Mutations in the ‘DRY’ motif of the CB₁ cannabinoid receptor result in biased receptor variants

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Abstract

The role of the highly conserved ‘DRY’ motif in the signaling of the CB₁ cannabinoid receptor (CB₁R) was investigated by inducing single-, double-, and triple-alanine mutations into this site of the receptor. We found that the CB₁R-R3.50A mutant displays a partial decrease in its ability to activate heterotrimeric G₃ proteins (~80% of WT CB₁R (CB₁R-WT)). Moreover, this mutant showed an enhanced basal β-arrestin₂ (β-arr₂) recruitment. More strikingly, the double-mutant CB₁R-D3.49A/R3.50A was biased toward β-arres, as it gained a robustly increased β-arr₁ and β-arr₂ recruitment ability compared with the WT receptor, while its G-protein activation was decreased. In contrast, the double-mutant CB₁R-R3.50A/Y3.51A proved to be G-protein-biased, as it was practically unable to recruit β-arres in response to agonist stimulus, while still activating G-proteins, although at a reduced level (~70% of CB₁R-WT). Agonist-induced ERK1/2 activation of the CB₁R mutants showed a good correlation with their β-arr recruitment ability but not with their G-protein activation or inhibition of cAMP accumulation. Our results suggest that G-protein activation and β-arr binding of the CB₁R are mediated by distinct receptor conformations, and the conserved ‘DRY’ motif plays different roles in the stabilization of these conformations, thus mediating both G-protein- and β-arr-mediated functions of CB₁R.

Key Words

- G-proteins
- signal transduction
- mutations
- receptors

Introduction

Seven transmembrane receptors (7TMRs) constitute the largest family of plasma membrane receptors. Most of their intracellular effects are mediated via direct coupling to heterotrimeric G-proteins. To understand the molecular details of 7TMR activation and G-protein coupling, identification of key structural elements regulating these processes is critically important. Using mutational analyses as well as recent high-resolution X-ray crystal structure data, such structural features have been extensively mapped (Venkatakrishnan et al. 2013). Among these, the conserved Asp-Arg-Tyr (DRY) motif, located at the beginning of the second intracellular loop (ICL2), seems to play a central role both in the activation and the G-protein coupling of class A (rhodopsin-like) 7TMRs (Rasmussen et al. 2011). Nevertheless, the exact nature of this regulatory role is still not completely understood. For instance, although the Arg residue (R3.50) is suggested to directly interact with the G-protein α subunit in the active 7TMR conformation, its nonconservative mutations in many cases fail to impair G-protein coupling of the receptor (Fanelli et al. 1999, Rhee et al. 2000, Rovati et al. 2007). Furthermore, Asp (D3.49) is believed to stabilize...
inactive receptor conformation by forming a salt-bridge with the neighboring R3.50 (Scheer et al. 1996, 1997, Ballesteros et al. 1998, 2001, Li et al. 2001); however, its mutations can also result in completely diverse phenotypes, depending on the investigated receptor (Rovati et al. 2007). Therefore, the exact role of the DRY motif obviously shows receptor-specific differences, and its detailed analysis for a particular 7TMR seems reasonable.

Besides G-proteins, β-arrestins (β-arrs) are also able to directly bind to the intracellular surface of an activated 7TMR, leading to the desensitization and internalization of the receptor (Shenoy & Lefkowitz 2011). Moreover, receptor-bound β-arrs can also serve as a starting-point for G-protein-independent signaling pathways, such as the activation of the p42/44 MAPK cascade or Src kinases (Wei et al. 2003, DeWire et al. 2007).

Many data suggest that the β-arr-bound conformation of 7TMRs may differ from the one mediating their G-protein activation, a fact being implicitly exploited by several functionally selective 7TMR ligands as well as by functionally selective 7TMR mutants, which are able to induce β-arr recruitment without affecting G-protein coupling or vice versa (Reiter et al. 2012). However, in the lack of a high-resolution crystal structure describing a 7TMR in its β-arr-bound form, relatively little is known about the receptor–arrestin binding interface. According to the prevailing idea, arrestins utilize two distinct sites to bind to 7TMRs, one of which is a ‘phosphorylation sensor’, recognizing Ser/Thr-phosphorylated C-terminus of the receptor (Gurevich & Benovic 1993, Gurevich & Gurevich 2006). The other site is a so-called ‘activation sensor’, which recognizes the active 7TMR conformation, independently of receptor phosphorylation (Gurevich & Gurevich 2006). The 7TMR elements constituting the docking site for the arrestin ‘activation sensor’ are less understood. The second ICL2, beginning with the DRY motif, has been proposed to play such a role (Huttenrauch et al. 2002, Marion et al. 2006). Furthermore, complementary roles for the DRY motif and receptor C-terminus in the regulation of β-arr binding have been described (Kim & Caron 2008). In addition, mutations of R3.50 in many cases result in basal β-arr binding and subsequent constitutively desensitized phenotype of 7TMRs (Barak et al. 2001, Wilbanks et al. 2002). Thus, the conserved DRY motif seems to be involved not only in G-protein coupling, but also in β-arr binding of 7TMRs.

