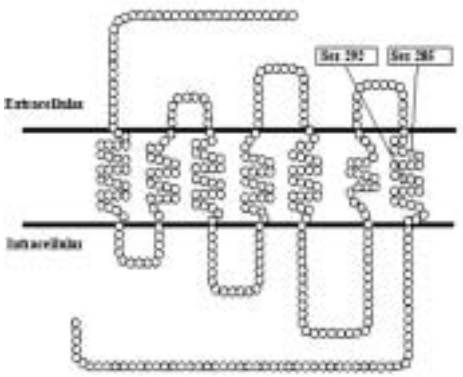

Fig. 1. Location of S285 and S292 in the CB2 cannbinoid receptor.

Results

To assess the role of serine residues in the binding of ligand and in signaling at the CB2 cannabinoid receptor, I replaced Ser-285 and Ser-292 with Ala.

Three representative cannabinoids were applied in the signaling assay: 1) classical cannabinoid, [HU-210, (-)-11-hydroxy-△8-tetrahydrocannabinol-dimethyl-heptyl], 2) nonclassical cannabinoid, [CP55940, (-)-3-[2-hydroxyl-4-(1,1-dimethylheptyl) phenyl] -4-[3-hydroxyl propyl] cyclohexan- 1-ol], 3) aminoalkylindole, [WIN55,212-2,(R)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl) methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl) methanone](Fig. 2).

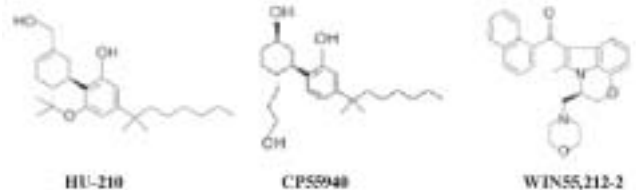

Fig. 2. Cannabinoid receptor agonists used in the study. (Structure of cannabinoid agonists, HU-210, CP55940 and WIN55,212-2)

Protein expression of wild type and mutant CB2 receptors

Fig. 3 depicts a Western blot using HA antibody (HA 11) in homogenates of whole cells transiently transfected with the cDNAs of wild type CB2 receptor, S285A and S292A mutants. The specific immunoreactive species had a relative molecular mass of 41 kDa, which is consistent with that predicted for the human CB2 receptor protein (Nowell et al.,

1998). The somewhat higher molecular weight of the second immunoreactive band (43 kDa) could represent a glycosylated form of the receptor. To get the similar level of protein expression, COS-7 cells were transfected with cDNAs of wild type CB2 receptor, 2 μg/plate and mutant CB2 receptor, 4 μg/plate. HA-tagged CB2 receptor did not affect either in the binding of agonist or in signaling such as inhibition of AC-V activity (data not shown, Rhee et al, 2000).

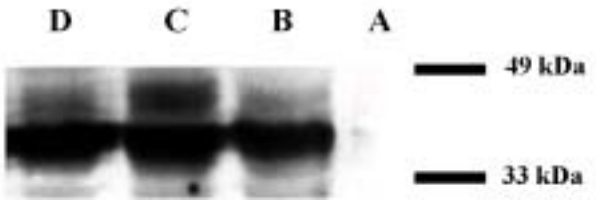

Fig. 3. Western blot analysis of receptor expressed in membranes from COS-7 cells transfected with wild-type or mutant human CB2 cDNA. Cells were transfected with 4 μg of cDNA for each construct, except that 2 μg of the wild type construct was used. Lane A shows the mock transfection (pcDNA 3), Lane B the positive control (HA-CB2), Lane C the S285A mutant, Lane D the S292A mutant. The amount of protein used was 10-20 μg, as determined by the Bradford method. Immunoreactive bands were detected by chemiluminescence (ECL, Amersham).

Role of S285 and S292 in CB2 binding

Using homologous competition binding of HU-243 to determine the binding properties, It is found (Fig. 4) that wild type CB2 receptor bind [^{3}H]HU-243 with 1.2 $\pm$ 0.4 pM of IC_{50}, S285A mutant binds [^{3}H]HU-243 with 15.3 $\pm$ 0.3 pM of IC_{50}, and S292A mutant binds [^{3}H]HU-243 with 4.5 $\pm$ 0.3 pM of IC_{50}. Interestingly, a relatively significant reduction in the binding affinity of S285A to HU-243 and a small reduction in binding affinity of S292A to HU-243 were observed, compared to the wild type receptor.

