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

Pál Gyombolai^{1,2}, András D Tóth¹, Dániel Tímár¹, Gábor Turu¹ and László Hunyady^{1,2}

¹Department of Physiology, Faculty of Medicine, Semmelweis University, PO Box 259, H-1444 Budapest, Hungary

²MTA-SE Laboratory of Molecular Physiology, Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary

Correspondence
should be addressed
to L Hunyady
Email
Hunyady@puskin.sote.hu

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 G_o 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 -arrestins, 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 -arrestins 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

*Journal of Molecular
Endocrinology*
(2015) 54, 75–89

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 (Venkatakrisnan *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 -arrests) 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 -arrests 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 G_{i/o} proteins, and include inhibition of cAMP production,

activation of GIRK potassium channels, inhibition of Ca_v 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 -arrests 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 -arrests (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 β_2 -adrenergic receptor-G α_s 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, G α_i -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 -arr 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 -arr-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 -arr 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 -arr2 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 β_1 and γ_{11} G-protein subunits were purchased from the Missouri S&T cDNA Resource Center (Rolla, MO, USA). β -arr2-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 eYFP in CB₁R-eYFP (kindly provided by Zsolt Lenkei (Centre National de la Recherche

Scientifique)) to the sequence of mVenus using AgeI and NotI restriction enzymes. The α_o -Rluc and YFP- β_1 constructs were created from α_o -CFP (kindly provided by Dr N Gautam (Azpiazu & Gautam 2004)), and β_1 , respectively, as described previously (Turu *et al.* 2007). The β -arr2-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 μ g/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 μ g/well CB₁R-mVenus or 0.5 μ g/well β -arr2-GFP and 2 μ g/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.

Figure 1

Cellular distribution of WT and mutant mVenus-tagged CB₁R variants. (A, B, C, D, E, F, G, and H) CHO cells expressing mVenus-tagged CB₁R variants are visualized using confocal microscopy. (A) CB₁R-WT-mVenus; (B) CB₁R-ARY-mVenus; (C) CB₁R-DAY-mVenus; (D) CB₁R-DRA-mVenus; (E) CB₁R-DAA-mVenus; (F) CB₁R-ARA-mVenus; (G) CB₁R-AAY-mVenus; (H) CB₁R-AAA-mVenus. Images are representative from three independent experiments.

Scale bar = 10 μm. (I) PM/total receptor BRET showing the fraction of mVenus-tagged CB₁R variants residing on the plasma membrane. 0% reflects no net BRET interaction and 100% reflects normalized BRET interaction of CB₁R-WT-mVenus. Data are mean ± s.e.m., *n* = 3, **P* < 0.05, NS, non-significant.

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, −8.0 (only by WIN55), −7.5, −7.0, −6.5, −6.0, −5.5, and −5.0 (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):

$$\beta = \log \left(\left(\frac{E_{\max,1}}{EC_{50,1}} \frac{EC_{50,2}}{E_{\max,2}} \right)_{\text{mut}} \times \left(\frac{E_{\max,2}}{EC_{50,2}} \frac{EC_{50,1}}{E_{\max,1}} \right)_{\text{ref}} \right)$$

where $E_{\max,1}$, $EC_{50,1}$, $E_{\max,2}$, and $EC_{50,2}$ are $E_{\max}$ and EC_{50} 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 in 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 (CB₁R-ARY-mVenus) and CB₁R-D3.49A/Y3.51A-mVenus (CB₁R-ARA-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-AAY) 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).

Figure 2

Functional analysis of the CB₁R-DAY mutant. (A and B) Dose–response curves showing G-protein activation of CB₁R-WT (grey curve) and CB₁R-DAY (black curve) in CHO cells under basal and different (A) WIN55- or (B) 2-AG-stimulated conditions, as detected by G_o-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 $\pm$ s.e.m., $n=3-8$. (C, D, E, F, G, and H) Confocal images showing distribution of β-arr2-GFP in CHO cells co-expressing CB₁R-WT (C and D) or CB₁R-DAY (E, F, G, and H), under control conditions (C, E, and G) and 10 min after WIN55 (1 μM, D, and F) or AM251

(10 μM, H) treatment. Arrows indicate β-arr2-GFP puncta at the plasma membrane. Images are representative from at least four independent experiments. Scale bar = 10 μm. (I and J) Dose–response curves showing recruitment of β-arr2 to the plasma membrane by CB₁R-WT (grey curve) and CB₁R-DAY (black curve) in CHO cells under basal and different (I) WIN55- or (J) 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 WIN55- (I) or 2-AG- (J) induced response (E_{max}) of CB₁R-WT. Data are mean $\pm$ s.e.m., $n=4-7$.