The CB₁ cannabinoid receptor (CB₁R) belongs to the 7TMR superfamily. The signaling pathways originating from CB₁R are mediated mainly via heterotrimeric G₁/o proteins, and include inhibition of cAMP production, activation of GIRK potassium channels, inhibition of Ca₂⁺ calcium channels, and activation of MAPK cascades (Turu & Hunyady 2010). Moreover, CB₁R shows basal G-protein activation and constitutive internalization under diverse cellular conditions (Leterrier et al. 2006, McDonald et al. 2007, Turu et al. 2007). Like most other 7TMRs, CB₁R also recruits β-arr following activation, which leads to the desensitization and internalization of the receptor (Kouznetsova et al. 2002, Daigle et al. 2008, Gyombolai et al. 2013). The binding between β-arrs and CB₁R is relatively weak, and the affinity of the receptor for β-arr2 (β-arr2) is substantially higher than that for β-arr1 (β-arr1) (Gyombolai et al. 2013). Furthermore, β-arr1 recruitment of CB₁R appears to be agonist-dependent (Laprairie et al. 2014, Flores-Otero et al. 2014). Interestingly, in addition to canonical G-protein-mediated intracellular effects, recent data have suggested that the existence of β-arr-mediated, G-protein-independent signaling of CB₁R, i.e. the p42/44 MAPK (ERK1/2) activation of the receptor seems to be at least partly mediated by β-arrs (Ahn et al. 2013a, Mahavadi et al. 2014).

Through these cellular events, CB₁R is involved in the regulation of many important physiological and pathophysiological processes, such as memory, learning, pain sensation, metabolic regulation, or the regulation of vascular tone (Pacher et al. 2006). Moreover, several natural and synthetic cannabinoid ligands are known to stabilize distinct active CB₁R conformations, i.e. prove to be functionally selective (Glass & Northup 1999, Mukhopadhyay & Howlett 2001, Ahn et al. 2013a). Thus, investigation of the structural elements responsible for G-protein- and β-arr-mediated CB₁R functions has a major physiological and pharmacological impact. Accordingly, a number of studies have aimed to identify such regulatory motifs of CB₁R. A detailed computational model based on the crystal structure of the β₂-adrenergic receptor-Gαs complex, combined with mutational data, suggested that distinct residues in the ICL2 and ICL3 regions of the CB₁R may be involved in the stabilization of the active, Gαs-coupled receptor conformation (Shim et al. 2013). Two other recent studies have analyzed the role of several intramolecular salt-bridges, which may stabilize inactive, partially active, and fully active CB₁R conformations (Ahn et al. 2013b, Scott et al. 2013). According to this model, D3.49 and R3.50 residues form salt-bridges with K4.41 and D6.30, respectively, which (together with a D2.63+K3.28 salt-bridge) may keep the receptor in a partially active conformation under basal conditions.

Less is known about the structural features governing the β-arr binding of CB₁R. The C-terminal Ser/Thr...
phosphorylation of the receptor seems to play a role, because alanine mutations of these residues impaired agonist-induced β-arrest recruitment and subsequent internalization of CB1R (Daigle et al. 2008).

Although the above studies clearly provide important insights into the molecular details of CB1R function, none of them assessed the role of the DRY motif in CB1R function directly, i.e. through mutational analysis. More importantly, none of the available studies have aimed to identify β-arrest-regulatory motifs of CB1R other than the receptor C-terminus. Therefore, our goal was to analyze the role of the conserved DRY sequence in the G-protein activation and β-arrest binding of CB1R. We introduced single-, double-, and triple-alanine mutations into this site of CB1R and applied functional assays directly measuring G-protein activation, β-arrest2 recruitment, and intracellular signaling of WT and mutant CB1R variants.

Materials and methods

Materials

The cDNA of the rat vascular CB1R was provided by Zsolt Lenkei (Centre National de la Recherche Scientifique, Paris, France). cDNAs of human β1 and γ11 G-protein subunits were purchased from the Missouri S&T cDNA Resource Center (Rolla, MO, USA). β-arrest2-eGFP cDNA was kindly provided by Dr Marc G Caron (Duke University, Durham, NC, USA). Molecular biology enzymes were obtained from Fermentas (Vilnius, Lithuania) and Stratagene (La Jolla, CA, USA). Fetal bovine serum (FBS), OptiMEM, Lipofectamine 2000, and PBS–EDTA were from Invitrogen. CHO-K1 and HeLa cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Coelenterazine h was from Regis Technologies (Morton Grove, IL, USA). WIN55,212-2, 2-arachidonoyl glycerol and AM251 were from Tocris (Bristol, UK). Cell culture dishes and plates for bioluminescence resonance energy transfer (BRET) measurements were from Greiner (Kremsmunster, Austria). Anti-pERK1/2, anti-ERK1/2, and HRP-conjugated anti-rabbit and anti-mouse antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Unless otherwise stated, all other chemicals and reagents were from Sigma.

Plasmid constructs and site-directed mutagenesis

The mVenus-tagged rat CB1R (CB1R–mVenus) was created by exchanging the sequence of eYPF in CB1R-eYPF (kindly provided by Zsolt Lenkei (Centre National de la Recherche Scientifique)) to the sequence of mVenus using AgeI and NotI restriction enzymes. The αo-Rluc and YFP-β1 constructs were created from αoA-CFP (kindly provided by Dr N Gautam (Azpiazu & Gautam 2004)), and β1 respectively, as described previously (Turu et al. 2007). The β-arrest2-Rluc was constructed as described previously (Turu et al. 2006). The plasma membrane-targeted mVenus (MP-mVenus) was constructed as described previously (Varnai et al. 2007). The plasma membrane-targeted super Renilla luciferase (MP-SLUC) was generated from MP-mVenus by replacing the mVenus coding sequence with the cDNA of super Renilla luciferase (Woo & von Arnim 2008). The EPAC-based BRET sensor was constructed as described previously (Erdelyi et al. 2014). Mutations in the DRY motif of CB1R or CB1R-mVenus were inserted by the QuikChange Site-directed Mutagenesis Kit (Stratagene), according to manufacturer’s suggestions. The sequences of all constructs were verified using automated DNA sequencing.

