Angiotensin type 1A receptor regulates β-arrestin binding of the β_2-adrenergic receptor via heterodimerization

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Abstract

Heterodimerization between angiotensin type 1A receptor (AT_1R) and β_2-adrenergic receptor (β_2AR) has been shown to modulate G protein-mediated effects of these receptors. Activation of G protein-coupled receptors (GPCRs) leads to β-arrestin binding, desensitization, internalization and G protein-independent signaling of GPCRs. Our aim was to study the effect of heterodimerization on β-arrestin coupling. We found that β-arrestin binding of β_2AR is affected by activation of AT_1Rs. Costimulation with angiotensin II and isoproterenol markedly enhanced the interaction between β_2AR and β-arrestins, by prolonging the lifespan of β_2AR-induced β-arrestin2 clusters at the plasma membrane. While candesartan, a conventional AT_1R antagonist, had no effect on the β-arrestin2 binding to β_2AR, TRV120023, a β-arrestin biased agonist, enhanced the interaction.

These findings reveal a new crosstalk mechanism between AT_1R and β_2AR, and suggest that enhanced β-arrestin2 binding to β_2AR can contribute to the pharmacological effects of biased AT_1R agonists.

Highlights:

Heterodimerization between AT_1R and β_2AR enhances β-arrestin coupling of β_2AR.

Heterodimerization increases the lifespan of β-arrestin2 clusters after β_2AR stimulation.

Biased AT_1R ligands alter the function of heterodimerized β_2AR.

Keywords: GPCR, heterodimerization, arrestin, BRET, biased signaling

Abbreviations: G protein-coupled receptors (GPCR), Angiotensin type 1A receptor (AT_1R), β_2-adrenergic receptors (β_2AR), serotonin 2C receptor ($5HT_{2C}R$), angiotensin II (AngII), isoproterenol (ISO), *Renilla* luciferase (Rluc), Super *Renilla* luciferase (Sluc)

1. Introduction

G protein-coupled receptors (GPCRs) are the largest plasma membrane receptor superfamily, and according to estimations ~40% of the marketed drugs target GPCRs (Whalen, Rajagopal and Lefkowitz, 2011). Although the monomeric form of GPCRs is functional, a large number of evidence has accumulated demonstrating that they are also capable to form higher order complexes (Milligan, 2013). A very intriguing finding is that dimerization or oligomerization can greatly influence the signaling properties of GPCRs (Ferre, Casado, Devi et al., 2014). It has been reported that GPCR dimerization can result in altered ligand binding, receptor conformation or effector functions (Smith and Milligan, 2010,Szidonya, Cserzo and Hunyady, 2008). Heterodimerization between GPCRs widens the number of the possible physiological receptor crosstalk mechanisms, and helps fine tune receptor functions (Ferre, Baler, Bouvier et al., 2009,Jonas, Rivero-Muller, Huhtaniemi et al., 2013,Rivero-Muller, Jonas, Hanyaloglu et al., 2013). On the other hand, receptor dimerization can also cause unexpected drug interactions.

Angiotensin type 1A receptor (AT_1R) and β-adrenergic receptors (βAR) play crucial role in the regulation of heart function and vascular tone under physiological and pathophysiological conditions, therefore they are pivotal drug targets in cardiovascular diseases, including heart failure or hypertension (Whalen et al., 2011). Moreover, they were shown to form dimeric complexes and the blockade of either protomer with an antagonist can result in simultaneous hindering of the other protomer's G protein activation (Barki-Harrington et al., 2003).

In addition to G proteins, β-arrestin molecules are also considered to be effector proteins of GPCRs. β-arrestins govern GPCR desensitization, endocytosis and also participate in G protein-independent signaling pathways (Shenoy and Lefkowitz,

2011). β-arrestins regulate β_2AR function via several mechanisms. β-arrestin2 induces desensitization and internalization of β_2AR, and these effects have been linked to tachyphylaxis of β_2-adrenergic agonists (Deshpande, Theriot, Penn et al., 2008). This phenomenon greatly limits the use of β_2-agonist drugs in the treatment of bronchial asthma. β-arrestins also mediate signaling of β_2AR. β-arrestin2 initiates the activation of MAPK cascade independently of G protein activation (Shenoy, Drake, Nelson et al., 2006), and β-arrestins promote cardiomyocyte contraction (Carr, Schilling, Song et al., 2016). Chronic activation of β_2-adrenergic receptor by catecholamines leads to DNA damage via β-arrestin1 (Hara, Kovacs, Whalen et al., 2011). β-arrestin1 facilitates the MDM2 promoted ubiquitination and degradation of p53. In the absence of β-arrestin1 this effect of β_2AR is greatly abrogated. These examples show the central role of β-arrestins in the function of β_2AR.