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-AAAY, 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 G_o protein subunits (α_o -Rluc and YFP-β_{1γ11}) (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

Table 1 Parameters of G_o BRET and β -arrestin2 BRET dose–response curves for the different CB₁R variants. Bottom and $E_{\max}$ values are expressed as % of $E_{\max}$ of CB₁R-WT. Data are mean $\pm$ S.E.M., $n=3$ –8

Receptor	WIN55			2-AG		
	pEC ₅₀	Bottom	$E_{\max}$	pEC ₅₀	Bottom	$E_{\max}$
G _o BRET						
CB ₁ R-WT	-7.9 ± 0.1	37 ± 2	100	-7.4 ± 0.1	50 ± 2	100
CB ₁ R-DAY	-7.9 ± 0.2	37 ± 4	$83 \pm 4^*$	-7.4 ± 0.3	42 ± 6	$78 \pm 4^*$
CB ₁ R-DRA	$-6.8 \pm 0.3^*$	$66 \pm 3^*$	97 ± 4	-7.5 ± 0.2	$64 \pm 2^*$	$87 \pm 1^*$
CB ₁ R-DAA	-7.3 ± 0.2	44 ± 3	$75 \pm 3^*$	-7.3 ± 0.4	54 ± 4	$73 \pm 2^*$
CB ₁ R-AAAY	$-7.2 \pm 0.2^*$	0 ± 4	$64 \pm 4^*$	$-7.0 \pm 0.2^*$	$17 \pm 4^*$	$78 \pm 4^*$
β -arrestin2 BRET						
CB ₁ R-WT	-6.8 ± 0.1	1 ± 3	100	-5.5 ± 0.0	2 ± 1	100
CB ₁ R-DAY	$-7.5 \pm 0.2^*$	25 ± 4	$62 \pm 3^*$	$-6.7 \pm 0.3^*$	$17 \pm 4^*$	$45 \pm 3^*$
CB ₁ R-DRA	$-7.2 \pm 0.2^*$	14 ± 2	$43 \pm 2^*$	$-6.4 \pm 0.2^*$	$10 \pm 2^*$	$35 \pm 2^*$
CB ₁ R-DAA	$> -5.0^*$	21 ± 2	ND	$> -4.5^*$	$15 \pm 5^*$	ND
CB ₁ R-AAAY	$-7.2 \pm 0.1^*$	33 ± 11	$284 \pm 10^*$	$-6.2 \pm 0.1^*$	$26 \pm 6^*$	$145 \pm 8^*$

* $P < 0.05$ vs CB₁R-WT. ND, not detectable.

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 G_o BRET and β -arr 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-arachidonoyl glycerol (2-AG) showed that the CB₁R-DAY mutant is impaired, but not completely disrupted in its ability to activate G_o 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 -arr2 recruitment of CB₁R-DAY was investigated. GFP-tagged β -arr2 (β -arr2-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 -arr2-GFP and CB₁R-DAY, β -arr2-GFP was recruited to the plasma membrane in punctate structures already in resting cells, indicating an increased basal β -arr2 recruitment of CB₁R-DAY (Fig. 2E and G). Such basal recruitment of β -arr2-GFP could not be observed with CB₁R-WT (Fig. 2C). This basal recruitment of β -arr2 was the consequence of a partially active receptor conformation, because treatment with the CB₁R inverse agonist AM251 (10 μ M, 10 min) resulted in the disappearance of most of the β -arr2 puncta from the plasma membrane (Fig. 2H).

After addition of the CB₁R agonist WIN55 (1 μ M, 10 min), further translocation of β -arr2-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 -arr2-GFP recruitment of the CB₁R-WT (Fig. 2D).