Cell culture and transfection

CHO or HeLa cells (passage numbers 5–15) were maintained in Ham’s F12 or DMEM, respectively, supplemented with 10% FBS (Invitrogen), 100 µg/ml streptomycin, and 100 IU/ml penicillin in 5% CO2 at 37 °C. For confocal microscopy experiments, cells were grown on glass coverslips in six-well plates and transfected with the indicated constructs using Lipofectamine 2000 in OptiMEM following the manufacturer’s instructions. For BRET and western blotting experiments, cells were grown on six-well plates and transfected with the indicated constructs using Lipofectamine 2000 in OptiMEM following the manufacturer’s instructions.

BRET measurements

A detailed description of the BRET measurements applied here is provided in Supplementary Methods, see section on supplementary data given at the end of this article.

Confocal laser-scanning microscopy

The cells were grown on glass coverslips and transfected with the appropriate constructs (using 2 µg/well CB1R-mVenus or 0.5 µg/well β-arrest2-GFP and 2 µg/well CB1R). The cells were analyzed 22–26 h later in a modified Krebs–Ringer buffer (see above), using a Zeiss LSM 710 confocal laser-scanning microscope.
Western blotting analysis

A detailed description of the western blotting measurements applied here is provided in Supplementary Methods.

Statistical analysis

The dose–response curves for G-protein, β-arr, and EPAC BRET measurements were fitted and statistically compared using the built-in algorithms of GraphPad Prism 4.03 (GraphPad Software, Inc, San Diego, CA, USA). Equimolar comparison was carried out by plotting the points of G-protein and β-arr2 BRET dose–response curves for vehicle, K8.0 (only by WIN55), K7.5, K7.0, K6.5, K6.0, K5.5, and K5.0 (only by 2-AG) log[WIN55] or log[2-AG] (M) treatments of the same receptor against each other. Equiactive comparison was carried out by determining the bias factor (β) using the equation (Rajagopal et al. 2011):

$$\beta = \log \left( \frac{E_{\text{max},1} \ E_{C50,2}}{E_{C50,1} \ E_{C50,2}^2} \right)_\text{mut} \times \left( \frac{E_{\text{max},2} \ E_{C50,1}}{E_{C50,2} \ E_{C50,1}^2} \right)_\text{ref}$$

where $E_{\text{max},1}$, $E_{C50,1}$, $E_{\text{max},2}$, and $E_{C50,2}$ are $E_{\text{max}}$ and $E_{C50}$ values from G-protein and β-arr BRET dose–response curves, respectively, using CB1R-WT as reference receptor. Quantified western blotting data were evaluated with two-way ANOVA combined with Holm–Sidak’s post hoc test, using the software SigmaStat for Windows 3.5 (Systat Software, Inc., Richmond, CA, USA), and a $P$ value $<0.05$ was considered to be statistically significant.

Results

Plasma membrane localization of the CB1R mutants

To investigate whether any of the mutations inserted into the DRY motif of CB1R affects the proper plasma membrane localization of the receptor, CHO cells expressing mVenus-tagged CB1R variants were analyzed by confocal microscopy. In resting cells, CB1R-mVenus is localized both at the plasma membrane and intracellular vesicles, consistent with the constitutive internalization of CB1R (Fig. 1A). Importantly, D3.49A mutation strongly impaired plasma membrane localization of CB1R, with most of the receptors being retained in the endoplasmic reticulum of the cells (CB1R-D3.49A-mVenus and CB1R-D3.49A/Y3.51A-mVenus, Fig. 1B and F, respectively). Interestingly, this effect of the D3.49A mutation was reversed by co-mutation of R3.50, as the double-mutant CB1R-D3.49A/R3.50A (CB1R-ARY) and the triple-mutant CB1R-D3.49A/R3.50A/Y3.51A (CB1R-AAA) both showed proper plasma membrane localization (Fig. 1G and H, respectively). The other three mutants, i.e. CB1R-R3.50A (CB1R-DAY), CB1R-Y3.51A (CB1R-DRA), and CB1R-R3.50A/Y3.51A (CB1R-DAA) displayed a cellular distribution roughly similar to that of the WT receptor (Fig. 1C, D, and E, respectively).
As analysis of confocal images is not always sensitive enough to detect fine changes in receptor distribution, we also applied a more quantitative approach here, i.e. we measured the BRET interaction levels between CB1R-mVenus and plasma membrane-targeted SLUC protein. The fraction of the receptors residing on the plasma membrane of nonstimulated cells (PM/total receptor BRET) was found to be similar in cells expressing CB1R-WT, CB1R-AAY, or CB1R-AAA, whereas CB1R-DAY, CB1R-DRA, and CB1R-DAA showed ca. 40% reduction in plasma membrane localization. Furthermore, in accordance with the confocal images, the plasma membrane localization of CB1R-ARY and CB1R-ARA was shown strongly reduced (Fig. 1I).

As the plasma membrane localization of the CB1R-ARY and CB1R-ARA mutants was severely disrupted, these two mutants were not characterized in the subsequent studies.

