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Allosteric interactions within the AT_1 angiotensin receptor homodimer: Role of the conserved DRY motif

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ABSTRACT

G protein coupled receptor (GPCR) dimerization has a remarkable impact on the diversity of receptor signaling. Allosteric communication between the protomers of the dimer can alter ligand binding, receptor conformation and interactions with different effector proteins. In this study we investigated the allosteric interactions between wild type and mutant protomers of type 1 angiotensin receptor (AT_1R) dimers transiently expressed in CHO cells. In our experimental setup, one protomer of the dimer was selectively stimulated and the β-arrestin2 binding and conformation alteration of the other protomer was followed. The interaction between β-arrestin2 and the non-stimulated protomer was monitored through a bioluminescence resonance energy transfer (BRET) based method. To measure the conformational alterations in the non-stimulated protomer directly, we also used a BRET based intramolecular receptor biosensor, which was created by inserting yellow fluorescent protein (YFP) into the 3rd intracellular loop of AT_1R and fusing Renilla luciferase (RLuc) to its C terminal region. We have detected β-arrestin2 binding, and altered conformation of the non-stimulated protomer. The cooperative ligand binding of the receptor homodimer was also observed by radioligand dissociation experiments. Mutation of the conserved DRY sequence in the activated protomer, which is also required for G protein activation, abolished all the observed allosteric effects. These data suggest that allosteric interactions in the homodimers of AT_1R significantly affect the function of the non-stimulated protomer, and the conserved DRY motif has a crucial role in these interactions.

1. Introduction

G protein-coupled receptors (GPCRs) are one of the largest and most intensively studied groups of signaling molecules. A wide range of physiological processes like sensory perception, neurotransmission, hormonal regulation, immune defense mechanisms are mediated by GPCRs. Although GPCRs have been considered for a long time as monomeric entities, during the last two decades evidences have accumulated suggesting that GPCRs can form dimeric, or higher ordered oligomeric structures [1]. The possible interactions between the protomers of the oligomer can notably increase the diversity of GPCR signaling [2–4].

To concept and importance of GPCR dimerization was established in the case of class C GPCRs, like $GABA_B$ receptors. $GABA_{B1}$ and $GABA_{B2}$ receptors form obligatory heterodimers, where the dimerization is also required for proper surface expression and signaling [5,6]. In contrast to the widely accepted concept of Class C GPCR oligomerization, the occurrence and functional importance of Class A (rhodopsin like) GPCR oligomerization is still debated [7]. Recent studies show that monomeric β_2 adrenergic [8], μ opiate receptors [9] and rhodopsin [10] reconstructed in lipid vesicles are capable to couple and activate G proteins, showing that dimerization is not strictly required for Class A GPCR signaling. However, a growing list of data indicates that Class A GPCRs are able to form dimers or higher ordered oligomers, also in vivo [11,12], and that oligomerization has important effects on receptor signaling.

The most interesting consequence of GPCR dimerization is its effect on receptor signaling. To achieve this, an allosteric communication between the protomers of the dimer is required: the activation of one protomer has to influence the conformation, and thus signaling properties of the other protomer. Recent data indicate that allosteric interactions within a GPCR dimer can alter ligand binding, receptor conformation and the interactions with different effector proteins such as heterotrimeric G protein or arrestins [3,13–15]. While the concept of intradimeric allosteric effects is widely accepted in general, the molecular mechanism behind it is not clearly understood.

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Type 1 angiotensin receptor (AT_1R) is a Class A GPCR, responsible for most of the known physiological and pathophysiological actions of the peptide hormone angiotensin II (AngII). AT_1R is coupled to Gq/11 heterotrimeric G proteins, and can initiate a wild range of G protein dependent and independent signaling mechanisms [16]. Recent studies have reported that AT_1R is able to form homooligomers, as well as heterodimeric complexes with type 2 angiotensin receptor (AT_2R), MAS receptor, B_2 bradykinin receptor and β_2 adrenergic receptor [17]. In these complexes the signaling of AT_1R to Gq heterotrimeric protein is either enhanced (B_2R) or attenuated (AT_2R, MAS). In our previous work we showed that inhibiting one protomer of AT_1R dimer with a non-peptide antagonist "cross-inhibits" the G protein coupling of the other. [18].

In this study, we investigated the allosteric communication between the protomers of AT_1 receptor homodimer. We used a system, where we were able to stimulate selectively one protomer of the dimer, while the activation process of the other protomer was followed on three different levels of receptor activation: ligand binding, receptor conformation alteration and β-arrestin2 binding. Ligand biding was investigated through radio ligand dissociation experiments, and the alterations of receptor conformation and β-arrestin2 binding were measured in a bioluminescent resonance energy transfer (BRET) based assays. Here we show that agonist binding of one protomer is followed by increased ligand dissociation, altered conformation and β-arrestin2 binding of the other protomer. To investigate the molecular mechanisms behind the observed allosteric effects, selected mutants of AT_1 receptor were used. We found that mutation of the conserved DRY sequence (which was previously known to influence active conformation and G protein activation of the receptor [19]) in the activated protomer abolished all the observed allosteric effects, suggesting a crucial role of this motif in the intradimeric interactions.

2. Material and methods

2.1. Materials

Molecular biology enzymes were obtained from Fermentas (Burlington, Canada), Stratagene (La Jolla, CA), and Invitrogen. Cell culture dishes and plates for BRET measurements were purchased from Greiner Bio-One GmbH (Kremsmunster, Austria). Cell culture media were purchased from Gibco and Lonza. Lipofectamine 2000 and coelenterazine h were from Invitrogen. Candesartan was a gift from AstraZeneca (Mölndal, Sweden). ^{125}I-AngII was provided by Dr. R.C. Speth (Univ. Mississippi, University, MS). Angiotensin II and U-73122 were purchased from Sigma. CHO cells were from American Type Culture Collection (Manassas, VA).

2.2. DNA constructs

We used differently mutated and labeled rat AT_{1a} receptors (Agtr1a, Gene ID: 24180) in our experiments. Constructions of WT-AT_1R-YFP [20], DRY/AAY-AT_1R-YFP [21], WT-AT_1R [21], DRY/AAY-AT_1R [19], CR-AT_1R [21], CR-DRY/AAY-AT_1R [21] and β-arrestin2-RLuc [20] have been described previously. AT_1R-RLuc was created by replacing the yellow fluorescent protein (YFP) of AT_1R-YFP for humanized Renilla luciferase. PM-YFP was created by fusing the N terminal sequence of Lyn kinase to the N terminal of YFP molecule in pEYFP-N1 vector [22,23]. TSTS/A-AT_1R-YFP and CR-TSTS/A-AT_1R were created by mutating T332, S335, T336 and S338 into alanine in AT_1R-YFP or CR-AT_1R by site directed mutagenesis. AT_1R-BS was created by inserting PvuI and Bsp1407I restriction sites between the N231 and K232 amino acids of AT_1R-RLuc (in the 3rd IC loop) with site directed mutagenesis, and YFP was inserted between these restriction sites.