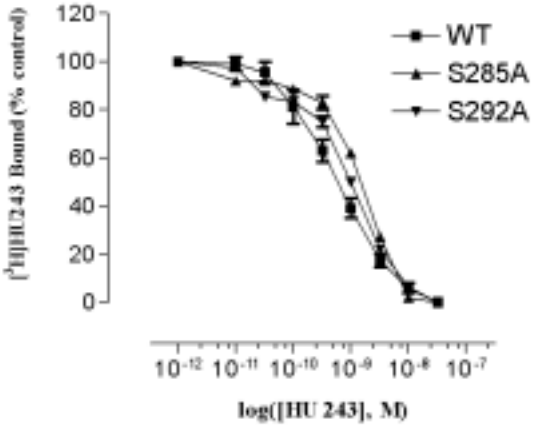

Fig. 4. Specific binding of mutants using [^{3}H]HU-243. COS cells were transfected with the cDNAs of wild type CB2 (2 μg/plate) and various mutants (4 μg/plate). The whole membrane homogenate was prepared after 72 h of transfection, and binding affinity was determined as described in Materials and Methods. The data represent the means $\pm$SEM of two experiments.

Role of S285 and S292 in CB2 signaling

I then analyzed the capacity of wild type CB2 receptor, and S285A and S292A mutants to inhibit AC-V activity. The COS cells were cotransfected with rabbit AC type V together with either wild type receptor or the mutated receptors, and the effect of increasing concentrations of HU-210 or WIN55,212-2 on forskolin-stimulated AC activity was determined. The results (Fig. 5) show that in wild type receptor HU-210 inhibit the activity of AC type V by EC_{50} of 0.86 $\pm$ 0.25 nM, confirming the functional expression of CB2 in COS cells. S285A mutant showed an similar AC inhibition pattern to that obtained with wild type receptor with the EC_{50} of 0.83 $\pm$ 0.12 nM. Interestingly, It has been shown that signaling by S292A was impaired, as the EC_{50} for AC inhibition by HU-210 was shifted to the right by 1 order of magnitude, compared to the wild type receptor. Similarly, CP55940 inhibit the AC activity in the same order between wild type receptor and S285A mutant receptor (EC_{50} of 1.2 $\pm$ 0,2 and 1.1 $\pm$ 0.1 nM, respcetively). However, in S292A mutants, CP55940 inhibit the activity of AC type V with less efficiency compared to the wild type receptor (EC_{50} of 3.9 $\pm$ 0.6). Surprisingly, WIN55,212-2, structurally distinct from classical and nonclassical cannabinoids (e.g., HU-210 and CP55940, see Fig. 2), inhibit the activity of AC type V with the same order of EC_{50} in wild type receptor, and S285A and S292A mutants (0.7 $\pm$ 0.2, 1.2 $\pm$ 0.4, and 0.8 $\pm$ 0.3 nM, respectively).

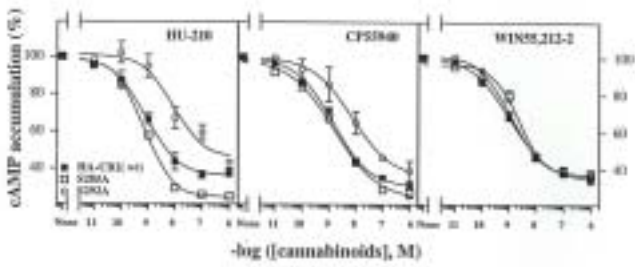

Fig. 5. AC inhibition in the S285A and the S292A mutants.
COS cells cotransfected with cDNAs of AC type V and with cDNAs of either wild-type CB2 (2 μg/plate), S285A, or S292A mutants (4 μg/plate) were stimulated with 1 μM FS in the presence of various concentrations of HU-210, CP55940, or WIN55,212-2. The data represent the means $\pm$SEM of two experiments.

Discussion

Strictly conserved residues located within the TM segments play an essential role in maintaining the structure

of the receptor, perhaps by determining protein folding, whereas those residues conserved only among major classes of receptors may play a role in defining their unique functional properties (Savarese and Fraser, 1992; Baldwin, 1994).

In this study, I have replaced Ser-285 (which is only conserved in CB1 and CB2 receptor) with Ala, and Ser-292 (which is highly conserved in GPCR superfamily) with Ala. Several laboratories have been shown that amino acids involved in ligand binding of CB2 receptor are located in TM II (Tao and Abood, 1998), TM III (Chin et al, 1999; Rhee et al, 2000; Tao et al, 1999), TM IV (Rhee et al, 2000), TM V (Rhee et al, in preparation), TM VI (Tao et al, 1999, Rhee et al, in preparation), and TM VII (Feng and Song; 2001). Based upon these results, we can speculate that the binding site of CB2 cannabinoid receptor is consisted of almost all TM segments.