**R3.50A mutation partially affects CB1R function**

R3.50 is the most conserved residue within the DRY motif, therefore we first tested the functionality of the CB1R-DAY mutant. The G-protein activation of the receptor was directly monitored by measuring BRET changes between heterotrimeric Go protein subunits (αo-Rluc and YFP-β1γ11) (Turu et al. 2007), co-expressed with WT or mutant CB1R. In control experiments measuring BRET donor and acceptor partner expression directly (i.e., through luminescence and fluorescence counts respectively), no significant changes were detected between these values.
When tested with the different CB1R mutants, suggesting that the observed changes in BRET were not due to alterations in BRET partner stoichiometry. This applies to all Gα BRET and β-arrestin2 BRET experiments presented in this study (data not shown). The dose–response curves performed with the synthetic CB1R agonist WIN55,212-2 (WIN55) or with the endocannabinoid 2-arachidonoylglycerol (2-AG) showed that the CB1R-DAY mutant is impaired, but not completely disrupted in its ability to activate Gα proteins. Moreover, CB1R-DAY shows a basal G-protein activation similar to that of CB1R-WT (Fig. 2A and B). The EC50 value of CB1R-DAY was also similar to that of CB1R-WT, indicating that the G-protein binding of CB1R is not affected by the R3.50A mutation (Table 1).

Next, the β-arrestin2 recruitment of CB1R-DAY was investigated. GFP-tagged β-arrestin2 (β-arrestin2-GFP) was co-expressed with CB1R-DAY in CHO cells, and its distribution was analyzed under confocal microscopy. Interestingly, we found that in cells co-expressing β-arrestin2-GFP and CB1R-DAY, β-arrestin2-GFP was recruited to the plasma membrane in punctate structures already in resting cells, indicating an increased basal β-arrestin2 recruitment of CB1R-DAY (Fig. 2E and G). Such basal recruitment of β-arrestin2-GFP could not be observed with CB1R-WT (Fig. 2C). This basal recruitment of β-arrestin2 was the consequence of a partially active receptor conformation, because treatment with the CB1R inverse agonist AM251 (10 μM, 10 min) resulted in the disappearance of most of the β-arrestin2 puncta from the plasma membrane (Fig. 2H).

After addition of the CB1R agonist WIN55 (1 μM, 10 min), further translocation of β-arrestin2-GFP to the plasma membrane could be observed in the case of CB1R-DAY (Fig. 2F); however, this did not reach the level of β-arrestin2-GFP recruitment of the CB1R-WT (Fig. 2D).

To evaluate β-arrestin2 recruitment in a more quantitative manner, translocation of β-arrestin2 to the receptors was followed by monitoring BRET changes between β-arrestin2-Rluc and plasma membrane-targeted mVenus (MP-mVenus). With this assay, β-arrestin2 recruitment to the investigated receptor can be monitored without tagging the receptor itself directly, which is advantageous because the detected BRET changes are not influenced by possible orientational changes resulting from the introduced receptor mutations. Furthermore, BRET signal in this assay is only affected via receptors residing on the plasma membrane, i.e. BRET ratios are not disturbed by intracellular receptor population.

Dose–response curves performed with WIN55 in this β-arrestin2 BRET assay were in good accordance with the data obtained by confocal microscopy, i.e. the increased basal β-arrestin2 recruitment of CB1R-DAY, as well as a lower β-arrestin2 recruitment in response to agonist stimulus were detectable (Fig. 2I). Similar results were obtained with the endocannabinoid 2-AG (Fig. 2J).

Table 1: Parameters of Gα BRET and β-arrestin2 BRET dose–response curves for the different CB1R variants. Bottom and Emax values are expressed as % of Emax of CB1R-WT. Data are mean ± s.e.m., n = 3–8

<table>
<thead>
<tr>
<th>Receptor</th>
<th>WIN55</th>
<th>2-AG</th>
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<tbody>
<tr>
<td></td>
<td>pEC50</td>
<td>Bottom</td>
</tr>
<tr>
<td>CB1R-WT</td>
<td>−7.9±0.2</td>
<td>37±2</td>
</tr>
<tr>
<td>CB1R-DAY</td>
<td>−7.9±0.2</td>
<td>37±4</td>
</tr>
<tr>
<td>CB1R-DRA</td>
<td>−6.8±0.3*</td>
<td>66±3*</td>
</tr>
<tr>
<td>CB1R-DAA</td>
<td>−7.3±0.2</td>
<td>44±3</td>
</tr>
<tr>
<td>CB1R-AAY</td>
<td>−7.2±0.2*</td>
<td>0±4</td>
</tr>
</tbody>
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*P<0.05 vs CB1R-WT. ND, not detectable.

Y3.51A mutation increases constitutive activity of CB1R

Among the three residues of the DRY motif, Y3.51 is the least conserved, and relatively little is known about its role in 7TMR signaling. To obtain data about its role in CB1R regulation, we tested the CB1R-DRA mutant under our experimental settings. Interestingly, although the maximal G-protein activation of this mutant was only marginally impaired (i.e., a significant change in Emax...
was detectable only upon 2-AG stimuli), the G-protein BRET dose–response analysis indicated an elevated basal G-protein activation for this mutant (Fig. 3A, B and Table 1). Confocal microscopy analysis showed that, similarly to the CB₁R-DAY mutant, basal β-arr2 recruitment of CB₁R-DRA occurs (Fig. 3C and E), which could be reversed by inverse agonist treatment (Fig. 3F). Agonist-induced β-arr2-GFP translocation to the plasma membrane was very weak (Fig. 3D). β-arr2 BRET analysis was in accordance with confocal data, namely, dose–response curve showed elevated basal β-arr2 recruitment together with a significantly impaired agonist-induced β-arr2 translocation (Fig. 3G and H).

**Enhanced β-arr2 recruitment and reduced G-protein activation of the CB₁R-AAY mutant**

Next, we investigated the signaling properties of the double-mutant CB₁R-AAY. The G-protein activation was monitored by the BRET assay described previously. Dose–response curves carried out with WIN55 or 2-AG showed that the CB₁R-AAY mutant has an impaired Go activation ability (Fig. 4A and B), which is reflected both in the $E_{\text{max}}$ and the pEC₅₀ values of these interactions (Table 1). Moreover, basal G-protein activation of this mutant was significantly lowered (Fig. 4A, B and Table 1).