2.3. Cell culture and transfection

The experiments were performed on Chinese hamster ovary (CHO) cells. CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin. The cells were cultured in plastic dishes, trypsinized 24 h prior to transfection, and plated on 6-well plates at a density of 5×10^5 cells/well (for BRET measurements) or on 24-well plates at a density of 10^5 cells/well (for ligand dissociation experiments). Transient transfection was performed with Lipofectamine 2000 according to the manufacturer's protocol. The total DNA amount was 4 μg/well in the case of 6-well plates and 1 μg/well in the case of 24-well plates, while the amount of Lipofectamine 2000 was 2 μl/well or 0.5 μl/well, respectively.

2.4. BRET measurements

BRET measurements were performed 24 h after transfection. CHO cells were detached with Versene, centrifuged, suspended in a modified Krebs-Ringer buffer containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM glucose, 10 mM sodium Hepes, pH 7.4 and transferred to white 96-well plates. The cell density was approximately 100 000 cells/well. Coelenterazine h was added at a final concentration of 5 μM, and readings were collected using a Mithras LB 940 Multilabel Reader (Berthold Technologies, Bad Wildbad, Germany). BRET ratio was defined as (emission at 530 nm)/(emission at 485 nm). In BRET titration experiments YFP fluorescence was measured before the addition of coelenterazine h, also with Mithras LB 940 Multilabel Reader (excitation at 485 nm, emission at 530 nm). Normalized BRET ratio for BRET titration experiments was calculated by subtracting the BRET ratio of only AT_1R-RLuc expressing cells from the measured BRET ratio of AT_1R-RLuc and acceptor (AT_1R-YFP or cytoplasmatic YFP) expressing cells. In the β-arrestin2 binding and AT_1R-BS experiments data are shown as the difference between stimulated and non-stimulated points.

2.5. Ligand dissociation assay

The dissociation of ^{125}I labeled AngII was measured 24 h after transfection at 4 °C to prevent receptor signaling and internalization. Radioligand (~0.01 nM) was added in 0.5 ml of Hepes buffered Ham's F12 medium. After 2 h of incubation cells were washed two times with ice cold PBS, and dissociation was initiated by replacing the medium with 2 ml Hepes buffered Ham's F12 medium, or 1 μM cold AngII in 2 ml Hepes buffered Ham's F12 medium. After different time elapsed, cells were washed two times with ice cold PBS to remove dissociated ^{125}I-AngII, and bound radioactivity was measured by γ-spectrometry after solubilization with 0.5 M NaOH/0.05% SDS. CPM was normalized for the maximal radioactivity measured at the beginning of dissociation.

2.6. Figures, statistical analysis

Figures were created by GraphPad Prism. All data are presented as means of 3–8 experiments ± SEM. We performed two-way analysis of variance in our radioligand dissociation experiments, ** means significant ($p < 0.001$) interaction between receptor type (WT-AT_1R or DRY/AAY-AT_1R) and treatment (cold AngII or only dilution).

3. Results

3.1. AT_1Rs form constitutive homodimers in CHO cells

AT_1R homodimerization has been reported by different groups previously [17,18]. To verify the homodimerization in our system

we performed BRET titration experiments. In this method BRET ratio (which depends on the level of interaction between donor and acceptor molecules) is plotted as a function of acceptor/donor ratio. In the case of specific interaction (e.g. dimerization) this plot shows a saturation curve, while in the case of a non-specific interaction it shows a linear relationship [24–26].

CHO cells were transfected with Renilla luciferase labeled AT_1R (AT_1R-Rluc), yellow fluorescent protein labeled AT_1R (AT_1R-YFP) and empty pcDNA3.1 vector. The amount of AT1R-RLuc coding plasmid was maintained constant, while the amount of AT_1R-YFP coding plasmid was varied to achieve different acceptor/donor ratios. The amount of total transfected DNA was also held constant with addition of varying amount of empty pcDNA3.1 plasmid. The measured BRET ratio was plotted as a function of total YFP fluorescence/RLuc luminescence. In the case of AT_1R-RLuc/AT_1R-YFP interaction we observed a saturation curve (Fig. 1A), which is consistent with the specific interaction between AT_1Rs. The BRET ratio between AT_1R-RLuc and AT_1R-YFP was not altered by stimulating with 100 nM AngII, suggesting that homodimerization is not affected by the activation state of the receptors (data not shown). When BRET ratio was measured between AT_1R-RLuc and plasma membrane marker PM-YFP, we observed a lineal relationship (Fig. 1B) indicating the non-specific nature of this interaction.

3.2. General approach to investigate intradimeric interactions within a homodimer using an antagonist resistant receptor

To detect the allosteric interactions between the protomers of the AT_1R homodimer we should stimulate selectively one protomer of the dimer, while detecting the effects of this stimulation on the other protomer. Selective stimulation in a homodimer is problematic, because agonist stimulus activates both protomers, so the consequences of direct stimulation and allosteric effects are indistinguishable. To resolve this problem, we used an antagonist resistant mutant receptor, described previously by our group. S109Y-AT_1R does not bind the non-peptide antagonist candesartan, while the main properties of this mutant receptor (such as surface expression, AngII binding, G protein dependent and independent signaling) are unaltered [21]. In our system, this candesartan resistant (CR-AT_1R) mutant receptor was co-expressed with candesartan sensitive, fluorescent labeled (AT_1R-labeled) AT_1Rs in CHO cells. Labeling of the candesartan sensitive receptors allowed us to perform specific functional assays to follow their activation, while in the presence of candesartan we could stimulate selectively the CR receptors. In the cells co-expressing these two types of receptors, three subpopulations of receptor dimers were expected to occur: dimers composed of two CR-AT_1R, two AT_1R-labeled and CR-AT_1R/AT_1R-labeled dimers (Fig. 2) (of course this is an over simplified model, there may be also monomers and heterodimers with endogenous GPCRs present in the cells, but we can assume that most of the receptors in our overexpression system are present in these mentioned three forms). In the absence of candesartan, AngII stimulus can activate both receptors, so we can detect the activation of dimers containing labeled receptors (Fig. 2A). Pre-treatment of the CHO cells with the non-peptide AT_1R antagonist candesartan blocks AT_1R-labeled, but leaves the CR-AT_1Rs intact (Fig. 2B). Dimers containing two CR-AT_1Rs are activated by AngII, but due to the lack of labeling produce no signal. Dimers composed of two AT_1R-labeled are blocked by candesartan, so they also do not produce signal. However, in CR-AT_1R/AT_1R-labeled dimers we can stimulate the CR protomer (protomer S for "stimulated") and detect the activation process of the labeled protomer (protomer L for "labeled"). So the measured changes in our assays in the presence of candesartan are specific for the CR-AT_1R/AT_1R-labeled dimers and represent an interaction between protomer S and protomer L. It is important to note, that the "homodimers" used in our experiments are not real homodimers (they are not composed of two fully identical protomers), but the protomers of these dimers are always mutated or labeled AT_1Rs, so we are referring these dimers as homodimers in our article.