Activation of G proteins by AT_1R is considered to evoke deleterious effects in several pathophysiological conditions. However, stimulation of the G protein-independent, β-arrestin-mediated signaling pathways through AT_1R has been shown to have beneficial outcomes (Hunyady and Catt, 2006,Whalen et al., 2011). The clinically used conventional AT_1R antagonist drugs antagonize both pathways, so the desired β-arrestin-mediated favorable effects are also blocked. Thus, it is proposed, that ligands which are able to antagonize the G protein activation of a GPCR, but still able to induce the β-arrestin dependent signaling, could be prosperous drugs in many pathological circumstances(Whalen et al., 2011). Such β-arrestin biased agonist ligands have been already discovered for AT_1R. The first such ligand was [Sar^1,Ile^4,Ile^8]-AngII, however its clinical use was seriously hindered because of its poor receptor affinity (Wei, Ahn, Shenoy et al., 2003). Since then, new peptides with higher affinity, like TRV120023 or TRV120027, have been developed, which offered the possibility of the clinical application (Rajagopal, Ahn, Rominger et al., 2011,Szakadati, Toth, Olah et al., 2015,Violin, Crombie, Soergel et al., 2014).

In this study, we investigated the consequences of angiotensin type 1A receptor-β2-adrenergic receptor (β_2AR) heterodimerization on β-arrestin binding using a bioluminescence resonance energy transfer (BRET)-based approach. We found that dimerization alters the β-arrestin binding of β_2AR. The physiological AT_1R agonist angiotensin II or the β-arrestin biased AT_1R ligand TRV120023, but not the unbiased AT_1R inverse agonist candesartan could potentiate β-arrestin coupling to

the β_2AR. These findings reveal a possible new physiological crosstalk mechanism between AT_1R and β_2AR.

2. Materials and methods

2.1. Materials

The AT_1R, AT_1R-DRY/AAY (Gaborik, Jagadeesh, Zhang et al., 2003), AT_1R-Δ319 (Hunyady, Bor, Balla et al., 1994), β_2AR, β_2AR-Sluc, untagged $5HT_{2C}$R-VGV (I156V, N158G, I160V), $5HT_{2C}$R-VGV-Sluc, PM-mRFP (mRFP fused to plasma membrane target sequence of Lyn) (Toth, Toth, Gulyas et al., 2012), AT_1R-Rluc (Szakadati et al., 2015), AT_1R-Venus, β-arrestin1-Venus, β-arrestin2-Venus (Gyombolai, Boros, Hunyady et al., 2013), β-arrestin2-Rluc, β_2AR-Venus (Turu, Szidonya, Gaborik et al., 2006), Cameleon D3 Ca^{2+}-BRET sensor (Gulyas, Toth, Toth et al., 2015), EPAC cAMP-BRET sensor (Erdelyi, Balla, Patocs et al., 2014) and L10-Venus (Venus fused to plasma membrane target sequence of Lck) (Toth, Gulyas, Toth et al., 2016) constructs were previously described. $5HT_{2C}$R-Venus was generated by replacing the Super *Renilla* luciferase (Sluc) tag to monomeric Venus (Venus) in the $5HT_{2C}$R-Sluc construct. To create the Cerulean tagged β_2AR construct, the Sluc tag of β_2AR-Sluc was replaced with Cerulean. To generate the YFP-β-arrestin2 construct the cDNA of rat β-arrestin2 was cloned into pEYFP-N1 vector between *AgeI* and *KpnI* restriction sites. The plasmids encoding HA epitope-tagged wild type and K44A mutant dynamin-2A were kindly provided by Dr. K. Nakayama (Tsukuba Science City, Ibaraki, Japan).

