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Role of the highly conserved Asp-Arg-Tyr motif in signal transduction of the CB_2 cannabinoid receptor

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Abstract The DRY motif, at the junction of transmembrane helix 3 and intracellular loop 2 of G protein-coupled receptors, is highly conserved. Mutations were introduced into the CB_2 cannabinoid receptor to study the role of this motif in CB_2 signaling. D mutations (DRY130–132AAA and D130A) markedly reduced binding of cannabinoid agonists, while no significant reduction was observed with R131A or Y132A. Mutating R (R131A) only partially reduced, and mutating Y (Y132A) more efficiently reduced the cannabinoid-induced inhibition of adenylyl cyclase. Thus, in CB_2, D130 is involved in agonist binding, whereas Y seems to have a role in receptor downstream signaling.

Key words: Cannabinoid; CB_2 receptor; G protein; Site-directed mutagenesis

1. Introduction

Two subtypes of cannabinoid receptors, CB_1 and CB_2, have been cloned to date [1,2]. Both CB_1 and CB_2 are members of the seven transmembrane (TM) domain G protein-coupled receptor (GPCR) superfamily. While CB_1 is mainly present in the central nervous system, the CB_2 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 [2,3]. Moreover, CB_2-like cannabinoid receptors have recently been found on peripheral nerve terminals [4,5]. Both CB_1 and CB_2 act via $G_{i/o}$ to inhibit adenylyl cyclase (AC) [6–8]. Relatively little is known about the structure of the CB_2 receptor and the molecular interactions involved in its ligand binding and signal transduction.

The DRY motif at the junction of TM3 and the second intracellular loop (ICL2) is highly conserved in most GPCRs ([9–12]; see Fig. 1) and is a candidate site for the interaction of the receptor with the associated G protein [12,13]. The D residue (residue 3.49 in the nomenclature of Ballesteros and Weinstein [14]) is found in 94% of the rhodopsin-like GPCRs [9]. A few receptors (e.g. rhodopsin, thyroid stimulating hormone receptor, follicle stimulating hormone receptor) were reported to have E instead of D at this site. Thus, this motif has been defined as the E/DRY motif, see [15]. Mutagenesis of this site in various GPCRs (e.g. rhodopsin, GnRH, α_{1B}-adrenergic and m_1-muscarinic receptors) revealed that the D3.49 residue has a role in receptor expression [16,17], internalization [16,18] and constitutive receptor activation [15,19,20]. However, different effects were observed with different receptors. For example, mutations at D3.49 of the α_{1B}-adrenergic receptor induced constitutive activation of inositol phosphate production [19,20], but did not seem to directly affect G protein signaling of the m_1-muscarinic receptor [17]. The R3.50 (in the nomenclature of Ballesteros and Weinstein) is completely conserved in all GPCRs and appears to affect the receptor interaction with the coupled G proteins [9,11,21]. The Y3.51 of this E/DRY motif is found in 79% of the rhodopsin-like GPCRs [9] and is claimed to be involved in m_1-muscarinic receptor expression [17] and in the internalization of the bradykinin B2 receptor [22,23].

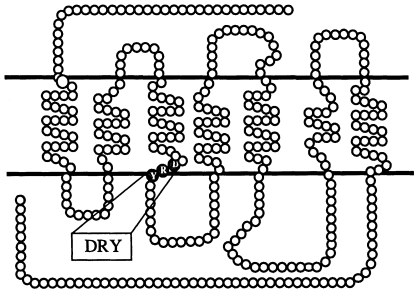
In this study, we investigated the role of the DRY motif in ligand binding and signal transduction of the CB_2 cannabinoid receptor. We found that D130 is essential for the capacity of the CB_2 receptor to bind cannabinoid agonists, whereas Y132 has a role in the receptor downstream signaling.