The β-arr2 recruitment of CB₁R-AAY was investigated also by β-arr2-GFP co-expression using confocal microscope. We found that, similarly to CB₁R-DAY and CB₁R-DRA, CB₁R-AAY recruited β-arr2-GFP to the plasma membrane in nonstimulated cells (Fig. 4C and E). The basal β-arr2 recruitment could be reversed on treatment with inverse agonist AM251 (Fig. 4F). A very robust translocation of β-arr2-GFP to the plasma membrane was observed on addition of WIN55, with practically no β-arr2-GFP remaining in the cytoplasm (Fig. 4D).

We further evaluated the β-arr2 recruitment of CB₁R-AAY with the BRET-based method described previously. WIN55 and 2-AG dose–response curves showed that, in addition to the increased basal β-arr2 recruitment of CB₁R-AAY, this mutant gained a substantially increased ability to recruit β-arr2 upon agonist stimulus, as shown

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**Figure 3**

Functional analysis of the CB₁R-DRA mutant. (A and B) Dose–response curves showing G-protein activation of CB₁R-WT (grey curve) and CB₁R-DRA (black curve) in CHO cells under basal and different (A) WIN55- or (B) 2-AG-stimulated conditions, as detected by Go-protein BRET. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μM), and 100% reflects maximal (A) WIN55- or (B) 2-AG-induced response ($E_{\text{max}}$) of CB₁R-WT. Data are mean ± S.E.M., n = 4–8. (C, D, E, and F) Confocal images showing distribution of β-arr2-GFP in CHO cells co-expressing CB₁R-DRA, under control conditions (C and E) and 10 min after WIN55 (1 μM, D) or AM251 (10 μM, F) treatment. Arrows indicate β-arr2-GFP puncta at the plasma membrane. Images are representative from at least four independent experiments. Scale bar = 10 μm. (G and H) Dose–response curves showing recruitment of β-arr2 to the plasma membrane by CB₁R-WT (grey curve) and CB₁R-DRA (black curve) in CHO cells under basal and different (G) WIN55- or (H) 2-AG-stimulated conditions, as detected by BRET between β-arr2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μM), and 100% reflects maximal (G) WIN55- or (H) 2-AG-induced response ($E_{\text{max}}$) of CB₁R-WT. Data are mean ± S.E.M., n = 4–7.
by the significant left- and upward shift of the curves (Fig. 4G, H and Table 1). These results suggest that the signaling of this mutant is shifted from G-protein activation toward β-arr2 recruitment, and therefore CB1R-AAY can be considered as a β-arr2-biased mutant.

The characteristics of the triple mutant CB1R-AAA were very similar to that of CB1R-AAY, i.e. a decrease in basal and agonist-induced G-protein activation, as well as an increase in basal and agonist-induced β-arr2 recruitment was observed (data not shown).

The CB1R-DAA mutant is G-protein-biased

In the next set of experiments, the functional characteristics of the CB1R-DAA double-mutant receptor were analyzed. The dose–response curves obtained by Go protein BRET assay showed that the CB1R-DAA mutant can activate G-proteins at a lowered level, although pEC50 values as well as basal G-protein activation remained unaffected (Fig. 5A, B and Table 1).

Confocal microscopy analysis of β-arr2-GFP co-expressed with CB1R-DAA showed that this mutant, similarly to the CB1R-DAY, CB1R-DRA, and CB1R-AAY mutants, recruited β-arr2-GFP to the plasma membrane under control conditions (Fig. 5C and E), and this was reversed by AM251 treatment (Fig. 5F). Interestingly, no further translocation of β-arr2-GFP could be detected in these cells upon addition of the CB1R agonist WIN55 (Fig. 5D). These results were strengthened by β-arr2 BRET measurements, showing a basal β-arr2 recruitment for CB1R-DAA, which, however, cannot be enhanced by WIN55 or 2-AG treatment (Fig. 5G and H). These results suggest that, in contrast to CB1R-AAY, the signaling of CB1R-DAA is shifted from β-arr2 recruitment toward G-protein activation, and therefore CB1R-DAA can be considered as a G-protein-biased mutant.

β-arr1 recruitment of CB1R-AAY mutant is robustly enhanced

In our previous study, we could not detect significant β-arr1 coupling to the CB1R upon WIN55 stimulus; however, others have suggested that CB1R-dependent β-arr1 recruitment can be present and may regulate ERK1/2 activation of CB1R (Flores-Otero et al. 2014).