Cell culture reagents were from Invitrogen (Carlsbad, CA). Cell culture dishes and white 96-well plates for BRET measurements were obtained from Greiner (Kremsmunster, Austria). TRV120023 (Sar-Arg-Val-Tyr-Lys-His-Pro-Ala-OH) was synthetized by Proteogenix (Schiltigheim, France). Coelenterazine h was purchased from Regis Technologies (Morton Grove, IL). Unless otherwise stated, all other chemicals and reagents were from Sigma (St. Louis, MO).

2.2. Cell culture and transfections

HEK 293T and COS-7 cells were cultured in DMEM supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum in 5% CO_2 at 37 ºC. For the BRET-experiments, the transfection was performed on cell suspension using Lipofectamine 2000 in OptiMEM according to the manufacturer's instructions,

thereafter the cells were plated on polylysine covered white 96-well plates. The measurements were performed 24 or 48 hours after transfection of HEK 293T and COS-7 cells, respectively.

CHO cells were cultured in Ham's F12 supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% FBS. The day before transfection the cells were plated on 6-well plates, the transfection was achieved using Lipofectamine 2000 according to manufacturer's protocol.

For confocal microscopy experiments, HEK 293T cells were grown on glass coverslips in 6-well plates the day before transfection, and were transfected with plasmids encoding β_2AR-Cerulean, AT_1R-Δ319 and β-arrestin2-Venus (1 µg, 4 µg, and 0.5 µg pro well, respectively) using Lipofectamine 2000. The experiments were performed the day after transfection.

2.3. Bioluminescence resonance energy transfer (BRET) measurements

After a washing step, the medium of HEK 293T or COS-7 cells was changed to modified Kreb's-Ringer medium containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM glucose 10 mM, pH 7.4 Na-HEPES, unless otherwise stated. 5 µM coelenterazine h, as *Renilla* luciferase substrate, was added to cells, thereafter luminescence was measured at 480 nm and 530 nm wavelengths by a Thermoscientific Varioskan Flash Reader (Perkin Elmer). BRET ratio was calculated by dividing the emission collected at 530 nm with the emission measured at 480 nm.

BRET signal of CHO cells was measured in cell suspension using Mithras LB 940 multilabel reader (Berthold Technologies), as earlier described (Gyombolai, Toth, Timar et al., 2014).

For the statistical analysis Two-Way-ANOVA tests were performed. An effect was considered statistically significant, when the p value of the interaction between the two treatments was less than 0,05.

2.4. BRET-titration experiments

Increasing amount of donor (Sluc containing) and acceptor (Venus containing) proteins were expressed in HEK 293T cells. Similarly to the conventional BRET experiments, before measurement the medium was changed to modified Kreb's-Ringer medium. Before addition of 5 µM coelenterazine h, Venus fluorescence was measured by excitation at 510 nm and emission collected at 535 nm. After

coelenterazine h treatment, luminescence was measured using 480 nm and 530 nm filters and total luminescence was determined without filter. The data analysis in details was earlier described (Szalai, Hoffmann, Prokop et al., 2014). Briefly, measured points were grouped into high/low luminescence group by the median luminescence value for β_2AR-Sluc and AT_1R-Venus expressing cells. The effect of luminescence on the measured BRET ratio was evaluated by covariance analysis, forcing the regression line through the origin.

2.5. Confocal laser-scanning microscopy and image analysis

The media of the cells were changed to modified Kreb's-Ringer medium. Time-series images were taken every 10 seconds for 190 seconds from the bottom of the cells with a Zeiss LSM 710 confocal laser-scanning microscope using a 63x objective at 37 °C. Size of the images was 79.38 x 79.38 μm with 1024x1024 resolution. Individual cells were selected and cropped in Fiji ImageJ software and processed for further analysis. β-arrestin puncta were identified on images with neural network algorithm using Keras and sklearn libraries in python programming language (https://github.com/fchollet/keras, http://scikit-learn.org/). Stacks of images were sliced into samples with sliding window of 20x20 pixels and each sample was classified as β-arrestin puncta or background. Classifier was trained on examples which were selected from images taken in separate experiments. 2809 negative and 664 positive samples were used total which were randomly divided into 2326 training and 1147 cross-validation examples. With a network with two hidden layers, the cross-validation resulted in an average of 98 percent both for precision and recall. After classification of the samples, the original sized binary image was reconstructed and was further processed for tracking with trackpy library (https://github.com/soft-matter/trackpy). Particles present on the tenth image were selected with size of at least 5 pixels and were tracked with memory set to 3. Duration of puncta was determined and the puncta were divided into two subgroups based on their lifespan. The distributions were statistically compared with Fischer's exact test.