2. Materials and methods

2.1. Materials

[2-^{3}H]Adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). The phosphodiesterase inhibitors, 1-methyl-3-isobutylxanthine (IBMX) and RO-20-1724,

A. The topographical location of DRY motif



B. Conservation pattern of E/DRY motif

CB2	TAI DRY LC
CB1	TAI DRY IS
D2 dopamine	ISI DRY TA
D4 dopamine	ISV DRF VA
α2 adrenergic	ISL DRY WS
Somatostatin 1	LSV DRY VA
A1 adenosine	IAV DRY LR
A3 adenosine	IAV DRY LR
β1 adrenergic	IAL DRY LA
β2 adrenergic	IAV DRY FA
Rhodopsin	LAI ERY VV
FSH	ITL ERW HT
m1 muscarinic	ISF DRY FS
MSH	IAV DRY IS
Bradykinin B2	VSI DRY LA

Fig. 1. Location of the DRY motif in various GPCRs. (A) General structure of the CB_2 receptor. (B) Amino acid sequences in the E/DRY area of several GPCRs, as obtained from [32,42,43].

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were from Calbiochem (La Jolla, CA, USA). Forskolin (FS), cAMP and fatty acid-free bovine serum albumin (FAF–BSA) were from Sigma (St. Louis, MO, USA). The cannabinoid agonists, HU-210, WIN55,212-2 and [^{3}H]HU-243 were kindly provided by Dr. R. Mechoulam (Jerusalem, Israel). Tissue culture reagents were from Life Technologies (Gaithersburg, MD, USA).

2.2. Plasmids

Rabbit AC type V (AC-V) and β-gal cDNAs in the pXMD1 vector [24] were described previously [7,25]. The human CB_2 cDNA in pCDM8 [2] was kindly provided by Dr. S. Munro (Cambridge, UK).

2.3. Construction of hemagglutinin (HA)-tagged human CB_2 in pcDNA3

The following oligonucleotide primers (P1 and P2) were synthesized and used to amplify (by PCR) an 1100 bp fragment containing the entire human CB_2 coding sequence (using the human CB_2 cDNA in pCDM8 as a template): P1, 5′-GCGGATCCGAGGAATGCTGGGTG-3′ sense primer; P2, 5′-GCGCGGCCGCTCAGCAATCAGAGAG-3′ antisense primer. P1 is homologous to the cDNA sequence at the CB_2 coding start site and was engineered to contain a unique *Bam*HI site (underlined) for in-frame subcloning into a *Bgl*II site at the carboxy end of the HA sequence in HA-pcDNA3 [26]. The P2 sequence was designed to introduce a unique *Not*I site (underlined) for ligation into the multiple cloning site of pcDNA3. The 1100 bp PCR product (following 25 cycles of 1 min denaturation at 92°C, 1 min annealing at 45°C and 1 min extension at 72°C) was digested with *Bam*HI and *Not*I and cloned into the *Bgl*II and *Not*I sites of HA-pcDNA3. The DNA sequence was confirmed by sequencing at the Weizmann Institute's DNA Sequencing Unit.

2.4. Preparation of point mutations in HA-CB_2

Mutations were introduced using the PCR-overlap extension method as previously described [27]. In brief, two general primers were designed for PCR that cover the region in HA-CB_2 where the mutations were planned. The 5′ general primer 5′-TAATACGACTCACTATAGGG-3′ (complementing part of the T7 sequence of pcDNA3) and the 3′ general primer 5′-TTGACCTGGTCACTGAGCGTAGT-3′ (complementing a coding sequence in CB_2 beyond the mutation) were used in conjunction with internal sense and matching antisense primers that contained the desired mutation. Three PCR reactions were run, the first two providing the 5′ and 3′ ends of the mutated fragment, and the third consecutive reaction joining the separate fragments. The final PCR product was cut with *Bam*HI and *Bst*EII (unique sites, one in the multiple cloning site of pcDNA3 and the other in HA-CB_2, beyond the area of the mutation), the mutated fragment cloned into HA-CB_2 and the DNA sequences of the various HA-CB_2 mutants confirmed.