To evaluate β -arr2 recruitment in a more quantitative manner, translocation of β -arr2 to the receptors was followed by monitoring BRET changes between β -arr2-*luc* and plasma membrane-targeted mVenus (MP-mVenus). With this assay, β -arr2 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 -arr2 BRET assay were in good accordance with the data obtained by confocal microscopy, i.e. the increased basal β -arr2 recruitment of CB₁R-DAY, as well as a lower β -arr2 recruitment in response to agonist stimulus were detectable (Fig. 2I). Similar results were obtained with the endocannabinoid 2-AG (Fig. 2J).

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 $E_{\max}$

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-AAAY mutant

Next, we investigated the signaling properties of the double-mutant CB₁R-AAAY. 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-AAAY mutant has an impaired G_o activation ability (Fig. 4A and B), which is reflected both in the $E_{\max}$ 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-AAAY was investigated also by β -arr2-GFP co-expression using confocal microscope. We found that, similarly to CB₁R-DAY and CB₁R-DRA, CB₁R-AAAY 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-AAAY 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-AAAY, this mutant gained a substantially increased ability to recruit β -arr2 upon agonist stimulus, as shown

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 G_o-protein BRET. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μ M), and 100% reflects maximal (A) WIN55- or (B) 2-AG-induced response ($E_{\max}$) of CB₁R-WT. Data are mean $\pm$ 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 μ M, D) or AM251 (10 μ M, F) treatment. Arrows indicate β -arr2-GFP puncta at the plasma membrane. Images are representative from at least four independent experiments. Scale bar = 10 μ m. (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 μ M), and 100% reflects maximal (G) WIN55- or (H) 2-AG-induced response ($E_{\max}$) of CB₁R-WT. Data are mean $\pm$ 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-AAAY can be considered as a β -arr2-biased mutant.

The characteristics of the triple mutant CB₁R-AAA were very similar to that of CB₁R-AAAY, 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 G₀ 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-AAAY 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-AAAY, 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-AAAY 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-AAAY mutant. (A and B) Dose-response curves showing G-protein activation of CB₁R-WT (grey curve) and CB₁R-AAAY (black curve) in CHO cells under basal and different (A) WIN55- or (B) 2-AG-stimulated conditions, as detected by G₀-protein BRET. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μ M), and 100% reflects maximal (A) WIN55- or (B) 2-AG-induced response ($E_{\max}$) of CB₁R-WT. Data are mean $\pm$ 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-AAAY, under control conditions (C and E) and 10 min after WIN55 (1 μ M, D) or AM251 (10 μ M, F) treatment. Images are representative from at least three independent experiments. Scale bar 10 μ m. (G and H) Dose-response curves showing recruitment of β -arr2 to the plasma membrane by CB₁R-WT (grey curve) and CB₁R-AAAY (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 μ M), and 100% reflects maximal (G) WIN55- or (H) 2-AG-induced response ($E_{\max}$) of CB₁R-WT. Data are mean $\pm$ s.e.m., $n=4-7$.

Laprairie *et al.* 2014). To test whether DRY mutations of CB₁R affect the recruitment of β -arr1, we applied the same BRET-based approach as above, i.e. the plasma membrane translocation of β -arr1-Rluc was monitored, and dose-response curves were performed using WIN55 and 2-AG as agonists. Our results show that agonist-induced β -arr1 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 -arr1, 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 -arr1 (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 -arr2 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 -arr2 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 -arr2 assays. Importantly, the points for CB₁R-AAY are substantially shifted to left and upwards on these graphs, representing bias toward β -arr2 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 E_{max} values from G-protein and β -arr2 dose-response curves (Rajagopal *et al.* 2011). In case of the reference receptor (CB₁R-WT), this

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 G₀-protein BRET. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μ M), and 100% reflects maximal (A) WIN55- or (B) 2-AG-induced response (E_{max}) of CB₁R-WT. Data are mean $\pm$ 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-DAA, under control conditions (C and E) and 10 min after WIN55 (1 μ M, D) or AM251 (10 μ M, F) treatment. Images are representative from at least three independent experiments. Scale bar 10 μ m. (G and H) Dose-response curves showing recruitment of β -arr2 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 -arr2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μ M), and 100% reflects maximal (G) WIN55- or (H) 2-AG-induced response (E_{max}) of CB₁R-WT. Data are mean $\pm$ S.E.M., $n=4-7$.