We and others have already shown that, using site-directed mutagenesis technique, amino acid containing aromatic side chain in various TM domain have an important role in ligand binding in CB2 cannabinoid (Rhee et al., 2000; Song et al., 1999), In addition, Reggio et al (1998) have suggested that the cannabinoid agonist WIN55, 212-2 interacts with both CB1 and CB2 with aromatic stacking. Moreover, Song et al (1999) have suggested that two regions of aromatic stacking interaction between receptor and cannabinoid ligands exist; one region is composed of aromatic amino acid in the second TM segment, and a second region is in the third, fourth, and fifth TM region. Moreover, they have shown that phenylalanine in the fifth TM region is important for selectivity of aminoalkylindole derivatives WIN55,212-2 for CB2 receptor, thereby suggesting for aromatic interaction or agonist docking site (Song et al., 1999). Tao et al (1999) have reported that the double mutant, K109AS112G in the region of TM III, retains the ability to bind WIN55,212-2 but loses affinity for classical cannabinoids, such as $\triangle^9$-THC and CP55940.

On the aspect of cannabinoid structure, it has been reported (Reggio et al, 1990) that, in elegant structure-activity relationship study, phenolic hydroxyl group is essential for the pharmacological activities of the classical cannabinoid ligand, possibly because this hydrogen can participate in a hydrogen bonding interaction with cannabinoid receptor. Therefore, it is inferred that Ser-285 and Ser-292 take a part in the formation of binding pocket in the CB2 cannabinoid receptor. Furthermore. the binding pocket of CB2 cannabinoid receptor could be composed of hydrogen bonding interaction and aromatic stacking interaction. Although IC_{50} of S285A was somewhat higher than that of S292A in ligand binding assay, the capability of AC inhibition by HU-210 and CP55940 was reversed in those mutants. In this regard, it is plausible that Ser-292 is more involved in the conformational change of receptor after agonist binding. Upon agonist binding, conformational change of CB2 cannabinoid receptor occur, thereby dissociating $G_{\alpha i}$ subunit from $G_{\beta\gamma}$ dimers. Resulting $G_{\alpha i}$ subunit and $G_{\beta\gamma}$ dimers signal into the downstream effectors (e.g., the inhibition of AC). Interestingly, WIN55,212-2 inhibits the AC activity of wild type and two mutant receptors with the same order, and binding affinity of WIN55,212-2 in those mutant receptors is under study.

On the other hand, there are several reports for sudying the role of serine residues in the signal transduction of GPCR superfamily: it has been reported that serine residues in TM domain contribute to binding of agonist and/or receptor activation in β-adrenergic receptor (Strader et al, 1989), β_2-adrenergic receptor (Ambrosio et al, 2000; Liapakis et al, 2000), D_2 dopamine receptor (Cox et al, 1992; Mansour et al, 1992), and α_1-adrenergic receptor (Hwa and Perez, 1996). It is well studied that in catecholamine receptor, such as dopamine receptor and adrenergic receptor, serine residues in the TM domain contribute to the binding of agonists and activation of the receptor, suggesting that receptor serine residue form specific hydrogen bonds with each of the catechol ring hydroxyl groups of catacholamine ligands (Ambrosio et al, 2000; Liapakis et al., 2000; Mansour et al., 1992; Strader et al., 1989; Wiens et al., 1998). Interestingly, Strader et al (1989) have reported that in β-adrenergic receptor specific hydrogen- bonding interaction between serine 204 and 207 of the TM V and hydroxyl group of catecholamine have been identified.

In a line with that, present results suggest that hydroxyl group of Serine-292 take a part in the interaction of hydrogen bonds with cannabinoid ligand. Whereas HU-210 and CP55940 contain aromatic side chain and phenolic hydroxyl group, WIN55,212-2 contains only aromatic side chain without hydroxyl group. Therefore, the replacement of Ser-292 into Ala partially impair the ligand binding and cannabinoid (i.e., HU-210 and CP55940)-induced AC inhibition but not aminoalkylindole derivatives (i.e., WIN55,212-2)-induced AC inhibition.

In conclusion, my present results support the difference of binding site between classical cannabinoid and aminoalkylindoes WIN55,212-2, and it is the first report that hydrogen bond interaction site (i.e., Ser-292) in CB2 cannabinoid receptor is observed.

Acknowledgments

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