2.5. Transient cell transfection

24 h before transfection, a confluent 10 cm plate of COS-7 cells in Dulbecco's modified Eagle's 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°C, was trypsinized and split into five 10 cm plates. The cells were transfected, using the DEAE-dextran chloroquine method [28], with wild-type (wt) HA-CB_2 cDNA (2 μg/plate) or mutant cDNAs (4 μg/plate), and where indicated, with AC-V cDNA (1.5 μg/plate). pXMD1-gal cDNA was used for mock DNA transfection. For binding studies, 72 h after transfection, the COS cells were washed twice with phosphate-buffered saline (PBS), scraped, centrifuged at 3000 rpm for 10 min and the cell pellets stored at −70°C. For AC assay, 48 h after transfection, the COS 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. Transfection efficiency was normally in the range of 40–80%, as determined by staining the cells for the activity of transfected β-galactosidase [29].

2.6. AC activity

The assay was performed in triplicate as described previously [7,8]. In brief, cells cultured in 24-well plates were incubated for 2 h 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), 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 2 μM FS. After 10 min at 37°C, 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 [30]. 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.

2.7. Binding of [^{3}H]HU-243

This assay was performed as described previously [8,31]. Frozen pellets of transfected cells were homogenized in 2 ml/plate of binding buffer and 100 μl aliquots of crude cell homogenates containing 20–30 μg protein (as determined by the Bradford method) were mixed with 300 pM of [^{3}H]HU-243, 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 2 mg/ml FAF–BSA. The binding mixtures were incubated at 30°C for 1.5 h 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 from all values. To study the binding affinities of the cannabinoid agonists HU-210 and WIN55,212-2, the indicated concentrations of these materials were added to the binding mixtures together with the [^{3}H]HU-243. The K_i values for HU-210 and WIN55,212-2 were determined by the formula $K_i = IC_{50}/1+([[^3H]HU\text{-}243]/K_d)$ [31].

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western immunoblotting

COS-7 cells transfected with the various HA-tagged CB_2 cDNAs in 10 cm culture plates were harvested with cold PBS and spun down at 3000 rpm (at 4°C for 10 min), and the cell pellets were mixed with 100 μl/plate of Laemmli sample buffer, sonicated and frozen at −20°C. Samples of 20 μl were incubated for 5 min at 100°C with dithiothreitol (0.1 M final) prior to loading onto 1.5 mm thick 10% polyacrylamide gel. Following electrophoresis, proteins were blotted onto a nitrocellulose membrane and the blot blocked in PBS containing 5% fat-free milk and 0.5% Tween-20, followed by 1.5 h incubation with anti-HA monoclonal antibody (HA.11, Boehringer Mannheim) diluted 1:1000 in 5% fat-free milk and 0.5% Tween-20. Blots were washed three times with PBS containing 0.3% Tween-20 and incubated for 1 h with horseradish peroxidase-coupled rat anti-mouse antibodies (Jackson Immunoresearch Laboratories) diluted 1:20 000 in 5% fat-free milk plus 0.5% Tween-20. The blot was extensively washed and peroxidase activity was observed by the enhanced chemiluminescence technique (Amersham).

2.9. Data analysis

Data presented in the text and figures are mean values ± S.E.M. Statistical analysis of the data was done using the Student's *t*-test. Dose–response curves were plotted using the Sigma Plot 4.11 program.

3. Results and discussion

There is evidence that the E/DRY sequence located at the junction of TM3 and ICL2 of many GPCRs has a role in various receptor functions, including receptor expression, constitutive receptor activation, G protein coupling, receptor sequestration and receptor internalization [11,19,20,22,23,32]. However, the role of this motif in the signal transduction of the CB_2 receptor has not yet been examined. We have therefore mutated the CB_2 receptor (tagged with a HA epitope at its N-terminus) at this site, replacing either individual amino acids or all three of them with alanines, and examined the cannabinoid binding activity and the signal transduction capacity of the mutants versus HA-CB_2 (wt). HA-tagging of the CB_2 receptor did not affect agonist binding, nor did it affect the modulation of AC activity by cannabinoid agonists (data not shown).