Figure 6

Dose-response curves showing β -arrestin1 recruitment of CB₁R mutants. (A and B) Dose-response curves showing recruitment of β -arr1 to the plasma membrane by CB₁R-WT (black circles), CB₁R-DAY (white diamonds), CB₁R-DRA (white circles), CB₁R-DAA (white squares), or CB₁R-AAY (white triangles) in CHO cells under basal and different (A) WIN55- or (B) 2-AG-stimulated conditions, as detected by BRET between β -arr1-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μ M), and 100% reflects maximal (A) WIN55- or (B) 2-AG-induced response (E_{max}) of CB₁R-AAY. Data are mean $\pm$ s.e.m., $n=3$. * $P<0.05$ vs vehicle treatment.

bias factor is by definition 0. In the case of CB₁R-DAA, the β -values were 1.42 or 1.61 (for WIN55 or 2-AG stimuli respectively), whereas the same values for CB₁R-AAY were -1.54 or -1.42 , representing more than tenfold bias of these two mutants toward G-protein activation and β -arr2 recruitment respectively (Fig. 7C).

Taken together, our detailed bias analysis indicated that CB₁R-AAY and CB₁R-DAA can be considered as β -arr-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, G_{i/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 -arr2, failed to induce the inhibition of cAMP accumulation in response to agonist stimulus (Fig. 8A).

Figure 7

Bias analysis showing functional selectivity of CB₁R mutants. (A and B) Equipotential comparison of CB₁R-WT (black points), CB₁R-DAA (grey squares), and CB₁R-AAY (white triangles) functions. For each receptor, responses from G-protein and β -arr2 BRET dose-response curves, elicited by the same (A) WIN55 or (B) 2-AG concentration, were plotted against each other. The left- and upward shift of CB₁R-AAY points represents bias toward β -arr2 recruitment, whereas the downward shift of CB₁R-DAA points indicates G-protein bias. Data are mean $\pm$ s.e.m. (C) Equiactive comparison of CB₁R-WT, CB₁R-DAA, and CB₁R-AAY functions. The biased factor (β) was calculated for each receptor, based upon EC_{50} and E_{max} values of G-protein and β -arr2 BRET dose-response curves obtained with WIN55 (black bars) or 2-AG (grey bars) stimuli, using the equation described in Materials and methods. CB₁R-WT was used as reference receptor. Positive values indicate bias toward G-protein signaling, whereas negative values reflect β -arrestin bias. Data are mean $\pm$ s.d.

Figure 8

Functional assays measuring intracellular signaling of WT and mutant CB₁R variants. (A) Dose-response curves showing the inhibition of forskolin-induced cAMP accumulation in CHO cells expressing CB₁R-WT, CB₁R-DAA, or CB₁R-AAY under basal and different WIN55-stimulated conditions, measured by the BRET changes in an EPAC-based intramolecular BRET sensor. BRET was measured 30 min after stimulus. 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251, 10 μ M), and 100% reflects maximal WIN55-induced response (E_{max}) of CB₁R-WT. Data are mean $\pm$ s.e.m., $n=6$. * $P<0.05$ vs basal state, # $P<0.05$ vs CB₁R-WT. (B) Amounts of phosphorylated (pERK1/2) and total ERK1/2 proteins detected by western blot in CHO cells expressing CB₁R-WT, CB₁R-DAA, or CB₁R-AAY, after 0, 5, or 20 min of WIN55 (1 μ M) treatment. Images are representative from four independent experiments. (C) Quantification of western blotting data. 0% reflects background intensity, and 100% reflects WIN55-induced pERK1/2 intensity of CB₁R-WT at 5 min. Data are mean $\pm$ s.e.m., $n=4$. * $P<0.05$ vs CB₁R-WT at 5 min, # $P<0.05$ vs CB₁R-WT at 20 min.

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-Roperh *et al.* 2002, Davis *et al.* 2003, Dalton & Howlett 2012), is also mediated by β -arrestins (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 -arr 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 μ M). 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 -arr2-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 -arr2 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 -arr 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 $\sim$ 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 -arr2 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, α_{1B} adrenergic, and AT_{1A} 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 -arr2 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. 1I).