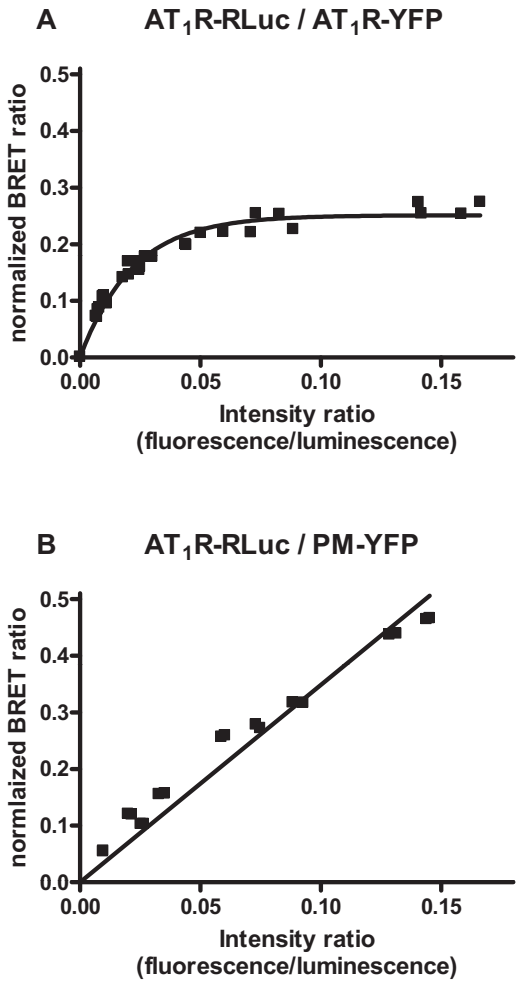

Fig. 1. Demonstration of AT_1R homodimerization is CHO cells using BRET titration experiments: CHO cell were transfected with AT_1R-RLuc and either with AT_1R-YFP (A) or with plasma membrane targeted PM-YFP (B). The amount of transfected AT_1R-RLuc coding plasmid was maintained constant, while different amount of acceptor (AT_1R-YFP or PM-YFP) coding plasmid was used to reach different acceptor/donor ratios. The total amount of transfected DNA was also maintained constant with the addition of varying amount of empty pcDNA3.1 plasmid. Total RLuc luminescence and YFP fluorescence were measured at the beginning of each experiment and intensity ratio was calculated as (total fluorescence)/(total luminescence). The luminescence intensities were much higher than fluorescence intensities with our experimental settings (even in the case of approximately equal protein expression levels), so the intensity ratio shows not directly the ratio of expressed acceptor and donor protein amount, but is proportional with it. Intensity ratio ~0.025 represents a 1:1 acceptor/donor ratio. The calculation of normalized BRET ratio is described in Section 2.

To detect the activation of protomer L, we used two different approaches: (A) the measurement of the interaction between the YFP-labeled protomer L and RLuc-labeled β-arrestin2 by BRET experiments, and (B) the measurement of the conformation changes of the protomer L using a newly developed, also BRET based intramolecular receptor biosensor.

3.3. β-Arrestin2 binds to the non-stimulated protomer of the activated AT_1R dimer

Arrestins bind to GPCRs following agonist stimulation, and play an important role in receptor desensitization, internalization and

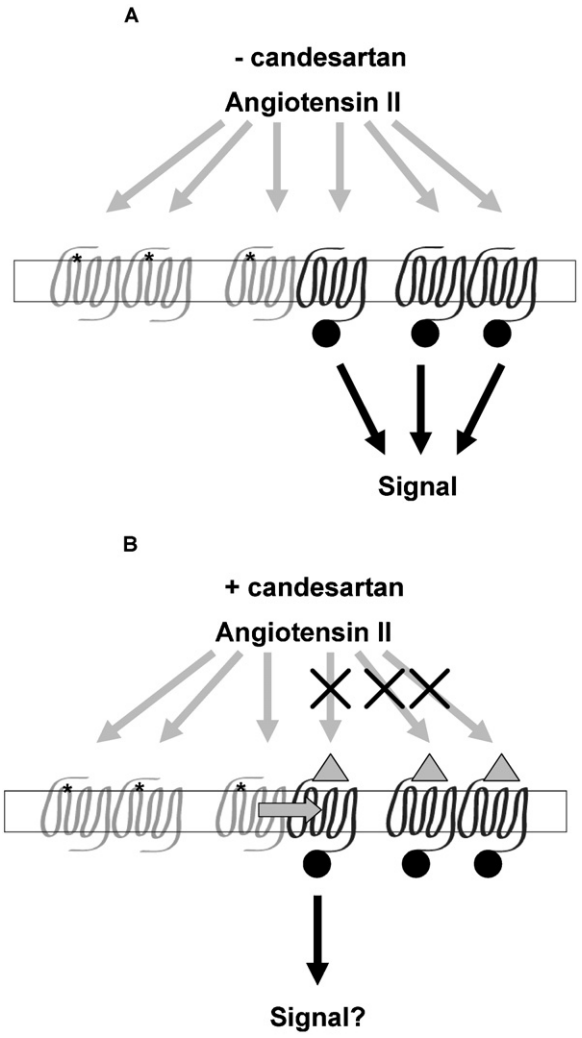

Fig. 2. Selective stimulation of one protomer in the AT_1R homodimer: CHO cells were co-transfected with candesartan resistant, unlabeled CR-AT_1R (gray, * shows the place of S109Y mutation) and candesartan sensitive, fluorescent labeled AT_1R-labeled (black). Labeling of AT_1R-labeled (black circle) allowed us to follow the activation of these receptors trough different functional assays. In the plasma membrane of CHO cells CR-AT_1R/CR-AT_1R, AT_1R-labeled/AT_1R-labeled and CR-AT_1R/AT_1R-labeled dimers were present. (A) Angiotensin II stimulus in the absence of candesartan was able to activate both CR-AT_1R and AT_1R-labeled, so we could detect the activation of the labeled receptors (black arrow). (B) In the presence of candesartan (gray triangles), AT_1-labeled were blocked, so we could only stimulate the unlabeled CR-AT_1 receptors. In this case CR-AT_1R/CR-AT_1R dimers and AT_1R-labeled/AT_1R-labeled dimers produced no signal (due the lack of labeling or activation, respectively), thus the only signal in this setting came from the CR-AT_1R/AT_1R-labeled dimer. In this dimer, we stimulated one protomer (CR-AT_1R: protomer S) and detected the activation of the other protomer (AT_1R-labeled: protomer L), thus the detected signal reported an interaction between the protomers (gray arrow).

