The role of β-arrestins in the regulation of CB_1 cannabinoid receptor function

Ph.D. thesis

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INTRODUCTION

7-transmembrane receptors (7TMRs)

7TMRs constitute the largest family of plasma membrane receptors. They have an outstanding physiological role – in fact it is hard to mention a regulatory process in the body where 7TMRs do not play an important role.

During their activity, 7TMRs couple to heterotrimeric G proteins, thus initiating a number of intracellular signaling pathways. Furthermore, G protein-independent signaling pathways of 7TMRs have been identified in the last decade.

The conserved 'DRY' motif (i.e. an Asp-Arg-Tyr amino acid triplet located at the boundary of the 3rd transmembrane helix and the 2nd intracellular loop of the receptor) is generally considered to be a key structural element mediating the G protein activation of 7TMRs. However, studies carried out with several 7TMRs have pointed to the fact that in distinct 7TMRs, the role of the DRY motif in receptor function can be different. Therefore, determining its functional characteristics in a particular receptor always needs direct investigations.

A key element in the adaptation of cells and tissues to altered circumstances is the active regulation of their sensitivity to external stimuli. This can be primarily achieved through the modification of receptor responsiveness. The regulation of 7TMR responsiveness involves three major levels: regulation of receptor production (i.e. downregulation and upregulation), modification of cell surface receptor numbers (via internalization and recycling) and altering receptor sensitivity (desensitization and resensitization).

7TMR internalization occurs mostly via the clathrin-mediated pathway. During this process, the activated receptor gets phosphorylated by G protein-coupled receptor kinases (GRKs), and the activated and phosphorylated receptor binds β-arrestin, which targets it to the clathrin-coated pit (CCP). The CCP invaginates together with the receptor, and then gets pinched off from the membrane, yielding an endocytic vesicle. The fate of the internalized receptor is determined by intracellular sorting mechanisms, the result of which is either degradation or recycling of the receptor back to the plasma membrane.

The β-arrestin proteins

β-arrestins are cytoplasmic regulatory proteins, which play key roles in the desensitization and internalization of 7TMRs. Furthermore, according to studies of the last 10-15 years, β-arrestins

are capable of initiating distinct, G protein-independent signaling pathways. Accordingly, the regulation of their function stays in the focus of today's physiological and pharmacological research. Namely, the G protein- and β-arrestin-dependent pathways of the same 7TMR may be activated separately, a phenomenon called biased signaling. This can lead to a major breakthrough in the optimization of the pharmacological profile of drugs acting on 7TMRs.

The two ubiquitously expressed isoforms of β-arrestins are β-arrestin1 (β-arr1) and β-arrestin2 (β-arr2). Based on the detailed analysis of the relationship between 7TMRs and β-arrestins, 7TMRs can be classified into two major groups: class „A" receptors bind primarily β-arr2, and this binding is transient, i.e. it occurs only at the plasma membrane. In contrast, class „B" 7TMRs bind β-arr1 and β-arr2 with similar affinity, and the binding is more stable, i.e. it is still present after the internalization of the receptor. The impact of these differences on the cellular signaling characteristics of the receptors has also been demonstrated.

The CB_1 cannabinoid receptor (CB_1R)

The CB_1R belongs to the 7TMR superfamily. The receptor is one of the key proteins constituting the endocannabinoid system, and mediates the major part of the known cannabinoid effects (together

with the other cannabinoid receptor, CB_2R, which is expressed primarily in immune cells). CB_1R is present in the central nervous system (where it modulates processes such as learning, memory, emotions and appetite regulation), in the circulatory system (where it participates in the regulation of vascular tone and cardiac function), in liver and adipose tissues (where it regulates metabolic processes of the body), and its expression and regulatory role has been described in many other tissues.

The principal characteristic of its intracellular signaling is he coupling to $G_{i/o}$ proteins. Thus, lowering of cytoplasmic cAMP levels, activation of K^+-channels, inhibition of Ca^{2+}-channels and activation of MAP-kinase cascades are its most important signaling effects.