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 -arr2 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 -arr1 and β -arr2 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 -arr-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 -arr-biased in a way that its G-protein activation is absent while its β -arr binding is present but certainly not increased (Wei *et al.* 2003, Balla *et al.* 2012), whereas CB₁R-AAY is β -arr-biased in that its β -arr 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 -arr1 recruitment to CB₁R-AAY, whereas β -arr1 translocation to CB₁R-WT was significant only upon 2-AG stimulus, but not after WIN55 treatment. Thus, it appears that the recruitment of β -arr1 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 -arr1 BRET upon 2-AG stimulus are in accordance with recent results showing higher β -arr1 recruitment by 2-AG compared with WIN55 (Laprairie *et al.* 2014). Taken together, recruitment of β -arr1 to CB₁R-WT is obviously lower than that of β -arr2, 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 $E_{\max}$ 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 G_o protein activation of CB₁R.

In contrast, CB₁R-DAA proved to be G-protein-biased, as its β -arr recruitment in response to agonist stimulus was

practically absent, but was still able to activate G-proteins, although at a lower level ($\sim 70\%$ 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 -arr recruitment, given the ca. 1:1 stoichiometry of receptor- β -arr complex. This is also supported by the fact that CB₁R-DAA still binds β -arr2 under basal conditions. Other 7TMRs described previously as biased mutants include the M₃-R3.50L designer muscarinic receptor (Nakajima & Wess 2012) and β_2 -AR-TYY, a triple-mutant β_2 -AR, which was rationally designed to be functionally selective (Shenoy *et al.* 2006). Interestingly, however, all of these mutants are β -arr-biased, i.e. they do not couple to G-proteins but still recruit β -arr, 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 -arrs in response to agonist stimulus, it still binds β -arr2 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 -arr2 constitutively. Thus, it seems that the absence of the conserved Arg residue can itself determine a receptor conformation that binds β -arr spontaneously. On the other hand, the agonist-induced β -arr2 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 -arr-bound conformations of a 7TMR are different. Moreover, receptor states responsible for constitutive and agonist-induced β -arr binding may also show differences.

We also demonstrate here that the agonist-induced ERK1/2 phosphorylation shows good correlation with the β -arr2 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 -arr-dependent CB₁R signaling, i.e. a β -arr-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-arrests 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 7TMR–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, Szczepek *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-AAY 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

- Ahn KH, Mahmoud MM, Shim JY & Kendall DA 2013a Distinct roles of β-arrestin 1 and β-arrestin 2 in ORG27569-induced biased signaling and internalization of the cannabinoid receptor 1 (CB1). *Journal of Biological Chemistry* **288** 9790–9800. (doi:10.1074/jbc.M112.438804)
- Ahn KH, Scott CE, Abrol R, Goddard WA III & Kendall DA 2013b Computationally-predicted CB1 cannabinoid receptor mutants show distinct patterns of salt-bridges that correlate with their level of constitutive activity reflected in G protein coupling levels, thermal stability, and ligand binding. *Proteins* **81** 1304–1317. (doi:10.1002/prot.24264)
- Azpiaz 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)
- Balla A, Toth DJ, Soltesz-Katona E, Szakadati G, Erdelyi LS, Varnai P & Hunyady L 2012 Mapping of the localization of type 1 angiotensin receptor in membrane microdomains using bioluminescence resonance energy transfer-based sensors. *Journal of Biological Chemistry* **287** 9090–9099. (doi:10.1074/jbc.M111.293944)
- Ballesteros J, Kitanovic S, Guarnieri F, Davies P, Fromme BJ, Konvicka K, Chi L, Millar RP, Davidson JS, Weinstein H *et al.* 1998 Functional microdomains in G-protein-coupled receptors. The conserved arginine–cage motif in the gonadotropin-releasing hormone receptor. *Journal of Biological Chemistry* **273** 10445–10453. (doi:10.1074/jbc.273.17.10445)
- Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U & Javitch JA 2001 Activation of the β 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *Journal of Biological Chemistry* **276** 29171–29177. (doi:10.1074/jbc.M103747200)
- Barak LS, Oakley RH, Laporte SA & Caron MG 2001 Constitutive arrestin-mediated desensitization of a human vasopressin receptor mutant associated with nephrogenic diabetes insipidus. *PNAS* **98** 93–98. (doi:10.1073/pnas.98.1.93)
- Daigle TL, Kwok ML & Mackie K 2008 Regulation of CB1 cannabinoid receptor internalization by a promiscuous phosphorylation-dependent mechanism. *Journal of Neurochemistry* **106** 70–82. (doi:10.1111/j.1471-4159.2008.05336.x)
- Dalton GD & Howlett AC 2012 Cannabinoid CB1 receptors transactivate multiple receptor tyrosine kinases and regulate serine/threonine kinases to activate ERK in neuronal cells. *British Journal of Pharmacology* **165** 2497–2511. (doi:10.1111/j.1476-5381.2011.01455.x)
- Davis MI, Ronesi J & Lovinger DM 2003 A predominant role for inhibition of the adenylate cyclase/protein kinase A pathway in ERK activation by cannabinoid receptor 1 in N1E-115 neuroblastoma cells. *Journal of Biological Chemistry* **278** 48973–48980. (doi:10.1074/jbc.M305697200)