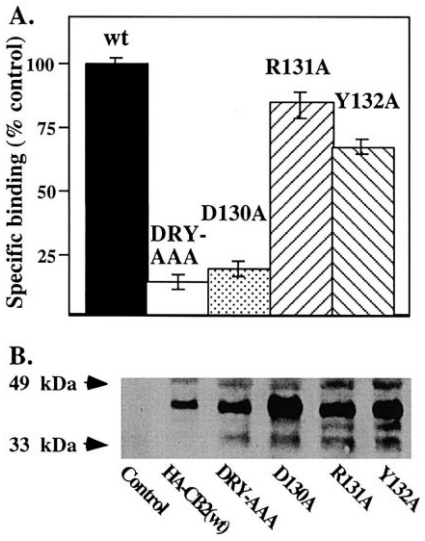

Fig. 2. Binding of [^{3}H]HU-243 and expression of HA-CB_2 (wt) and mutants. COS cells were transfected with the cDNAs of HA-CB_2 (wt) and the indicated mutants. (A) Binding of [^{3}H]HU-243 to equivalent aliquots of cell homogenates. Binding to HA-CB_2 (wt) is presented as 100%. The data represent the means ± S.E.M. of two experiments. (B) Western blot analysis showing the expression of the various mutants. Control represents mock-transfected cells.

3.1. Ligand binding to the CB_2 D130A mutant

Fig. 2A shows that compared to HA-CB_2 (wt), the DRY130–132AAA mutant retained only 15% of the binding of the high affinity cannabinoid agonist [^{3}H]HU-243, and the D130A mutant retained about 20%. On the other hand, the R131A and Y132A mutants retained almost normal binding activity of 80% and 70% specific binding, respectively. This implies that in CB_2, the D130 residue seems to be involved in the binding activity of the receptor, while R131 and Y132 would appear to be less important for this function. Even though both DRY130–132AAA and D130A showed only little cannabinoid agonist binding, we tested their signaling capacity and found that their activation by either HU-210 or WIN55,212-2 did not lead to any significant AC inhibition (data not shown). Thus, these mutants also appear to be defective in their downstream signaling.

Fig. 2B is a control experiment demonstrating by Western blot analysis that all the mutants used in this study are efficiently expressed, and thus the reduction in binding to the D130A mutant is not due to reduced expression. The molecular weight observed for the major immunoreactive band was 41 kDa, which is consistent with the amino acid composition of the HA-tagged receptor and with the molecular weight obtained for the expressed human CB_2 receptor protein [33].

This loss of binding by the D130A mutant, but not by the R131A mutant, in CB_2 is in agreement with the results obtained by others for the m_1-muscarinic receptor [17,21]. However, it should be noted that mutating the conserved D residue in the E/DRY motif led to different results with different receptors or receptor functions. A reduction in G protein coupling with retention of high affinity ligand binding was observed with β- and α_2-adrenergic receptors [34,35]. On the other hand, mutation of D122 in the m_1-muscarinic receptor led to a decrease in the level of antagonist binding to the receptor (depending on the amino acid replaced), but it was claimed that the D122 residue does not directly participate in the signaling [17]. Moreover, mutation of the D residue was shown to promote constitutive receptor activation in rhodopsin [36] and in the α_{1B}-adrenergic receptor [19,20], but not in the m_1-muscarinic receptor [17].

3.2. Ligand binding and signaling by the CB_2 R131A mutant

Fig. 3A,B shows the competitive binding curves for the cannabinoid agonists, HU-210 and WIN55,212-2, in COS cells transfected with cDNAs of either HA-CB_2 (wt) or the R131A mutant. The data show that HU-210 binds with a K_i of 145 ± 51 pM and 234 ± 52 pM for HA-CB_2 (wt) and the R131A mutant, respectively. WIN55,212-2 binds with a K_i of 1.0 ± 0.2 nM and 2.5 ± 0.3 nM for HA-CB_2 (wt) and the R131A mutant, respectively. Altogether, the R131A mutant binds well both HU-210 and WIN55,212-2, with affinities similar to those of wt receptor. It should be noted that the K_i values of HU-210 and WIN55,212-2 binding to HA-CB_2 (wt) as found here are in agreement with previous data obtained by us and others for the binding to the CB_2 receptor: 220 ± 180 pM [37], 147 ± 7 pM [38] and 170 ± 10 pM [31] for HU-210, and 280 ± 160 pM for WIN55,212-2 [37].