Figure 4

Functional analysis of the CB1R-AAY mutant. (A and B) Dose–response curves showing G-protein activation of CB1R-WT (grey curve) and CB1R-AAY (black curve) in CHO cells under basal and different (A) WIN55- or (B) 2-AG-stimulated conditions, as detected by Go-protein BRET. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μM), and 100% reflects maximal (A) WIN55- or (B) 2-AG-induced response (E_max) of CB1R-WT. Data are mean ± S.E.M., n = 3–8. (C, D, E, and F) Confocal images showing distribution of β-arr2-GFP in CHO cells co-expressing CB1R-AAY, under control conditions (C and E) and 10 min after WIN55 (1 μM, D) or AM251 (10 μM, F) treatment. Images are representative from at least three independent experiments. Scale bar 10 μm. (G and H) Dose–response curves showing recruitment of β-arr2 to the plasma membrane by CB1R-WT (grey curve) and CB1R-AAY (black curve) in CHO cells under basal and different (G) WIN55- or (H) 2-AG-stimulated conditions, as detected by BRET between β-arr2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μM), and 100% reflects maximal (G) WIN55- or (H) 2-AG-stimulated response (E_max) of CB1R-WT. Data are mean ± S.E.M., n = 4–7.
Laprairie et al. (2014). To test whether DRY mutations of CB1R affect the recruitment of β-arrestin 1, we applied the same BRET-based approach as above, i.e. the plasma membrane translocation of β-arrestin1-Rluc was monitored, and dose-response curves were performed using WIN55 and 2-AG as agonists. Our results show that agonist-induced β-arrestin1 recruitment is very low in cells expressing CB1R-WT, i.e. a significant increase could only be detected upon 2-AG treatment, whereas the changes obtained with WIN55 proved to be nonsignificant. Interestingly, the CB1R-AAY mutant displayed a robustly enhanced ability to recruit β-arrestin1, both upon WIN55 and 2-AG stimuli. All of the other three mutants (i.e., CB1R-DAY, CB1R-DRA, and CB1R-DAA) produced nonsignificant changes in the plasma membrane localization of β-arrestin1 (Fig. 6A and B).

Detailed data analysis strengthens biased signaling of DRY mutant CB1Rs

The above results suggest that distinct mutations in the conserved DRY motif of the CB1R can differentially affect G-protein activation and β-arrestin2 recruitment of the receptor. To assess this receptor bias in an exact manner, two different methods, proposed by Rajagopal et al. (2011), were applied to analyze data. First, ‘equimolar comparison’ was carried out, where G-protein and β-arrestin2 responses elicited by the same ligand concentrations are plotted against each other. In the case of the ‘reference receptor’, i.e. CB1R-WT, this analysis yields a roughly hyperbolic shape with both WIN55 and 2-AG (Fig. 7A and B, respectively, black circles), reflecting the difference in the amplification between G-protein and β-arrestin2 assays. Importantly, the points for CB1R-AAY are substantially shifted to left and upwards on these graphs, representing bias toward β-arrestin2 recruitment (Fig. 7A and B, white triangles). Furthermore, the points for CB1R-DAA are arranged along a horizontal line, demonstrating the bias of this receptor toward G-protein activation (Fig. 7A and B, grey squares). The other method was ‘equiactive comparison’, where the signaling of each receptor is characterized by a bias factor (β), based on the EC50 and Emax values from G-protein and β-arrestin2 dose–response curves (Rajagopal et al. 2011). In case of the reference receptor (CB1R-WT), this

![Graph](image-url)

**Figure 5**

Functional analysis of the CB1R-DAA mutant. (A and B) Dose–response curves showing G-protein activation of CB1R-WT (grey curve) and CB1R-DAA (black curve) in CHO cells under basal and different (A) WIN55- or (B) 2-AG-stimulated conditions, as detected by Go-protein BRET. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μM), and 100% reflects maximal (A) WIN55- or (B) 2-AG-induced response (Emax) of CB1R-WT. Data are mean ± S.E.M., n = 4–8. (C, D, E, and F) Confocal images showing distribution of β-arrestin2-GFP in CHO cells co-expressing CB1R-DAA under control conditions (C and E) and 10 min after WIN55 (1 μM, D) or AM251 (10 μM, F) treatment. Images are representative from at least three independent experiments. Scale bar 10 μm. (G and H) Dose–response curves showing recruitment of β-arrestin2 to the plasma membrane by CB1R-WT (grey curve) and CB1R-DAA (black triangles) in CHO cells under basal and different (G) WIN55- or (H) 2-AG-stimulated conditions, as detected by BRET between β-arrestin2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μM), and 100% reflects maximal (G) WIN55- or (H) 2-AG-induced response (Emax) of CB1R-WT. Data are mean ± S.E.M., n = 4–8.
that CB1R-AAY and CB1R-DAA can be considered as b-arrestin and G-protein activation bias of these two mutants toward G-protein activation respectively), whereas the same values for CB1R-AAY b-arrestin bias factor is by definition 0. In the case of CB1R-DAA, the β-values were −1.54 or −1.42, representing more than tenfold bias of these two mutants toward G-protein activation and β-arrestin2 recruitment respectively (Fig. 7C).

Taken together, our detailed bias analysis indicated that CB1R-AAY and CB1R-DAA can be considered as β-arrestin-biased and G-protein-biased mutants respectively.

**Functional assays reflect biased intracellular signaling of CB1R-AAY and CB1R-DAA**

Next, we wanted to assess whether the differences seen at the level of receptor–effector protein coupling are reflected in more distal intracellular signaling events initiated by CB1R activation. First, G1i/o protein-mediated signaling was assessed by measuring the inhibition of forskolin-induced cAMP accumulation under basal and CB1R-stimulated conditions, using an EPAC-based intramolecular BRET-sensor (Erdelyi et al. 2014). Our results showed that CB1R-WT inhibits cAMP accumulation under nonstimulated conditions, and this is substantially and dose-dependently enhanced upon treatment with WIN55 (Fig. 8A). Importantly, WIN55-induced cAMP inhibition of the G-protein-biased mutant CB1R-DAA was lower but still present, whereas CB1R-AAY, in accordance with its bias toward β-arrestin2, failed to induce the inhibition of cAMP accumulation in response to agonist stimulus (Fig. 8A).
Recent data have suggested that CB1R-induced activation of p42/44 MAPK (ERK1/2), which was formerly suggested to occur via G-protein-dependent pathways (Galve-Roperth et al. 2002, Davis et al. 2003, Dalton & Howlett 2012), is also mediated by β-arrests (Ahn et al. 2013a, Mahavadi et al. 2014). Therefore, we aimed to study how the ERK1/2 responses correlate with the G-protein activation and/or β-arrest recruitment of the biased CB1R mutants. Western blotting experiments carried out with cells expressing CB1R-WT showed a robust increase in the amount of phosphorylated ERK1/2 (pERK1/2) after 5 min treatment with WIN55 (1 μM). Moreover, lower but sustained pERK1/2 levels were also detectable after 20 min WIN55 treatment (Fig. 8B and C). Interestingly, we found that the β-arrest2-biased CB1R-AAY elicited pERK1/2 responses similar to CB1R-WT, both at 5- and 20-min stimulation, whereas the G-protein-biased CB1R-DAA produced significantly lower pERK1/2 responses than the WT receptor (Fig. 8B and C). Thus, ERK1/2 activation of the biased DRY mutants correlated well with their β-arrest2 recruitment ability, rather than with their G-protein activation.