- DeWire SM, Ahn S, Lefkowitz RJ & Shenoy SK 2007 β -arrestins and cell signaling. *Annual Review of Physiology* **69** 483–510. (doi:10.1146/annurev.physiol.69.022405.154749)
- Erdelyi LS, Balla A, Patocs A, Toth M, Varnai P & Hunyady L 2014 Altered agonist sensitivity of a mutant v2 receptor suggests a novel therapeutic strategy for nephrogenic diabetes insipidus. *Molecular Endocrinology* **28** 634–643. (doi:10.1210/me.2013-1424)
- Fanelli F, Barbier P, Zanchetta D, de Benedetti PG & Chini B 1999 Activation mechanism of human oxytocin receptor: a combined study of experimental and computer-simulated mutagenesis. *Molecular Pharmacology* **56** 214–225.
- Flores-Otero J, Ahn KH, Delgado-Peraza F, Mackie K, Kendall DA & Yudowski GA 2014 Ligand-specific endocytic dwell times control functional selectivity of the cannabinoid receptor 1. *Nature Communications* **5** 4589. (doi:10.1038/ncomms5589)
- Gaborik Z, Jagadeesh G, Zhang M, Spat A, Catt KJ & Hunyady L 2003 The role of a conserved region of the second intracellular loop in AT1 angiotensin receptor activation and signaling. *Endocrinology* **144** 2220–2228. (doi:10.1210/en.2002-0135)
- Galve-Roperh I, Rueda D, Gomez del Pulgar T, Velasco G & Guzman M 2002 Mechanism of extracellular signal-regulated kinase activation by the CB(1) cannabinoid receptor. *Molecular Pharmacology* **62** 1385–1392. (doi:10.1124/mol.62.6.1385)
- Glass M & Northup JK 1999 Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. *Molecular Pharmacology* **56** 1362–1369.
- Gurevich VV & Benovic JL 1993 Visual arrestin interaction with rhodopsin. Sequential multisite binding ensures strict selectivity toward light-activated phosphorylated rhodopsin. *Journal of Biological Chemistry* **268** 11628–11638.
- Gurevich VV & Gurevich EV 2006 The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacology & Therapeutics* **110** 465–502. (doi:10.1016/j.pharmthera.2005.09.008)
- Gyombolai P, Boros E, Hunyady L & Turu G 2013 Differential β -arrestin2 requirements for constitutive and agonist-induced internalization of the CB1 cannabinoid receptor. *Molecular and Cellular Endocrinology* **372** 116–127. (doi:10.1016/j.mce.2013.03.013)
- Huttenrauch F, Nitzki A, Lin FT, Honing S & Oppermann M 2002 β -arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif. *Journal of Biological Chemistry* **277** 30769–30777. (doi:10.1074/jbc.M204033200)
- Kim KM & Caron MG 2008 Complementary roles of the DRY motif and C-terminus tail of GPCRs for G protein coupling and β -arrestin interaction. *Biochemical and Biophysical Research Communications* **366** 42–47. (doi:10.1016/j.bbrc.2007.11.055)
- Kouznetsova M, Kelley B, Shen M & Thayer SA 2002 Desensitization of cannabinoid-mediated presynaptic inhibition of neurotransmission between rat hippocampal neurons in culture. *Molecular Pharmacology* **61** 477–485. (doi:10.1124/mol.61.3.477)
- Laprairie RB, Bagher AM, Kelly ME, Dupre DJ & Denovan-Wright EM 2014 Type 1 cannabinoid receptor ligands display functional selectivity in a cell culture model of striatal medium spiny projection neurons. *Journal of Biological Chemistry* **289** 24845–24862. (doi:10.1074/jbc.M114.557025)
- Leterrier C, Laine J, Darmon M, Boudin H, Rossier J & Lenkei Z 2006 Constitutive activation drives compartment-selective endocytosis and axonal targeting of type 1 cannabinoid receptors. *Journal of Neuroscience* **26** 3141–3153. (doi:10.1523/JNEUROSCI.5437-05.2006)
- Li J, Huang P, Chen C, de Riel JK, Weinstein H & Liu-Chen LY 2001 Constitutive activation of the mu opioid receptor by mutation of D3.49(164), but not D3.32(147): D3.49(164) is critical for stabilization of the inactive form of the receptor and for its expression. *Biochemistry* **40** 12039–12050. (doi:10.1021/bi0100945)