For determination of the β-arrestin binding phenotype, images were taken from the middle cross section of the cells 20-40 minutes after stimulation at 37 °C.

3. Results

3.1. *β_2AR and AT_1R form heterodimers*

The existence and the functional relevance of the β_2AR-AT_1R heterodimer have been reported earlier (Barki-Harrington et al., 2003). To verify the presence of heterodimerization between β_2AR and AT_1R, we performed BRET-titration experiments in HEK 293T cells. Sluc-tagged β_2AR was used as BRET donor and Venus-tagged AT_1R as BRET acceptor. In the classical BRET-titration experiments the amount of the donor molecule-encoding plasmid is held constant, while the acceptor-encoding plasmid is gradually increased. Despite of the constant amount of donor-encoding plasmid, the donor molecule expression was strongly dependent on the number of the acceptor molecule in our system, namely increased fluorescence levels led to a drop in the measured luminescence (Suppl. Fig. 1). Formerly we and others have shown that the correct interpretation of the classical approach is seriously hindered when the expression of the BRET donor is not maintained constant (Lan, Liu, Li et al., 2015,Szalai et al., 2014). With the use of computer simulations and *in vitro* experiments, we recently developed a new approach for the analysis of quantitative BRET data, where the BRET ratio is plotted as the function of the acceptor-labeled receptor expression at various donor receptor expression levels (Szalai et al., 2014). Briefly, we found that in case of non-specific interactions the BRET ratio is only dependent on the number of the acceptor molecules. In case of specific interactions, the BRET ratio is dependent both on the amount of acceptor and donor molecules (for more details see: (Szalai et al., 2014)). In our experiments, we confirmed the specific interaction between β_2AR and AT_1R, since a linear regression with lower steepness could be fitted on high luminesce points compared to the low luminescence points (Fig. 1A). On the other hand, we detected no specific interaction between Sluc-tagged β_2AR and Venus-tagged serotonin 2C receptor ($5HT_{2c}$R), as the donor expression did not influence the slope of the linear regression (Fig. 1B). This result shows that β_2AR forms heterodimer with AT_1R, but not with $5HT_{2c}$R.

3.2. *Activation of AT_1R influences the β-arrestin2 binding to β_2AR within a heteromer*

To investigate the crosstalk between the β_2AR-AT_1R heterodimer, we designed a BRET-based experimental approach. We cotransfected the cells with plasmids encoding Sluc-tagged β_2AR, C-terminally Venus-tagged β-arrestin2 and untagged

AT_1R. Using this experimental setup, we were able to selectively monitor the β-arrestin2 binding of the β_2AR and the impact of the AT_1R stimulation on the β_2AR-β-arrestin2 association (Fig. 2A). Isoproterenol (ISO, 10 μM), a β_2AR agonist, induced an increase in the BRET ratio, reflecting the β-arrestin2 binding to the β_2AR (Fig. 2B). Angiotensin II (AngII, 100 nM), which exerts its main physiological effects via AT_1R, alone induced only a slight increase in the BRET ratio. Strikingly, during simultaneous activation of the two receptors, the association between β-arrestin2 and β_2AR was significantly potentiated. Similar results were obtained in COS-7 and CHO cells (Suppl. Fig. 2A and B, in case of CHO cells β_2AR was tagged with acceptor and β-arrestin2 with donor). Since the BRET ratio is also dependent on the relative orientation of the donor and acceptor molecules, we tested the interaction with N-terminally YFP-tagged β-arrestin2 (Suppl. Fig. 3). We observed a similar effect indicating that the BRET increase does not originate from conformational changes, but reflects the increased interaction of β_2AR and β-arrestin2. The β-arrestin1 binding of β_2AR was also examined by BRET, and a very similar response was found (Suppl. Fig. 4). We have also investigated the dose-dependence of the ISO effect on β-arrestin2 binding to β_2AR in the presence or absence of AngII (Fig. 2C). We found that 100 nM AngII could increase the ISO-mediated β-arrestin2 binding already at lower ISO concentrations. In addition to the increased maximal response, AngII treatment also caused a left-shift in the β-arrestin2 binding curve (log EC50 (M) -7.48 vs -7.17, $p<0.05$, tested with Student's t-test), thus at lower ISO concentrations AngII raised the β_2AR-β-arrestin2 association more markedly.