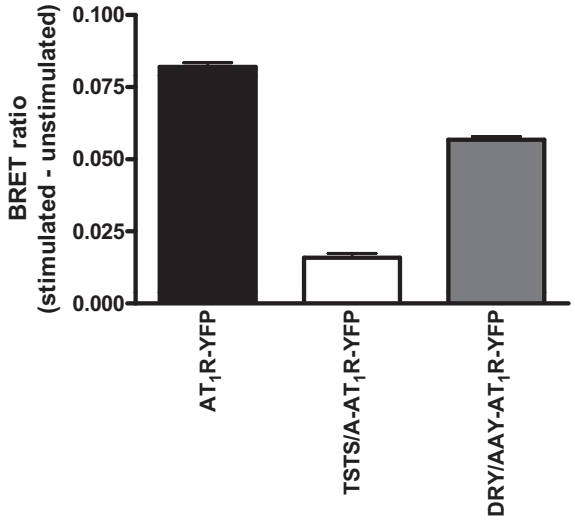

Fig. 3. β-arrestin2 binding of different AT_1R mutants: to characterize the β-arrestin2 binding ability of the different AT_1R mutants used in our experiments, we measured the interaction by BRET between β-arrestin2-RLuc and YFP labeled AT_1R or different AT_1R mutants (AT_1R-YFP, TSTS/A-AT_1R-YFP and DRY/AAY-AT_1R-YFP) coexpressed in CHO cells. The difference between BRET ratio of the stimulated (100 nM AngII) and non-stimulated (vehicle) points is plotted, measured 10 min after stimulation. $n = 3$, mean ± SEM.

as a scaffold protein promote the activation of different signaling cascades [27]. Measurement of the interaction between the non-stimulated protomer of the AT_1R dimer and β-arrestin2 allowed us to detect activation state of this protomer as a putative consequence of allosteric interactions within the receptor dimer.

First, we wanted to characterize the β-arrestin2 binding of the wild type AT_1R and the different AT_1R mutants used in our later experiments. We followed the receptor β-arrestin2 interaction by measuring the BRET ratio changes between AT_1R-YFP and β-arrestin2 labeled with Renilla luciferase (β-arrestin2-RLuc). After AngII stimulus (100 nM) the BRET signal measured between wild type AT_1R-YFP and β-arrestin2-RLuc increased (Fig. 3), showing the well known association between the activated receptor and β-arrestin2 [20]. This interaction is dependent on the phosphorylation state of different serine and threonine residues at the C terminal tail region of the receptor [28]. In the TSTS/A mutant of AT_1R-YFP these residues were mutated to alanine, which resulted in a decreased of BRET signal, due to the decreased β-arrestin2 activation of this mutant. The DRY/AAY-AT_1R-YFP was able to recruit β-arrestin2 comparable to the wild type receptor. This mutant receptor is mutated at the conserved DRY sequence at the cytoplasmatic side of the 3rd TM segment, cannot achieve fully active conformation, cannot activate heterotrimeric G protein, but capable to bind arrestins [19,29].

To investigate the effect of the activation of protomer S on the β-arrestin2 binding of protomer L we co-expressed candesartan resistant CR-AT_1R (protomer S) and candesartan sensitive WT-AT_1R-YFP (protomer L) with β-arrestin2-RLuc in CHO cells. Stimulation (AngII, 100 nM) in the absence of candesartan resulted in a robust increase of BRET signal (Fig. 4A), in accordance with the direct activation of WT-AT_1R-YFP in this case (Fig. 2A). When CHO cells were pre-incubated with the non-peptide antagonist candesartan (Cand 10 μM) AngII stimulus resulted in a smaller but significant increase of BRET signal, which was the consequence of interaction between the non-stimulated protomer L and β-arrestin2. When both protomers were candesartan sensitive, no changes of the BRET signal were observed (Fig. 4B), indicating that the applied candesartan dose was high enough, to fully block the candesartan sensitive receptors.

To evaluate the molecular mechanism behind the observed β-arrestin2 binding of the non-stimulated protomer L we inserted selected mutations into protomer S and/or protomer L and investigated the effects of these mutations on the measured BRET signal in the presence of candesartan.

To investigate the role of the arrestin binding of protomer S in the observed signal, we created CR-TSTS/A-AT_1R, which mutant is candesartan resistant, but its arrestin binding is impaired (Fig. 3). In CHO cells co-expressing CR-TSTS/A-AT_1R as protomer S with WT-AT_1R-YFP as protomer L we observed the same increase of BRET signal, as in the CR-AT_1R expressing CHO cells (Fig. 4C). These experiments indicate, that ability of protomer S to bind arrestin