- Mahavadi S, Sriwai W, Huang J, Grider JR & Murthy KS 2014 Inhibitory signaling by CB1 receptors in smooth muscle mediated by GRK5/ β -arrestin activation of ERK1/2 and Src kinase. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **306** G535–G545. (doi:10.1152/ajpgi.00397.2013)
- Marion S, Oakley RH, Kim KM, Caron MG & Barak LS 2006 A β -arrestin binding determinant common to the second intracellular loops of rhodopsin family G protein-coupled receptors. *Journal of Biological Chemistry* **281** 2932–2938. (doi:10.1074/jbc.M508074200)
- McDonald NA, Henstridge CM, Connolly CN & Irving AJ 2007 An essential role for constitutive endocytosis, but not activity, in the axonal targeting of the CB1 cannabinoid receptor. *Molecular Pharmacology* **71** 976–984. (doi:10.1124/mol.106.029348)
- Mukhopadhyay S & Howlett AC 2001 CB1 receptor–G protein association. Subtype selectivity is determined by distinct intracellular domains. *European Journal of Biochemistry / FEBS* **268** 499–505. (doi:10.1046/j.1432-1327.2001.01810.x)
- Nakajima K & Wess J 2012 Design and functional characterization of a novel, arrestin-biased designer G protein-coupled receptor. *Molecular Pharmacology* **82** 575–582. (doi:10.1124/mol.112.080358)
- Pacher P, Batkai S & Kunos G 2006 The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacological Reviews* **58** 389–462. (doi:10.1124/pr.58.3.2)
- Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, DeWire SM, Violin JD & Lefkowitz RJ 2011 Quantifying ligand bias at seven-transmembrane receptors. *Molecular Pharmacology* **80** 367–377. (doi:10.1124/mol.111.072801)
- Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D *et al.* 2011 Crystal structure of the β 2 adrenergic receptor–Gs protein complex. *Nature* **477** 549–555. (doi:10.1038/nature10361)
- Reiter E, Ahn S, Shukla AK & Lefkowitz RJ 2012 Molecular mechanism of β -arrestin-biased agonism at seven-transmembrane receptors. *Annual Review of Pharmacology and Toxicology* **52** 179–197. (doi:10.1146/annurev.pharmtox.010909.105800)
- Rhee MH, Nevo I, Levy R & Vogel Z 2000 Role of the highly conserved Asp-Arg-Tyr motif in signal transduction of the CB2 cannabinoid receptor. *FEBS Letters* **466** 300–304. (doi:10.1016/S0014-5793(00)01094-2)
- Rovati GE, Capra V & Neubig RR 2007 The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Molecular Pharmacology* **71** 959–964. (doi:10.1124/mol.106.029470)
- Scheer A, Fanelli F, Costa T, de Benedetti PG & Cotecchia S 1996 Constitutively active mutants of the α 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *EMBO Journal* **15** 3566–3578.
- Scheer A, Fanelli F, Costa T, de Benedetti PG & Cotecchia S 1997 The activation process of the α 1B-adrenergic receptor: potential role of protonation and hydrophobicity of a highly conserved aspartate. *PNAS* **94** 808–813. (doi:10.1073/pnas.94.3.808)
- Scott CE, Abrol R, Ahn KH, Kendall DA & Goddard WA III 2013 Molecular basis for dramatic changes in cannabinoid CB1 G protein-coupled receptor activation upon single and double point mutations. *Protein Science* **22** 101–113. (doi:10.1002/pro.2192)
- Shenoy SK & Lefkowitz RJ 2011 β -Arrestin-mediated receptor trafficking and signal transduction. *Trends in Pharmacological Sciences* **32** 521–533. (doi:10.1016/j.tips.2011.05.002)
- Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O & Lefkowitz RJ 2006 β -arrestin-dependent. G protein-independent ERK1/2 activation by the β 2 adrenergic receptor. *Journal of Biological Chemistry* **281** 1261–1273. (doi:10.1074/jbc.M506576200)
- Shim JY, Ahn KH & Kendall DA 2013 Molecular basis of cannabinoid CB1 receptor coupling to the G protein heterotrimer Gai $\beta\gamma$: identification of key CB1 contacts with the C-terminal helix α 5 of Gai. *Journal of Biological Chemistry* **288** 32449–32465. (doi:10.1074/jbc.M113.489153)
- Shukla AK, Westfield GH, Xiao K, Reis RI, Huang LY, Tripathi-Shukla P, Qian J, Li S, Blanc A, Oleskie AN *et al.* 2014 Visualization of arrestin