Since β-arrestin2 also binds to the AT_1R, one could assume that, in case of costimulation, β-arrestin2 translocates to the AT_1R, and nonspecific BRET is detected between β_2AR and membrane-translocated β-arrestin2. To rule out this possibility, we transfected a C-terminally truncated AT_1 receptor (AT_1R-Δ319), which is impaired in the ability of β-arrestin2 binding, because it lacks the major docking site of β-arrestins (Fig. 2D) (Balla, Toth, Soltesz-Katona et al., 2012,Qian, Pipolo and Thomas, 2001). In contrast to the small BRET ratio elevation when wild type AT_1R was used, AngII stimulation alone did not lead to any change in basal BRET signal. However, a significant increase in the BRET signal was still present after costimulation of β_2AR and AT_1R-Δ319, indicating that the association between β_2AR and β-arrestin2 was enhanced.

Next, we checked whether β_2AR could also influence the β-arrestin2 binding of the AT_1R. In these experiments the AT_1R was tagged with Rluc and the β_2AR was untagged (Suppl. Fig. 5). Nonetheless, the β_2AR stimulation with ISO (10 μM) had no significant effect on the BRET between AT_1R-Rluc and β-arrestin2-Venus after AngII treatment. We concluded that the strong β-arrestin2 binding of AT_1R cannot be further increased by β_2AR stimulation.

3.3. *Signaling pathways originating from AT_1R are not essential for the modulation of β_2AR signaling*

To reveal the underlying mechanism of the AT_1R induced potentiation of the β_2AR β-arrestin2 binding, we used an AT_1R mutant that is deficient in G protein activation (AT_1R-DRY/AAY) (Gaborik et al., 2003). After stimulation of this mutant with AngII the β-arrestin binding of the β_2AR was increased similar to the wild type AT_1R (Fig. 3A). However, the kinetics of the potentiation was slower compared to that of the wild type AT_1R.

Wild type AT_1R is coupled to $G_{q/11}$ proteins, thus after receptor activation the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) are produced by phospholipase C (Hunyady and Catt, 2006). IP_3 is responsible for the calcium release from the intracellular stores, while DAG is important in the activation of protein kinase C. However, administration of a specific inhibitor of protein kinase C (Bisindolylmaleimide I /BIM/,2 μM) or calcium depletion of the cells in calcium-free media with calcium chelator EGTA (100 μM) and 200 nM thapsigargin /TG/) could not block the AT_1R mediated increase in the β_2AR β-arrestin2 binding (Fig. 3B). Calcium depletion abolished the AngII-induced calcium signaling, which is shown using a calcium responsive BRET biosensor (Gulyas et al., 2015) (Suppl. Fig. 6). Coactivation of untagged $5HT_{2C}$R, which receptor is coupled to similar signaling pathways as AT_1R (Balla et al., 2012), but does not dimerize with β_2AR, could not induce the potentiation of β-arrestin binding (Suppl. Fig. 7). These results and the data obtained with the AT_1R-DRY/AAY mutant suggest that G protein activation is not necessary for this effect.

In the past years it has become evident that AT_1R can also signal in the absence of G protein activation. Among others, β-arrestin dependent Src and MAP kinase activation has been described (Fessart, Simaan and Laporte, 2005,Hunyady and Catt, 2006). Src (PP1, 1 μM) and MEK (PD98059, 20 μM) inhibitors did not interfere

with the increased BRET ratio during costimulation of the receptors (Fig. 3B). These results, and the fact that the stimulation of the β-arrestin binding deficient AT_1R mutant (AT_1R-Δ319) was capable to increase the β-arrestin2 binding of β_2AR, suggest that β-arrestin mediated signaling is not required for the observed phenomenon.

β-arrestin2 dissociates from β_2AR after its internalization (Oakley, Laporte, Holt et al., 1999), therefore an increased β_2AR-β-arrestin2 interaction could origin from the inhibition of receptor endocytosis. To block β_2AR endocytosis, we overexpressed a dominant negative mutant dynamin2A (dynamin2A-K44A), which has been shown to inhibit agonist induced internalization (Scarselli and Donaldson, 2009,Zhang, Ferguson, Barak et al., 1996). Indeed, the ISO-induced BRET signal was significantly elevated (Suppl. Fig. 8). However, the cotreatment with AngII and ISO still increased the BRET signal under these circumstances, showing that the observed effect cannot be explained by AT_1R induced blockade of β_2AR internalization.