**Discussion**

In this study, we evaluated the role of the conserved DRY motif in the function of the CB1R. Our goal was to assess its role in mediating basal and agonist-induced G-protein activation and β-arrest recruitment of CB1R, as well as to identify possible differences caused in these two main effector functions of the receptor. Interestingly, single-alanine mutation of the conserved Arg (R3.50A) resulted only in a ∼20% reduction in the G-protein coupling efficiency of CB1R, without affecting its basal G-protein activation. This may seem surprising, as crystal structure analysis as well as several mutational data have suggested a pivotal role for this residue in the G-protein coupling of 7TMRs (Zhu et al. 1994, Ballesteros et al. 1998, Rasmussen et al. 2011). However, several other 7TMRs exist, where similar nonconservative mutations of R3.50 failed to abolish G-protein activation of the receptor (Fanelli et al. 1999, Rovati et al. 2007). Thus, CB1R appears to belong to a subgroup of 7TMRs, where this conserved Arg residue plays no absolute role in the direct receptor–G-protein coupling. Furthermore, our results demonstrate a basal β-arrest2 recruitment of the CB1R-DAY mutant (or any double or triple mutant carrying the same mutation), which is in good accordance with previously published data showing similar characteristics for R3.50H mutants of V2 vasopressin, α1B adrenergic, and AT1A angiotensin II receptors (Wilbanks et al. 2002). This strengthens the idea that this conserved Arg somehow prevents arrestin binding in the inactive receptor conformation. Agonist-induced β-arrest2 recruitment of CB1R-DAY and CB1R-DRA was lowered, which is most likely to be caused by the lowered plasma membrane localization of these mutants (Fig. 11).

The most interesting finding of our study is the major difference between the functions of two double mutants, CB1R-DAA and CB1R-AAY. Although both mutants...
contain the R3.50A mutation, and accordingly show increased basal β-arrestin2 recruitment, their ultimate characteristics are further determined by the location of the second mutation. Thereby, a simultaneous lack of D3.49 and R3.50 residues seems to have a dominant-positive effect on both the β-arrestin1 and β-arrestin2 recruitment of CB1R (which is also supported by the fact that the triple-mutant CB1R-AAA functionally resembles CB1R-AAY). Thus, CB1R-AAY is a β-arrestin-biased 7TMR mutant. Interestingly, these characteristics of the CB1R-AAY are similar to those of the formerly described biased mutant angiotensin II receptor AT1-DRY/AAY (AT1R-AAY) (Gaborik et al. 2003, Wei et al. 2003). However, an important difference here is that AT1R-AAY is β-arrestin-biased in a way that its G-protein activation is absent while its β-arrestin binding is present but certainly not increased (Wei et al. 2003, Laprairie et al. 2012), whereas CB1R-AAY is β-arrestin-biased in that its β-arrestin recruitment is substantially increased, together with a lowered, but not abolished G-protein activating ability. Furthermore, we were able to detect a robustly enhanced β-arrestin1 recruitment to CB1R-AAY, whereas β-arrestin1 translocation to CB1R-WT was significant only upon 2-AG stimulus, but not after WIN55 treatment. Thus, it appears that the recruitment of β-arrestin1 to CB1R-WT is very weak, so that it challenges the limits of detectability via the (otherwise quite sensitive) BRET approach applied here. However, our results showing a significant increase in β-arrestin1 BRET upon 2-AG stimulus are in accordance with recent results showing higher β-arrestin1 recruitment by 2-AG compared with WIN55 (Laprairie et al. 2014). Taken together, recruitment of β-arrestin1 to CB1R-WT is obviously lower than that of β-arrestin2, but both are substantially enhanced in the CB1R-AAY mutant. Interestingly, basal G-protein activation of CB1R-AAY was absent, while the difference between vehicle-treated and WIN55-stimulated cells remained comparable with that of CB1R-WT (Fig. 4A), raising the question whether the reduced $E_{\text{max}}$ value of CB1R-AAY in this assay reflects a true loss of agonist-induced G-protein activation, or it is caused merely by the absence of basal activity, while WIN55-induced G-protein activation remains unaffected. However, repeating these experiments in HeLa cells, where basal activity of CB1R is minimal (Gyombolai et al. 2013), also showed substantially impaired WIN55-induced G-protein activation of CB1R-AAY (Supplementary Figure 1, see section on supplementary data given at the end of this article), suggesting that this mutation reduces not only the basal but also the WIN55-induced Go$_{i}$ protein activation of CB1R.