Internalization of CB_1R

It is known that CB_1R, similarly to most 7TMRs, gets internalized in response to agonist stimulus. This phenomenon has been demonstrated in tumor cell lines and also in cells endogenously expressing the receptor, however, the underlying mechanisms are only partially described. In addition, CB_1R displays constitutive internalization (i.e. basal internalization in the absence of agonist), which has also been described in many cell types, but the physiological importance of this process is not well understood.

Furthermore, it is not known whether the mechanisms of agonist-induced and constitutive CB_1R internalization are the same, or these processes are regulated differentially.

The relationship between CB_1R and β-arrestins

At the beginning of our work it was already known that CB_1R binds β-arr2 following agonist treatment. Furthermore, a role for β-arr2 in CB_1R desensitization (i.e. in the attenuation of the G protein-dependent signaling of the receptor) had also been described. However, no direct data was available concerning the role of β-arr2 in the agonist-induced internalization of CB_1R. Moreover, the relationship between CB_1R and β-arr1, and the affinities for the two β-arrestin isoforms were only poorly investigated. In addition, the role of β-arrestins in the constitutive CB_1R internalization was also not addressed, thus, no direct data was available on whether the mechanisms of these two forms of internalization are truly different.

OBJECTIVES

In our work we wanted to study the hitherto unrevealed details of the relationship between CB_1R and β-arrestins, focusing mainly on the affinity of the receptor for β-arrestins, the role of β-arrestins in CB_1R internalization and identification of newer regions that mediate the β-arrestin binding of the receptor. Thus, we wanted to answer the following questions:

1. How can the binding between CB_1R and β-arr1 be characterized? Is there a difference between the binding affinities of CB_1R for β-arr1 versus β-arr2?

2. What is the role of β-arr2 in the agonist-induced and constitutive internalization of CB_1R? Do these two processes truly differ from each other?

3. What is the role of the conserved DRY motif in the β-arrestin binding and G protein activation of CB_1R?

METHODS

Plasmid constructs and site-directed mutagenesis

For the investigation of CB_1R, untagged or C-terminally mVenus-, mCherry- or Sluc-tagged constructs of the receptor were used. To study receptor internalization, the HaloTag protein was fused N-terminally to the receptor. For the investigation of β-arr1 and β-arr2, untagged or C-terminally Rluc- or GFP- or RFP-tagged constructs were used. For G protein BRET measurements, Rluc-tagged α_o subunit, YFP-tagged β_1 subunit and untagged γ_{11} subunit were used. For internalization BRET, the C-terminally eYFP-tagged ICAM-1 construct, or plasma membrane targeted mVenus or Sluc constructs were used. The cAMP level was monitored using an EPAC-based intramolecular BRET sensor construct. The V54D point mutation into the different β-arr2 constructs, as well as the distinct mutations into the DRY motif of CB_1R and CB_1R-mVenus were inserted using the QuikChange® site-directed mutagenesis kit. The sequence of each construct was verified using automated DNA sequencing.

Cell cultures and transfection

The studies investigating the β-arr1 binding of CB_1R and CB_1R internalization were carried out in HeLa and Neuro-2a cells. For the investigation of the CB_1R-DRY mutants, CHO-K1 cells were used. Cells were transfected on 6-well plates (with glass coverslips in the case of confocal microscopy measurements) with Lipofectamine 2000 reagent, following the manufacturer's instructions.

Bioluminescence resonance energy transfer (BRET) measurements

For the BRET assay measuring the G_o protein activation of CB_1R, cells were transfected with Rluc-tagged α_o subunit, YFP-tagged β_1 subunit, untagged γ_{11} subunit and the (untagged) receptor. Measurements were carried out in a cell suspension, in 96-well plates, after the addition of the cell permeable substrate coelenterazine h. Fluorescence and bioluminescence intensities were recorded using a Mithras LB 940 plate reader.