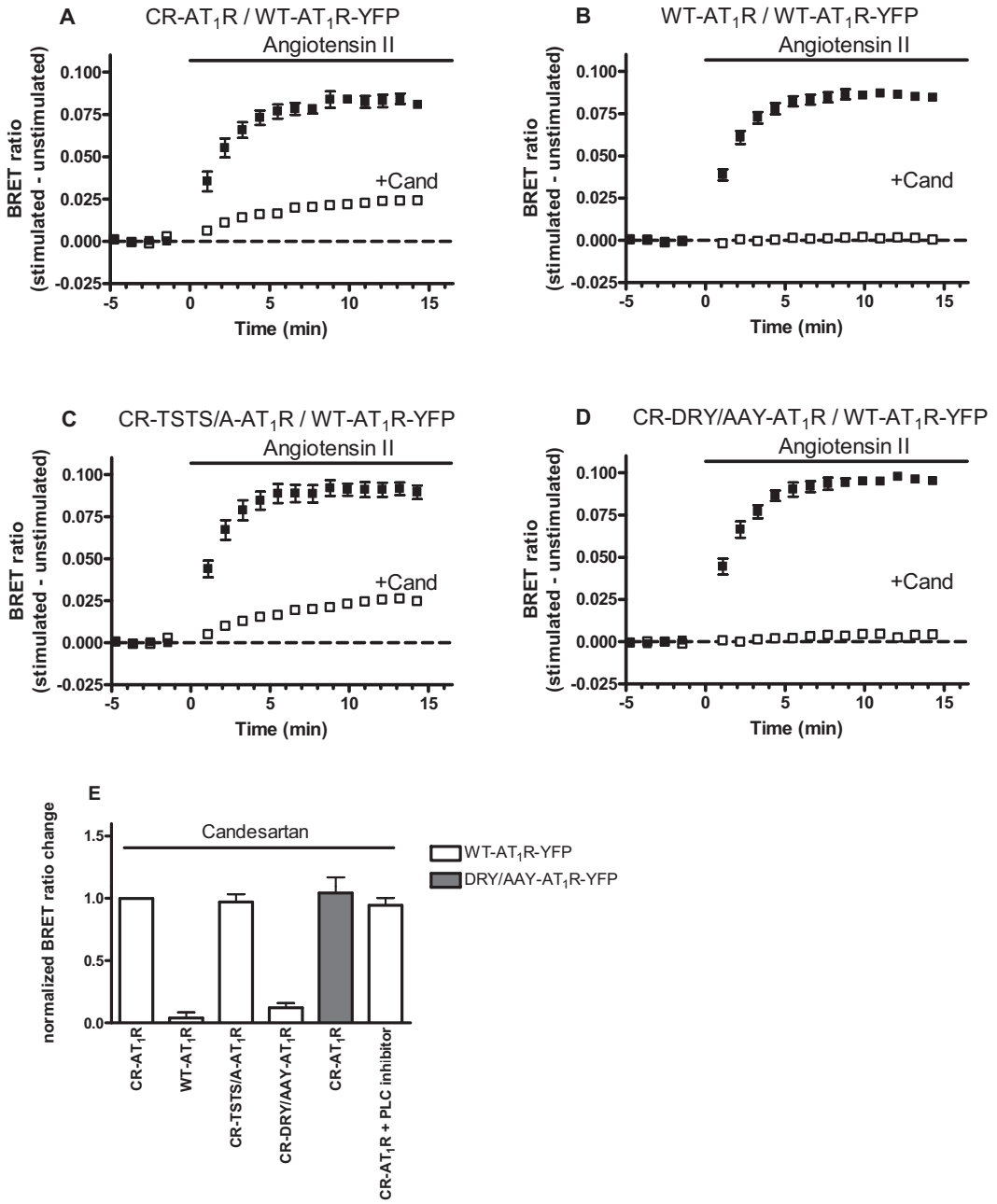

Fig. 4. Measurement of the allosteric interactions within AT_1R homodimer via β-arrestin2 binding: (A–D) CHO cells were co-transfected with β-arrestin2-RLuc, CR-AT_1R (A), WR-AT_1R (B), CR-TSTS/A-AT_1R (C) or CR-DRY/AAY-AT_1R (D) as protomer S and AT_1R-YFP as protomer L. Cells were pretreated for 5 min with 10 μM candesartan (□) or vehicle (■), stimulated with 100 nM AngII at 0 time point, and the difference between BRET ratio of the stimulated and non-stimulated (vehicle) points is plotted. (E) Column diagram of the BRET ratio changes (stimulated–unstimulated) measured in the presence of candesartan in different protomer S (horizontal axis) and protomer L (column color) expressing CHO cells. Measurements were performed after 5 min candesartan (and in one case 2 μM PLC inhibitor U-73122) treatment and 10 min angiotensin II stimuli, and BRET changes were normalized to CR-AT_1R/WT-AT1R-YFP expressing cells. $n = 4–8$, mean ± SEM.

plays no role in the β-arrestin2 binding of the non-stimulated protomer L.

The role of the fully active conformation of the activated protomer was investigated with the CR-DRY/AAY-AT_1R. This mutant is candesartan resistant, able to activate β-arrestin2 (Fig. 3), but cannot achieve fully active receptor conformation nor activate G protein. Expressing CR-DRY/AAY-AT_1R as protomer S resulted no increase of BRET ratio in candesartan pre-treated CHO cells (Fig. 4D). In contrast, when protomer L contained the DRY/AAY mutation (DRY/AAY-AT_1R-YFP), stimulation of the CR-AT_1R as protomer S was followed by the rise of BRET signal (Fig. 4E). Thus the intact DRY sequence of the activated protomer S, and thus fully active receptor conformation is required for the β-arrestin2 binding of protomer L.

We also wanted to examine the effects of signal transduction mechanisms on the observed signal. The main G protein dependent signaling mechanism activated by AT_1R is the activation of Gq/11 heterotrimeric G proteins and the subsequent activation of phospholipase C (PLC) β enzymes. We pre-treated CR-AT_1R (protomer S), WT-AT_1R-YFP (protomer L) and β-arrestin2-RLuc co-expressing cells with 2 μM PLC inhibitor U-73122 for 5 min. This concentration of U-73122 fully inhibited the 100 nM AngII

evoked Ca^{2+} signal (detected with FURA2) in AT_1R expressing cells (data not showed). In these cells we could not observe any inhibition of the BRET signal in the presence of candesartan (Fig. 4E), indicating that PLC dependent signaling mechanisms play no role in the β-arrestin binding of protomer L.

3.4. Conformational alterations in the non-stimulated protomer of the dimer

To investigate the allosteric effects within the receptor dimer directly on the level of receptor conformation, we created a BRET-based intramolecular AT_1R biosensor, similar to the sensor first described by Vilardaga et al. [30–32]. AT_1R-BS contained a YFP molecule in its 3rd intracellular loop, and Renilla luciferase was fused C terminally (Fig. 5A). Activation of AT_1R-BS results in changes in receptor conformation, thus it has an effect on the distance and/or orientation of the donor and acceptor, creating a BRET signal. Stimulation of AT_1R-BS with AngII (100 nM) is followed by a decrease of the BRET signal (Fig. 5B). Candesartan pre-treatment (10 μM) blocked the effects of AngII (Fig. 5C).

Co-expression of AT_1R-BS as protomer L with CR-AT_1R as protomer S allowed us to investigate the conformation alterations in the non-stimulated protomer. The kinetics of the BRET signal produced by AT_1R-BS changed remarkably by the co-expression of CR-AT_1R even in the absence of candesartan: AngII stimulus resulted in a fast increase of the signal, followed by the previously observed decrease of the BRET ratio (Fig. 6A). In the presence of candesartan only the initial rise of the BRET ratio was observed. The possible explanation for this phenomenon is that the initial rise of BRET signal is due the allosteric effect of the stimulated protomer, while the subsequent decrease is the consequence of the direct activation of AT_1R-BS (in the absence of candesartan).