Since the observed effect was independent on activation of the investigated signaling pathways, we concluded that it is mediated by heterodimerization between the β_2AR and AT_1R.

3.4. *β-arrestin2 binding of β_2AR is dependent on the expression of AT_1R*

Since β_2AR can be present in both monomeric and dimeric states, only a portion of β_2ARs interact with AT_1R. Presuming random pairing of the two receptors, the relative number of β_2AR-AT_1R heterodimers should be elevated by increasing the AT_1R-β_2AR expression ratio. Therefore, we increased the amount of the AT_1R encoding plasmid during the transfection, while keeping the amount of the β_2AR-Sluc plasmid constant. As shown in Fig. 4A and B, no AngII effect was detected in absence of AT_1R. By increasing the AT_1R:β_2AR DNA ratio, when compared to ISO stimulation, the costimulation with AngII and ISO caused gradually increased BRET signal. This elevation was not due to higher plasma membrane expression of β_2AR-Sluc, since ISO stimulation itself led to slightly decreased BRET signals (Fig. 4A). These results show that the magnitude of β-arrestin translocation in this system depends on the relative expression ratio of AT_1R and β_2AR, which is consistent with the role of heterodimers.

3.5. *Biased activation of AT_1R affects the β-arrestin2 binding of β_2AR*

It has been shown earlier that a conventional AT_1R antagonist can simultaneously block the G protein mediated signaling of both AT_1R and β_2AR (Barki-Harrington et al., 2003). Therefore, we investigated the effects of different AT_1R antagonists on the β-arrestin2 binding of the AT_1R-β_2AR heterodimer. The cotreatment with ISO and the unbiased antagonist candesartan (10 μM) had no effect on the β-arrestin2 translocation (Fig. 5A). However, when we costimulated the cells with the β-arrestin biased AT_1R agonist TRV120023, we detected an increase in the the β-arrestin2 binding of β_2AR, similarly to AngII-cotreatment (Fig. 5B), but the kinetics of the potentiation was slower. Other β-arrestin biased agonists (TRV120027 and [Sar^1,Ile^4,Ile^8]-AngII) induced a very similar response (data not shown). These results suggest, in good agreement with the data obtained with the G protein activation-deficient AT_1R mutant, that the β-arrestin activating conformation of AT_1 receptor enhances the β-arrestin2 binding of β_2AR.

3.6. *Coactivation of β_2AR and AT_1R increases the lifespan of β-arrestin2 clusters*

Upon β_2-adrenergic receptor activation, β-arrestin2 translocates to the plasma membrane and forms clusters at the clathrin coated pits via interaction with β2-adaptin (Laporte, Oakley, Holt et al., 2000). To address the mechanism of the increased β-arrestin2 binding, we measured the lifetime and intensity of β-arrestin2 puncta of cells expressing β_2AR-Cerulean, AT_1R-Δ319 and β-arrestin2-Venus by confocal microscopy (Fig. 6A). Images were taken every 10 seconds at the bottom of the cells, and the lifespan of the individual puncta was determined. The lifespan of these β-arrestin2-Venus dots was comparable to those detected in previous studies (Eichel, Jullie and von Zastrow, 2016). AngII treatment did not lead to detectable puncta formation, since AT1R-Δ319 lacks the major binding site for β-arrestin2 (data not shown). However, the longevity of β-arrestin2 puncta was altered upon costimulation with AngII and ISO, compared to ISO stimulation alone. After costimulation, the fraction of puncta with longer lifespan was increased (Fig. 6A and B). On the other hand, we found no difference between the average fluorescence intensity values of the puncta (Suppl. Fig. 9). These results indicate that the detected increase in β-arrestin2 binding is the consequence of the stabilized interaction between β_2AR and β-arrestin2. Increased β-arrestin2 localization at the plasma membrane upon costimulation was also found by BRET measurements between

plasma membrane targeted Venus and β-arrestin2-Rluc in cells expressing untagged β_2AR and AT_1R-Δ319 (Fig. 6C). The raise of bystander BRET rises from the enrichment of β-arrestin2 in the juxtamembrane region.