In the experiments measuring the β-arr1 and β-arr2 binding of the wild-type CB_1R, BRET was measured between mVenus-tagged receptor and Rluc-tagged β-arrestins, under the above circumstances. In the BRET titration experiments studying the affinity between receptors and β-arrestins, cells were transfected using the same

constructs, with varying transfection ratios of donor and acceptor BRET partners. The mVenus/Rluc emission ratio was then determined and average BRET signal changes after agonist stimulus were plotted against this ratio. When investigating the β-arr1 and β-arr2 binding of the different DRY mutant CB_1Rs, BRET was measured between plasma membrane targeted mVenus and β-arr1- or β-arr2-Rluc, with co-transfection of the untagged receptor, under the above circumstances. To study the plasma membrane localization of the different CB_1R variants, BRET was measured between plasma membrane targeted Sluc and mVenus-tagged receptor, in non-stimulated cells. To follow CB_1R internalization, Sluc-tagged CB_1R and eYFP-tagged ICAM-1 plasma membrane protein were used as BRET partners. The effect of the different CB_1R variants on forskolin-induced cAMP elevation was measured using EPAC-BRET, an intramolecular BRET sensor.

Confocal laser microscopy

For confocal microscopy experiments, cells were transfected with appropriately tagged constructs of the receptor, β-arr1 or β-arr2, and with siRNA (where indicated). Measurements were done using Zeiss LSM 510 or Zeiss LSM 710 laser confocal microscopes. Excitation wavelengths were 488 nm for eGFP and Halo-Alexa488, 514 nm for mVenus and 543 nm for mCherry and RFP.

Halo-labeling protocols

To measure agonist-induced internalization of the receptor, cells transfected with Halo-CB_1R were stained for 15 min with Halo-Alexa488, followed by a 30 min treatment with DMSO, WIN55 or WIN55 + AM251. Cells were fixed with 4% formaldehyde and then analyzed with confocal microscope.

To measure constitutive internalization of the receptor, cells were stained for 15 min with Halo-Alexa488, followed by 5 h 45 min incubation without any further treatment, or in the presence of DMSO, WIN55 or WIN55 + AM251. At the end of the 6 h period, cells were fixed and analyzed with confocal microscope.

Western blot measurements

For the Western blot analysis of β-arr2 and clathrin heavy chain, proteins were loaded on SDS polyacrylamide gel and blotted on PVDF membranes. Membranes were blocked and incubated with primary (anti-β-arr2 or anti-clathrin heavy chain), and secondary (HRP-conjugated) antibodies. Total protein amounts were controlled using anti-β-actin primary antibodies. For pERK1/2 Western blot measurements, cells were serum starved for 2 hours and then treated with WIN55 for 0, 5 or 30 min. The measurement was then carried

out similarly as above, using anti-pERK1/2 or anti-ERK1/2 primary antibodies and HRP-conjugated secondary antibodies. Antibodies were visualized using a chemiluminescent substrate. Western blot images were scanned and quantified using the ImageJ software.

Data analysis and statistical evaluation

Data were evaluated using two-way analysis of variance (combined with Holm-Sidak's post-hoc test). G protein and β-arrestin BRET dose-response curves were fitted and statistically compared using the built-in algorithms of the GraphPad Prism software. P values <0.05 were considered statistically significant. To evaluate biased signaling with the equimolar comparison, G protein and β-arr2 BRET dose-response curve points representing the same concentrations for the same receptor were plotted against each other. Equiactive comparison was carried out by determining a 'biased factor', using the E_{max} and EC_{50} values from G protein and β-arr2 BRET dose-response curves, choosing wild-type CB_1R as reference receptor.

RESULTS

Characterization of the relationship between CB_1R and β-arr1

The binding between CB_1R and β-arr1 was first investigated with confocal microscope. In these experiments, mCherry-tagged CB_1R and GFP-tagged β-arr1 or β-arr2 were expressed in the cells, and the redistribution of β-arrestins after agonist stimulus was followed. We observed that β-arr2-GFP, which showed diffuse cytoplasmic localization previously, translocates to the plasma membrane into punctuate structures after the addition of the synthetic CB_1R agonist WIN55. No such β-arr2-GFP puncta were detected in the inner regions of the cytoplasm. In the case of β-arr1-GFP, no redistribution could be detected upon WIN55-stimulus, neither at the plasma membrane, nor in the cytoplasm.