To further investigate the role of the DRY motif in the intradimeric interactions, CR-DRY/AAY-AT_1R as protomer S was co-expressed with AT_1R-BS. In this case, the signal was similar to that, when AT_1R-BS was expressed alone, but the initial rise of BRET signal was not observed (Fig. 6B). After candesartan pre-treatment no changes of BRET signal were observed. These data also indicate that the presence of DRY/AAY mutation is the activated protomer S eliminated the intradimeric interactions.

3.5. Ligand binding of the AT_1R homodimer: negative cooperativity

Cooperative ligand binding can also be the consequence of allosteric interactions within a GPCR dimer [33,34]. The ligand binding of one protomer can modulate positively or negatively the ligand affinity of the other protomer. To investigate the presence of cooperative ligand binding in the AT_1R homodimer, we performed radio ligand dissociation experiments with ^{125}I labeled AngII. CHO cells expressing WT-AT_1R were pre-incubated with 0.01 nM ^{125}I-AngII for 2 h. This low concentration of tracer bound only to a low portion of AT_1Rs (Kd of AT_1R for AngII is in the nanomolar range), so we could assume that only one protomer of a dimer is occupied by ^{125}I-AngII. The experiments were performed at 4 °C to inhibit receptor internalization. After 2 h the unbound tracer was washed out, and the dissociation of bound ^{125}I-AngII was measured in the absence and the presence of saturating concentration (1 μM) of cold AngII. We observed that the addition of unlabeled AngII

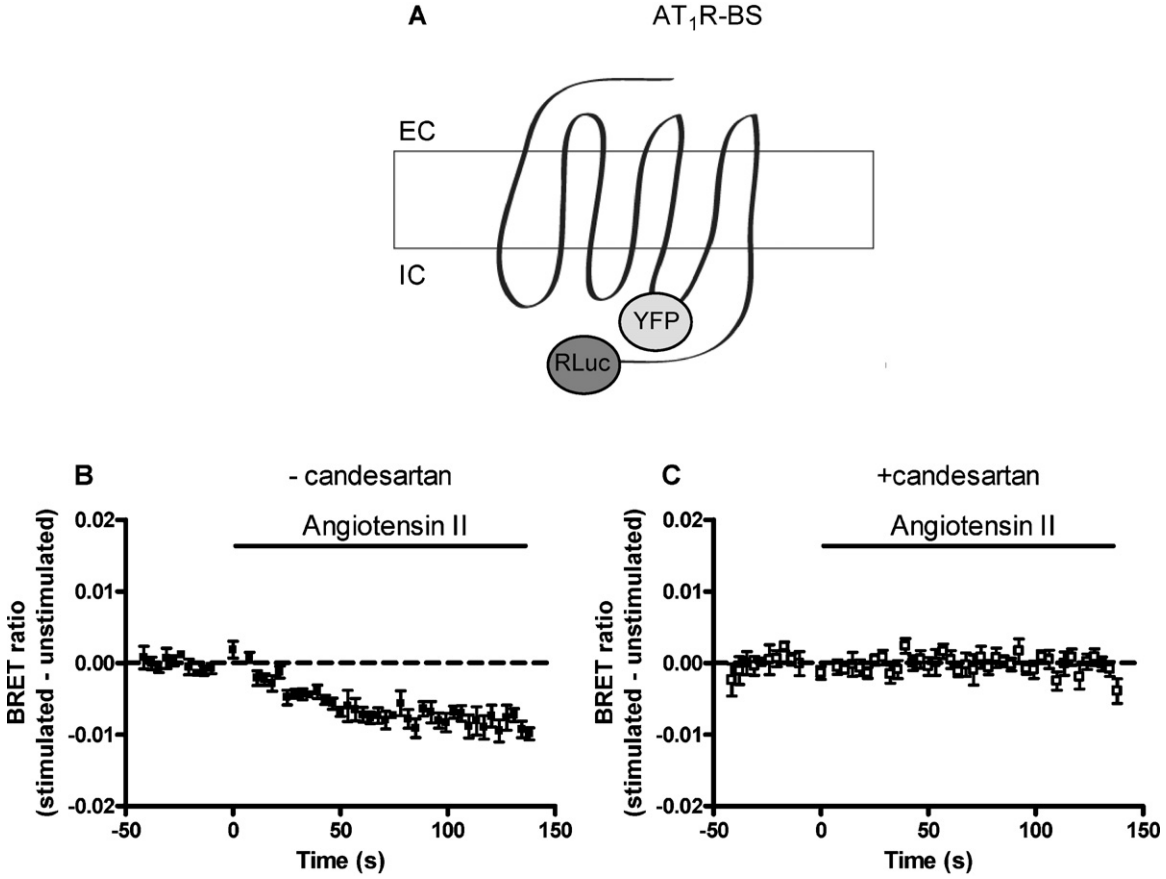

Fig. 5. AT_1R-BS reports directly the conformational alterations of the receptor: (A) to monitor the conformation of AT_1R, a BRET based intramolecular receptor biosensor was created, by inserting YFP into the 3rd IC loop of AT_1R between N231 and K232, and fusing RLuc to its C terminal region. (B) AngII stimulus (100 nM) is followed by a decrease of the BRET signal. (C) Candesartan pre-treatment (10 μM for 5 min) blocked the effects of angiotensin II. The difference between stimulated and non-stimulated points is plotted. $n = 3$, mean ± SEM.