- recruitment by a G-protein-coupled receptor. *Nature* **512** 218–222. (doi:10.1038/nature13430)
- Szcepek M, Beyriere F, Hofmann KP, Elgeti M, Kazmin R, Rose A, Bartl FJ, von Stetten D, Heck M, Sommer ME *et al.* 2014 Crystal structure of a common GPCR-binding interface for G protein and arrestin. *Nature Communications* **5** 4801. (doi:10.1038/ncomms5801)
- Turu G & Hunyady L 2010 Signal transduction of the CB1 cannabinoid receptor. *Journal of Molecular Endocrinology* **44** 75–85. (doi:10.1677/JME-08-0190)
- Turu G, Szidonya L, Gaborik Z, Buday L, Spat A, Clark AJ & Hunyady L 2006 Differential β -arrestin binding of AT1 and AT2 angiotensin receptors. *FEBS Letters* **580** 41–45. (doi:10.1016/j.febslet.2005.11.044)
- Turu G, Simon A, Gyombolai P, Szidonya L, Bagdy G, Lenkei Z & Hunyady L 2007 The role of diacylglycerol lipase in constitutive and angiotensin AT1 receptor-stimulated cannabinoid CB1 receptor activity. *Journal of Biological Chemistry* **282** 7753–7757. (doi:10.1074/jbc.C600318200)
- Varnai P, Toth B, Toth DJ, Hunyady L & 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)
- Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF & Babu MM 2013 Molecular signatures of G-protein-coupled receptors. *Nature* **494** 185–194. (doi:10.1038/nature11896)
- Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM & Lefkowitz RJ 2003 Independent β -arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *PNAS* **100** 10782–10787. (doi:10.1073/pnas.1834556100)
- Wilbanks AM, Laporte SA, Bohn LM, Barak LS & Caron MG 2002 Apparent loss-of-function mutant GPCRs revealed as constitutively desensitized receptors. *Biochemistry* **41** 11981–11989. (doi:10.1021/bi020275m)
- Woo J & von Arnim AG 2008 Mutational optimization of the coelenterazine-dependent luciferase from Renilla. *Plant Methods* **4** 23. (doi:10.1186/1746-4811-4-23)
- Zhu SZ, Wang SZ, Hu J & el Fakahany EE 1994 An arginine residue conserved in most G protein-coupled receptors is essential for the function of the m1 muscarinic receptor. *Molecular Pharmacology* **45** 517–523.

Received in final form 28 November 2014

Accepted 15 December 2014

Accepted Preprint published online 15 December 2014