The relationship between CB_1R and β-arr1 was further investigated with the generally more sensitive BRET method: BRET was measured between mVenus-tagged CB_1R and Rluc-tagged β-arr1 or β-arr2. In these experiments, we observed an increase of the BRET signal after WIN55-stimulus in case of β-arr2-Rluc, but such increase was absent with β-arr1-Rluc. The binding between the two proteins was further assessed using a BRET titration approach, which is appropriate for the precise affinity analysis of proteins. These measurements also showed that a binding between CB_1R and β-arr2

occurs, whereas binding to β-arr1 could not be detected in these experiments.

Detailed analysis of the agonist-induced and constitutive internalization of CB_1R

First, the role of β-arr2 in the agonist-induced internalization of CB_1R was investigated. To follow CB_1R internalization with microscope, the HaloTag technique was applied. With this approach, CB_1Rs residing in the plasma membrane can be selectively stained, and their internalization can be subsequently followed.

In case of the co-expression of a dominant-negative β-arr2 (β-arr2-V54D) mutant, the agonist-induced internalization of Halo-CB_1R was substantially lowered compared to cells expressing wild-type β-arr2. In cells transfected with β-arr2-specific siRNA, agonist-induced CB_1R internalization was also inhibited compared to control siRNA transfected cells. This phenomenon was further examined with BRET method, where the removal of the super Rluc-(Sluc-) tagged CB_1R from the plasma membrane was monitored upon agonist-stimulus (the YFP-tagged ICAM-1 protein was used as a plasma membrane marker). Based upon these measurements, the extent of internalization was decreased both by β-arr2-V54D expression and by β-arr2-specific siRNA transfection.

To assess the constitutive internalization of CB_1R, cells expressing Halo-CB_1R were Halo-stained and then left alone for 6 hours. The constitutive CB_1R internalization occurring during this period was not inhibited by the continuous presence of the CB_1R inverse agonist AM251; furthermore, neither β-arr2-V54D expression, nor the transfection of β-arr2-specific siRNA decreased its extent. The siRNA experiments were repeated in Neuro-2a mouse neuroblastoma cells which express CB_1Rs endogenously. In these experiments we also observed that the inhibition of β-arr2 expression has no effect on the constitutively occurring CB_1R internalization. The role of clathrin in the agonist-induced and constitutive CB_1R internalization was then investigated, using clathrin heavy chain-specific siRNA. We found that both forms of internalization could be inhibited by lowering the clathrin heavy chain protein levels.

Investigating the role of the conserved DRY motif in the function of CB_1R

To investigate the role of the conserved DRY region in CB_1R function, the three amino acids constituting the motif were mutated to alanine in every possible (simple, double and triple) combination. Thus, the following mutants were created: CB_1R-ARY, CB_1R-DAY, CB_1R-DRA, CB_1R-DAA, CB_1R-ARA, CB_1R-AAY and CB_1R-AAA.

When investigating the plasma membrane (PM) expression of the mutants, we found that PM expression of CB_1R-DAY, CB_1R-DRA and CB_1R-DAA is lowered but still present, whereas the PM localization of the CB_1R-ARY and CB_1R-ARA mutants is essentially absent.

The G protein activation of the mutants was monitored using a BRET assay measuring the dissociation of G_o protein subunits. The β-arr2 binding was assessed with confocal microscope as well as with a BRET assay measuring the translocation of β-arr2 to the PM. In each case, the effects of WIN55 (synthetic CB_1R agonist) and of 2-AG (endocannabinoid) were analyzed.

Our experiments carried out with the CB_1R-DAY mutant showed that the ability of this mutant to activate G proteins is decreased but not abolished. When investigating β-arr2 binding, we found that a basal β-arr2 binding appears by this mutant; however the β-arr2 binding following agonist-stimulus is lower than in wild-type receptor. In case of CB_1R-DRA mutant we found that the basal G protein-coupling is increased compared to the wild-type receptor, while agonist-induced G protein activation was slightly lowered. The β-arr2 binding was similar to CB_1R-DAY, i.e. appearance of basal β-arr2 binding as well as a decreased agonist-induced β-arr2 binding was observed. By investigation of CB_1R-AAY we observed that this mutant has a substantially impaired G protein activation, both under basal and stimulated conditions. The (basal and stimulated) β-arr2

binding of CB_1R-AAY was however significantly increased. The G protein activation of the CB_1R-DAA mutant was impaired but not absent. However, although a basal β-arr2 binding was characteristic for this mutant, we detected the complete loss of β-arr2 translocation upon agonist-stimulus.