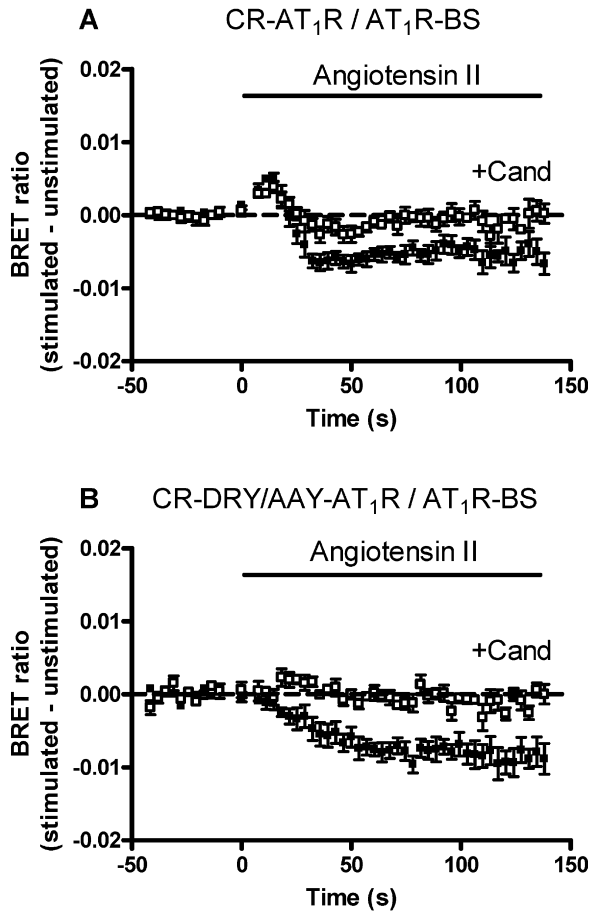

Fig. 6. Measurement of the allosteric interactions within AT_1R homodimer via AT_1R-BS: CHO cells were co-expressing either CR-AT_1R (A) or CR-DRY/AAY-AT_1R (B) as protomer S with AT_1R-BS as protomer L. Cells were pretreated for 5 min with 10 μM candesartan (□) or vehicle (■), stimulated with 100 nM AngII at 0 time point, and the difference between BRET ratio of the stimulated and non-stimulated (vehicle) points is plotted. $n = 3–5$, mean ± SEM.

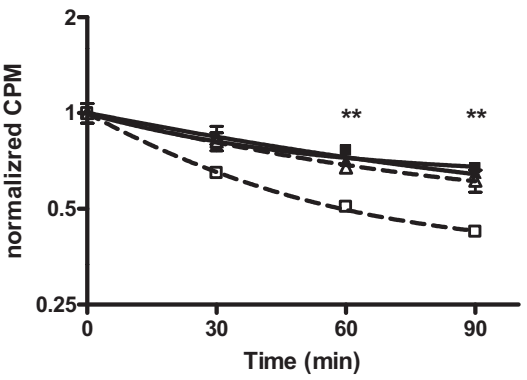

Fig. 7. Negative cooperative ligand binding of AT_1R homodimer: CHO cells expressing WT-AT_1R (■) or DRY/AAY-AT_1R (▲) were pre-incubated with 0.01 nM ^{125}I-AngII for 2 h at 4 °C. Unbound tracer was washed out, and dissociation was measured in the absence (black) and the presence (open) of 1 μM cold AngII. After different elapsed time the cells were washed to remove dissociated ^{125}I-AngII and the remained bound radioactivity was measured. (**: significant ($p < 0.001$) interaction between receptor-type and cold AngII treatment). $n = 3$, mean ± SEM.

accelerated the dissociation of the tracer (Fig. 7), showing negative cooperativity between the ligand binding of the protomers. When we performed the same experiments with DRY/AAY-AT_1R expressing CHO cells, we did not detect any effect of cold AngII on the dissociation kinetics (Fig. 7). These results also show the absence of allosteric interaction (negative cooperativity) between the protomers of AT_1R dimer, when DRY sequence is mutated.

4. Discussion

Dimerization or oligomerization of GPCRs is an emerging field of receptor research. Growing sets of data are indicating homo- and heterodimerization of different GPCRs, and the consequences of altered functions of these oligomers have important pharmacological perspectives. Allosteric interactions between the protomers of the dimers literally influence every aspect of receptor signaling, and are able to alter ligand binding, receptor conformation and interactions with different effector proteins (heterotrimeric G proteins, arrestins, RGS proteins etc.). Modulation of the signaling in a GPCR heterodimer through allosteric interactions between the protomers can increase the specificity and selectivity of different pharmacological therapies [35]. Understanding of the molecular mechanism behind these allosteric interactions can further facilitate the pharmacological interventions.

Here we demonstrated that activation of one protomer of the AT_1R homodimer lead to the β-arrestin2 binding, altered conformation and decreased ligand binding (increased dissociation) of the other protomer. Examining the effects of different mutations showed that the intact DRY sequence is required in the activated protomer for the allosteric interactions.

Detecting the interaction between the non-stimulated protomer of a dimer and β-arrestin2 through BRET has been previously showed to be a useful tool to study dimerization. Hansen et al. [36] showed that the co-expression of WT-AT_1R rescued the β-arrestin2 activation of the binding deficient K199A mutant AT_1R, which was unable to bind angiotensin II. This method was also used to provide evidence for the dimerization of AT_1R and the type 2 angiotensin receptor (AT_2R) [37], and to provide evidence against the dimerization between AT_1R and B_2 bradykinin receptor [36]. The rational of this approach is that β-arrestin2 binds to the stimulated protomers of the dimers, and due to the close molecular proximity of the other protomer an increase in the BRET signal occurs. Our experiments also showed an increased BRET signal between β-arrestin2-RLuc and the non-stimulated, YFP-labeled protomer L (Fig. 3). However, activation of the TSTS/A mutant receptor, which is unable to bind β-arrestin2, caused the same interaction between protomer L and β-arrestin2 (Fig. 4C) as the WT receptor (Fig. 4A), whereas stimulation of the DRY/AAY mutant receptor did not cause detectable interaction under similar conditions (Fig. 4D). These data suggest that β-arrestin2 binds directly to the non-stimulated protomer. However, it is important to note, that the possibility cannot be excluded that the observed interaction between protomer L and β-arrestin2 is a consequence of a signaling event, instead of a direct allosteric interaction between the protomers, because β-arrestin2 binding is a quite distant step of receptor activation process. To make this possibility less likely we performed experiments in the presence of PLC inhibitor U-73122. In this case we observed the same BRET signal between protomer L and β-arrestin2, which suggests that this interaction is independent the PLC dependent signaling mechanisms (which are the main signaling pathway of the Gq/11 coupled AT_1R).

We created a BRET-based intramolecular biosensor of AT_1R activation, which contained YFP in its third IC loop and RLuc fused to the C terminus, to directly monitor the early conformational changes of receptor activation, when signaling-mediated crosstalk is less likely. This sensor was created based on previously reported FRET-based sensors of α_{2a} adrenergic and parathyroid hormone receptor activation [30–32]. These sensors were able to detect very rapid (half time τ between 50 and 1000 ms) changes in FRET signal.