This R residue was found to be absolutely conserved within all of the members of the GPCR family [11,13] and its substitution in various GPCRs, such as the m_1-muscarinic and α_{1B}-adrenergic receptors, severely reduced the agonist-induced activation of phosphoinositide hydrolysis [19,21]. Oliveira et al. [11] suggested that this R residue serves as an Arg-switch, leading to activation of GPCRs (due to the shift of the

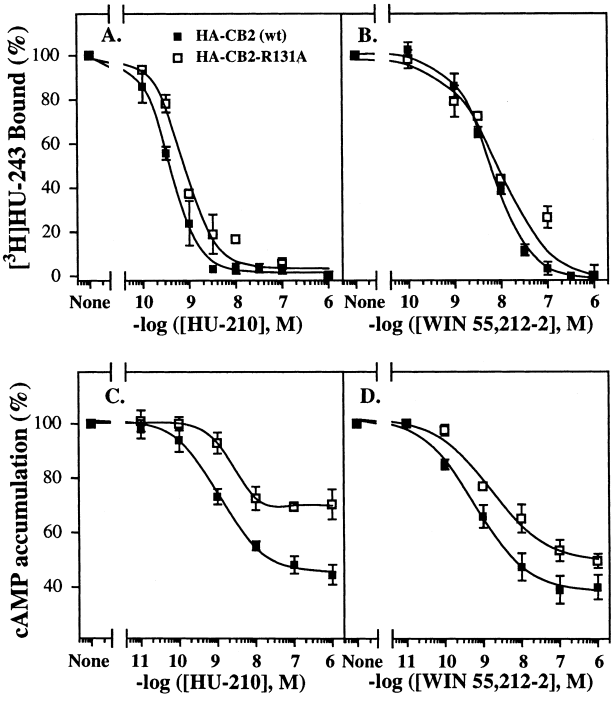

Fig. 3. Effect of the R131A mutation on cannabinoid binding and AC inhibition. (A,B) COS cells transfected with the cDNA of HA-CB_2 (wt) or of the R131A mutant were assayed for displacement of [^{3}H]HU-243 by increasing concentrations of HU-210 or WIN55,212-2. (C,D) COS cells transfected as in (A,B), but also cotransfected with AC-V, were assayed for the effects of various concentrations of HU-210 or WIN55,212-2 on FS-stimulated AC activity. Data represent the means ± S.E.M. of two experiments (A and B) or three experiments (C and D).

charged side chain upon agonist activation, thereby modulating G protein coupling). Indeed, a combination of mutagenesis and computational simulations on α_{1B}-adrenergic and GnRH receptors showed the importance of this Arg in signaling [19,32].

We therefore analyzed the signaling of the R131A HA-CB_2 mutant in comparison with HA-CB_2 (wt). Fig. 3C,D shows that G protein signaling by the R131A mutant is only weakly affected, as the EC_{50} values for the inhibition of transfected AC-V by WIN55,212-2 and by HU-210 were shifted to the right by less than 2-fold, in comparison to wt receptor. The EC_{50} values for WIN55,212-2 were 1.34 ± 0.27 vs. 0.73 ± 0.2 nM, respectively, and for HU-210, 1.26 ± 0.03 vs. 0.78 ± 0.18 nM, respectively. The maximal levels of inhibition of AC-V by HU-210 and WIN55,212-2 with the R131A mutant were somewhat reduced compared with HA-CB_2 (wt): 30% vs. 55% and 50% vs. 60%, respectively. Interestingly, AC signaling by HU-210 was more affected than by WIN55,212-2 in the R131A mutant. This difference between HU-210 and WIN55,212-2 is not completely clear but could possibly be explained by the finding that the binding site for classical cannabinoids (e.g. HU-210) is distinct from that for the aminoalkylindoles (e.g. WIN55,212-2) for both CB_1 and CB_2 receptors [39,40]. Altogether, mutating R131 in CB_2 only slightly affected signaling. This result differs from the observation made with the m_1-muscarinic, GnRH and α_{1B}-adrenergic receptors which, as described above, lost signaling capacity upon mutation of this site [17,19,32].