When investigating the β-arr1 binding of the receptors using BRET measurements, we found that the wild-type receptor does not cause a detectable β-arr1 translocation upon WIN55 stimulus, whereas the changes were statistically significant in response to 2-AG. At the same time, the CB_1R-AAY mutant recruited β-arr1 at a significantly increased extent with both agonists. None of the other mutants had a significant impact on the distribution of β-arr1.

The detailed analysis of the G protein activation and β-arr2 binding data of the CB_1R-AAY and CB_1R-DAA mutants (via equimolar and equiactive analyses) showed that the CB_1R-AAY is a β-arr2-biased mutant, whereas the CB_1R-DAA can be considered as a G protein-selective mutant. Further experiments measuring cAMP-inhibition and ERK1/2-phosphorylation showed that the cAMP-lowering effect of CB_1R-AAY was almost completely abolished, whereas its ERK1/2 activating ability was unchanged. In case of CB_1R-DAA, cAMP-lowering effect was present, while ERK1/2-phosphorylation proved to be substantially impaired.

CONCLUSIONS

Based upon our results we conclude that CB_1R binds β-arr2 isoform with substantially higher affinity than β-arr1, and the binding occurs only at or near the plasma membrane, i.e. it is transient. Based on this, we strengthen that CB_1R can be classified as a class 'A' 7TMR.

We have found that β-arr2 plays a role in the agonist-induced internalization of CB_1R, but the constitutive internalization of the receptor is a β-arr2-independent process. Moreover, both forms of internalization occur via a clathrin-mediated pathway.

We have shown that the conserved R3.50 amino acid does not play an exclusive role in the G protein-coupling of CB_1R, and that the absence of the R3.50 residue leads to a basal β-arr2 binding. We demonstrated that CB_1R-AAY is a β-arrestin-selective mutant, since its β-arr1 and β-arr2 binding is substantially increased, while its G protein activation is decreased. In contrast, CB_1R-DAA mutant is biased towards G proteins, since its agonist-induced β-arrestin binding is essentially absent, while its G protein activation is still present, although at a lowered level. Based on our results, the conserved DRY motif plays an important role not only in the G protein activation, but also in the β-arrestin binding of CB_1R.

LIST OF PUBLICATIONS

Publications related directly to the thesis

Gyombolai P, Tóth AD, Tímár D, Turu G, Hunyady L. (2015) Mutations in the 'DRY' motif of the CB_1 cannabinoid receptor result in biased receptor variants. *J Mol Endocrinol*, 54:(1) 75-89. **IF: 3.621**

Gyombolai P, Boros E, Hunyady L, Turu G. (2013) Differential β-arrestin2 requirements for constitutive and agonist-induced internalization of the CB_1 cannabinoid receptor. *Mol Cell Endocrinol*, 372:(1-2) 116-127. **IF: 4.241**

Other publications

Gyombolai P, Pap D, Turu G, Catt KJ, Bagdy G, Hunyady L. (2012) Regulation of endocannabinoid release by G proteins: A paracrine mechanism of G protein-coupled receptor action. *Mol Cell Endocrinol*, 353:(1-2) 29-36. **IF: 4.039** *(Review article)*

Turu G, Várnai P, **Gyombolai P**, Szidonya L, Offertáler L, Bagdy G, Kunos G, Hunyady L. (2009) Paracrine transactivation of the CB_1

cannabinoid receptor by AT_1 angiotensin and other $G_{q/11}$ protein-coupled receptors. *J Biol Chem*, 284:(25) 16914-16921. **IF: 5.328**

Turu G, Simon A, **Gyombolai P**, Szidonya L, Bagdy G, Lenkei Z, Hunyady L. (2007) The role of diacylglycerol lipase in constitutive and angiotensin AT_1 receptor-stimulated cannabinoid CB_1 receptor activity. *J Biol Chem*, 282:(11) 7753-7757. **IF: 5.581**