AT_1R-BS expressed in CHO cells showed an AngII-induced decrease of the BRET signal with upon a much slower kinetics ($\tau \sim 25$ s) (Fig. 5). However, our experimental conditions did not permit detection of extremely rapid changes in the BRET signal due to the limitations of our equipments and we also did not use very high concentrations of the agonist, which is necessary to minimize the time of ligand–receptor association [30]. A conformational sensor of B2 bradykinin receptor was also created by Chachisvilis et al. [38]. The activation kinetic of this sensor was relatively slow, similar to our receptor. These data raise the possibility that the activation kinetic of different GPCRs is notably different, and the activation of peptide receptors may be slower. One other possibility is that our sensor does not report the initial conformation alteration of AT_1R upon agonist stimuli, but the faster activation kinetic of AT_1R-BS than the activation kinetics of receptor–arrestin interaction ($\tau \sim 100$ s) argues against this possibility.

Co-expression of AT_1R-BS with CR-AT_1R allowed us to detect the conformational alterations in the non-stimulated protomer L of the dimer (Fig. 6). We detected an early increase of BRET the signal (Fig. 6A) in contrast to the decrease observed after direct stimulation of AT_1R-BS (Fig. 5B). This suggests a different conformation of the non-activated protomer than the directly stimulated one. Vilardaga et al. [32] showed similar results in case of the α_{2a} adrenergic receptor and μ opiate receptor heterodimer. The decreased FRET signal of the α_{2a} receptor sensor was partially inhibited by co-stimulation with a μ receptor agonist. An interesting phenomenon detected by our experiments is that the early increase of BRET signal (thought to be the consequence of allosteric interactions) has a faster kinetic than the decrease of BRET signal reporting the direct activation of AT_1R-BS (Figs. 5 and 6). It is possible, that AT_1R-BS has a slower activation kinetic than that of the wild type receptor (the YFP molecule inserted in the third IC loop can affect the conformation of the sensor, and make its activation slower). The allosteric effects on the non-stimulated protomer are evoked from the CR-AT_1R, so it is possible, that it represents the faster activation kinetic of the wild type receptor, while decrease of BRET signal represents the slower activation of AT_1R-BS.

We also observed negative cooperativity between the protomers of the AT_1R homodimer. Cold AngII significantly increased the dissociation kinetics of ^{125}I labeled AngII (Fig. 7). Although alternative interpretations can be also found in the literature, negative cooperativity is a characteristic hallmark of allosteric interactions in receptor dimers [33,34]. The increased dissociation of labeled AngII in the presence of unlabeled agonist could be also a consequence of the inhibition of rebinding, because in the absence of cold agonist dissociated ^{125}I-AngII could rebind to the receptor, while in the presence of saturating concentration of unlabeled AngII the rebinding is inhibited. However, the dilution of the specimen through dissociation made rebinding less likely, and also the lack of increased dissociation in the case of the DRY/AAY-AT_1R in the presence of cold AngII argues against this hypothesis. Chabre et al. [39] suggested in a recent article that negative cooperativity could be a consequence of competition for heterotrimeric G proteins, even in the absence of dimerization. The high affinity binding state of a receptor requires the heterotrimeric G protein–receptor interaction, so decreased ligand binding (increased dissociation) could represent the competition of receptors for the same pool of G proteins. However, our ligand binding experiments were performed at 4 °C, which makes G protein-mediated events less likely. In addition, the dissociation kinetics of the DRY/AAY-AT_1R mutant receptor was not affected by the presence of cold AngII, despite the high affinity of this receptor, which suggest that G protein interaction, but not activation, of this receptor may occur. Thus, the lack of negative cooperativity of DRY/AAY-AT_1R suggests that the negative cooperativity of WT-AT_1R is caused by allosteric interactions between the protomers, and not the consequence of competition for the heterotrimeric G proteins.

Our data suggests that activation of one promoter of the AT_1R dimer favors an altered receptor conformation of the non-stimulated protomer, which is different from the conformation of the activated protomer. This altered conformation results in increased ligand dissociation and promotes the receptor β-arrestin2 interaction. Previous studies also suggest functional asymmetry of GPCR dimers. In purified, 5-hydroxytryptophan labeled LTB4 leukotriene receptor Damian et al. [40] have reported that only the agonist bound protomer of the dimer is in active state, while the other is in an intermediate conformation [40]. Experiments using α_{2a} adrenergic receptor and μ opiate receptor heterodimer [32] and the D2 dopamine receptor homodimer [41] also showed that only one protomer of the dimer is in fully active conformation, while the other protomer modulates its activity.

Another interesting finding detected in our experiments was the complete absence the interactions when the stimulated protomer contained the DRY/AAY mutation. DRY sequence at the cytoplasmatic side of the 3rd TM domain is one of the most conserved sequences among GPCRs, playing a crucial role in receptor activation process [42]. Mutations in the DRY sequence can lead to various alterations in receptor function from constitutive activity to decreased receptor activity. Recent crystallography data also suggests direct interaction between this sequence and the α subunit of heterotrimeric G protein in the activated state of receptor [43]. In the case of AT_1R, the DRY/AAY-AT_1R is unable to achieve fully active conformation, is unable to activate heterotrimeric G protein but binds β-arrestin2 normally [19,29]. Simplest explanation for the absence of intradimeric interactions is that the fully active conformation of the activated protomer is required to initiate the allosteric effects to the non-activated receptor. Another, interesting possibility is that the heterotrimeric G protein plays a role in conducting the allosteric effects on the other protomer. It has been suggested that C terminal helices of α subunit and βγ subunit play a role in the receptor–heterotrimeric G protein interaction. The distance between these two regions is greater than the width of a monomeric receptor, thus it was hypothesized that these two regions interact with different protomers of the dimer [14]. This model makes it possible that activation of one protomer promotes a conformation alteration of the heterotrimeric G protein, and the interaction of the G protein with the non-stimulated protomer initiates its altered conformation. Based on this model, when DRY/AAY-AT_1R is the activated protomer, it cannot activate the heterotrimeric G protein, and therefore unable to promote the conformation alteration of the other protomer. The results of another study are also compatible with this model. Damian et al. [40] reported that the non-stimulated protomer of LTB4 receptor dimer is in different conformation in the presence or absence of heterotrimeric G protein in their in vitro system. The essential role of DRY sequence in intradimeric allosteric interactions also explains that in our previous study we could not detect transactivation of the IP_3 response using CR-DRY/AAY-AT_1R [18].

In summary we detected altered ligand binding, receptor conformation and β-arrestin2 binding in the non-stimulated protomer of AT_1R homodimer. These findings suggest that direct intradimeric allosteric interactions occur between the receptor protomers. The DRY/AAY mutant AT_1R did not show such interactions, suggesting that these allosteric effects require the fully active conformation, and/or G protein activation of the stimulated protomer, highlighting the molecular mechanisms behind the allosteric interactions.

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