3.3. Ligand binding and signaling by the CB_2 Y132A mutant

Relatively little is known about the role of the Y residue of the E/DRY motif in the GPCR superfamily. Replacing this residue with non-aromatic amino acids led to a reduction in the expression of the m_1-muscarinic receptor binding sites, with minimal effects on the signaling capability of the ligand binding receptors [17]. Fig. 4A,B shows the competitive binding curves for HU-210 and WIN55,212-2 in COS cells transfected with HA-CB_2 (wt) or the Y131A mutant. The data show that agonist binding affinity is not affected by this mutation. HU-210 binds with a K_i of 145 ± 51 pM and 160 ± 41 pM for HA-CB_2 (wt) and the Y132A mutant, respectively. WIN55,212-2 binds with a K_i of 1.0 ± 0.2 nM and 0.5 ± 0.2 nM for HA-CB_2 (wt) and the Y132A mutant, respectively. We next analyzed the signaling of the Y132A mutant. Fig. 4C,D shows that the signaling by Y132A was markedly, but not completely, reduced. The maximal inhibitions by HU-210 and by WIN55,212-2 for the Y132A mutant were reduced as compared with HA-CB_2 (wt) (26% vs. 55% and 28% vs. 60%, respectively).

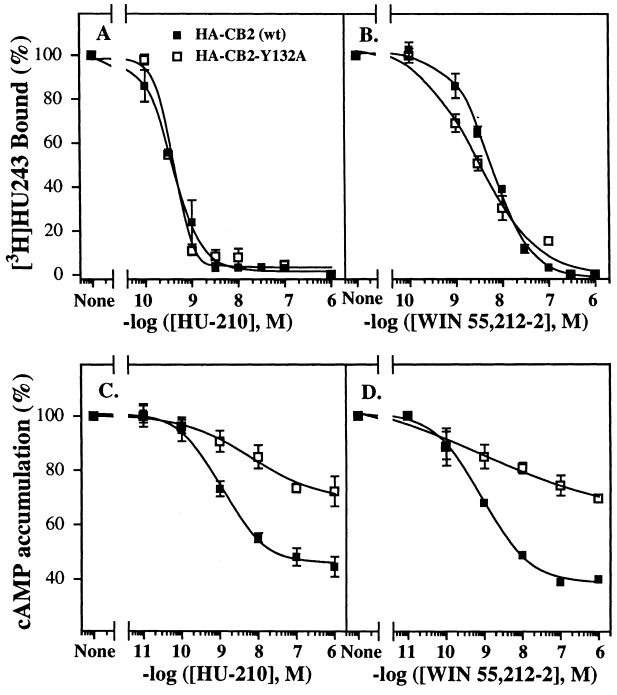

Fig. 4. Effect of the Y132A mutation on cannabinoid binding and AC inhibition. (A,B) COS cells transfected with the cDNAs of HA-CB_2 (wt) or of the Y132A mutant were assayed for displacement of [^{3}H]HU-243. (C,D) COS cells transfected as in (A,B), but also co-transfected with AC-V, were assayed for modulation of AC activity by cannabinoid agonists. For more details, see Fig. 3.

Reports regarding the role of this Y residue in signal transduction are controversial. Y3.51 was shown to have a role in the signaling and internalization of the bradykinin B2 receptor [22,23]. However, as described above, mutating this residue in the m_1-muscarinic receptor indicated that it is not critical for efficient signal transduction [17]. Moreover, converting the unique DRS sequence in the GnRH receptor to the conserved DRY sequence did not affect receptor signaling [41]. Here, we show that mutating this residue partially, but not completely, interferes with receptor signaling.

In summary, the E/DRY motif at the junction of TM3 and ICL2 is highly conserved in most members of the GPCR superfamily. Whereas D130 in CB_2 seems to be related to receptor intramolecular contact affecting ligand binding, Y132 appears to have a possible role in receptor downstream signaling.

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