In contrast, CB1R-DAA proved to be G-protein-biased, as its β-arrestin recruitment in response to agonist stimulus was practically absent, but was still able to activate G-proteins, although at a lower level (~70% of the WT CB1R). According to our data, plasma membrane expression of this mutant is ca. 40% lower than that of CB1R-WT. However, this extent of decrease is not likely to cause a complete loss of agonist-induced β-arrestin recruitment, given the ca. 1:1 stoichiometry of receptor–β-arrestin complex. This is also supported by the fact that CB1R-DAA still binds β-arrestin2 under basal conditions. Other 7TMRs described previously as biased mutants include the M$_1$-R3.50L designer muscarinic receptor (Nakajima & Wess 2012) and β2-AR-TYY, a triple-mutant β2-AR, which was rationally designed to be functionally selective (Shenoy et al. 2006). Interestingly, however, all of these mutants are β-arrestin-biased, i.e. they do not couple to G-proteins but still recruit β-arrestins, albeit at a lowered level. The CB1R-DAA mutant presented here is interesting in this respect, as it is biased toward G-protein activation, whereas its mutations affect a ‘classical’ G-protein-coupling region, i.e. the DRY motif. Intriguingly, although CB1R-DAA can hardly recruit β-arrestins in response to agonist stimulus, it still binds β-arrestin2 to some extent under nonstimulated conditions. This relies most probably on the presence of the R3.50A mutation, because, as mentioned previously, all of the CB1R mutants carrying this mutation recruited β-arrestin2 constitutively. Thus, it seems that the absence of the conserved Arg residue can itself determine a receptor conformation that binds β-arrestin spontaneously. On the other hand, the agonist-induced β-arrestin2 binding of the receptor can still be strongly influenced in both directions by co-mutations of the neighboring residues.

Taken together, our results obtained with the CB1R-AAY and CB1R-DAA mutants strongly support a model where the active G-protein-coupled and β-arrestin-bound conformations of a 7TMR are different. Moreover, receptor states responsible for constitutive and agonist-induced β-arrestin binding may also show differences.

We also demonstrate here that the agonist-induced ERK1/2 phosphorylation shows good correlation with the β-arrestin2 recruitment of our biased CB1R mutants, rather than their G-protein activation or their ability to inhibit forskolin-induced cAMP accumulation. These data are consistent with the recently emerging concept of β-arrestin-dependent CB1R signaling, i.e. a β-arrestin-mediated ERK1/2 phosphorylation following CB1R activation (Ahn et al. 2013a, Mahavadi et al. 2014).

One of the most interesting questions regarding the DRY mutants presented here is how (i.e. through which molecular structural rearrangements) the distinct mutations induce such large differences in the
β-arr-recruitment of CB₁R. One simple explanation would be that mutations of the DRY motif modify primarily the G-protein binding of the receptor, and their effects on the β-arr2 recruitment are merely secondary, resulting from the assumption that G-proteins and β-arrrs compete for the 7TMR binding. However, if this would be the only explanation, one should observe an indirect proportionality between the G-protein-and the β-arr binding abilities of the distinct mutants, which is actually not the case. Thus, mutations of the DRY motif most probably affect β-arr2 binding of CB₁R independently of its G-protein activation. Whether or not the DRY sequence itself is a part of the docking site for arrestins, can not be answered unequivocally based on our results. However, previously published data indicating that the ICL2 loop of 7TMRs, beginning with an intact DRY motif, is part of the β-arr binding site, add interesting aspects to our study (Huttenrauch et al. 2002, Marion et al. 2006). Moreover, two recent studies have provided important insights into the structural features within the 7TM-β-arr complex. Both of these studies point to an important interaction between the ‘finger loop’ region of β-arr and the receptor core, with the direct involvement of the DRY motif (Shukla et al. 2014, Szczepak et al. 2014). Combined with these data, our results show good fit with a model where DRY is directly involved in the β-arr binding of CB₁R. In addition, mutations in the DRY motif may also affect β-arr binding indirectly, i.e. by inducing structural rearrangements in the subsequent ICL2, resulting in diverse, sometimes completely opposite β-arr binding phenotypes. However, a more precise understanding of the intramolecular interactions that mediate these characteristics would require the high-resolution crystal structure data.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-14-0219.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This research was supported by Hungarian Scientific Research Fund (OTKA NK-100883), and a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Programme (PIOF-GA-2009-253628).

Author contribution statement
P G designed and carried out most of the experiments and wrote the manuscript. A D T carried out the β-arr2 BRET experiments, helped with data evaluation, and revised the manuscript. D T created the CB₁R-DAY mutant and carried out important control experiments. G T created the CB₁R-ΔAY mutant, helped with data interpretation, and revised the manuscript. L H managed the overall project, helped with data interpretation, and revised the manuscript.

Acknowledgements
The excellent technical assistance of Ilona Oláh, as well as the help of Bence Szalai with the statistical analyses and presentation of the data is greatly appreciated.

References
Azpiazu I & Gautam N 2004 A fluorescence resonance energy transfer-based sensor indicates that receptor access to a G protein is unrestricted in a living mammalian cell. Journal of Biological Chemistry 279 27709–27718. (doi:10.1074/jbc.M403712200)


Turu G & Hunyady I 2010 Signal transduction of the CB1 cannabinoid receptor. Journal of Molecular Endocrinology 44 75–85. (doi:10.1677/JME-08-0190)


Varnai P, Toth B, Toth DJ, Hunyady I & Balla T 2007 Visualization and manipulation of plasma membrane-endoplasmic reticulum contact sites indicates the presence of additional molecular components within the STIM1-Orai1 Complex. Journal of Biological Chemistry 282 29678–29690. (doi:10.1074/jbc.M704339200)


Received in final form 28 November 2014
Accepted 15 December 2014
Accepted Preprint published online 15 December 2014