β-arrestins dissociate from β_2AR before entering the early endosomes, therefore β_2AR is classified as a class A receptor (Oakley, Laporte, Holt et al., 2000). Nevertheless, we still could not observe β-arrestin2 at early endosomes after costimulation, therefore this more stable interaction is not strong enough to convert the interaction into a class B endocytic pattern (Suppl. Fig 10).

3.7. *Simultaneous activation of AT1R prolongs the β_2AR mediated cAMP signaling*

The generally considered main role of arrestins is the termination of G protein signaling (Shenoy and Lefkowitz, 2011).However, several studies have shown that noncanonical cAMP signaling arises from receptor-arrestin-G protein complexes (Feinstein, Wehbi, Ardura et al., 2011,Feinstein, Yui, Webber et al., 2013,Thomsen, Plouffe, Cahill et al., 2016,Wehbi, Stevenson, Feinstein et al., 2013). The magnitude of the noncanonical arrestin-dependent cAMP formation was associated with the stability of the receptor-arrestin interaction (Thomsen et al., 2016). Therefore, we investigated whether the sustained β-arrestin2 binding to β_2AR is accompanied by prolonged cAMP signaling. We coexpressed AT_1R and a BRET-based cAMP biosensor in HEK 293T cells (Erdelyi et al., 2014), and the cAMP signaling of endogenous β_2AR was monitored. Neither AngII nor TRV023 treatment alone could generate cAMP (Fig. 7A and C). Remarkably, cotreatment with AngII or TRV023 prolonged the ISO induced cAMP signal. Previously we have shown that calcium dependent pathways can potentiate the cAMP formation (Baukal, Hunyady, Catt et al., 1994). In calcium depleted cells we could still observe the prolonged cAMP signaling upon AngII and TRV023 costimulation, showing that the cAMP signaling was prolonged also via a calcium-independent way (Fig. 7B and C). These results show that AT_1R activity influences, in addition to β-arrestin binding, the G protein dependent signaling of β_2AR.

4. Discussion

Here we show that the β-arrestin binding of β_2AR is regulated by AT_1R coactivation. These results are in good agreement with an earlier report, where the

authors gave evidence that β_2AR and AT_1R are working as a functional unit (Barki-Harrington et al., 2003). Nowadays it is widely accepted that receptor dimerization has important impact on the properties of receptor signaling. In elegant studies, using ligand-binding deficient and signaling deficient luteinizing hormone receptors, dimerization was shown to rescue the defective GPCR function both *in vitro* and *in vivo* (Jonas, Fanelli, Huhtaniemi et al., 2015,Rivero-Muller, Chou, Ji et al., 2010). Altered G protein activating ability was shown in case of D_1-D_2 dopamine receptor heterodimer and its possible role was raised in the pathogenesis of major depression (Pei, Li, Wang et al., 2010,Rashid, So, Kong et al., 2007).

Several studies have shown that β-arrestin binding can be influenced by receptor heterodimerization. Altered β-arrestin binding was found in case of the V_1-V_2 vasopressin receptor dimer, the μ-δ opioid receptor dimer or the CXC chemokine receptor 2-α_{1A} adrenergic receptor heterodimer (Mustafa, See, Seeber et al., 2012,Rozenfeld and Devi, 2007,Terrillon, Barberis and Bouvier, 2004).

In our system, the stimulation of the untagged wild type AT_1R with AngII alone led to a slight increase of the BRET ratio between the Sluc-tagged β_2AR and Venus-labeled β-arrestin2. Since this increase was diminished when we used the β-arrestin binding-deficient AT_1R-Δ319 mutant, we concluded that this signal reflects the β-arrestin translocation to the untagged AT_1R resulting in nonspecific BRET between β-arrestin2 and β_2AR. It is worth noting that the AT_1R-Δ319 mutant was reported to bind β-arrestin2 very weakly (Anborgh, Seachrist, Dale et al., 2000), however it was not detectable under our experimental conditions.

A similar system, named BRET heteromer identification technology (BRET-HIT), was earlier introduced as a useful approach for GPCR heteromer detection (See, Seeber, Kocan et al., 2011). This system is based on the close proximity of the heteromer partners. Thus, the β-arrestin translocation to the untagged protomer can result in the elevation of the BRET ratio between the tagged protomer and β-arrestin, because the small distances in the molecular complex allow resonance energy transfer. In case of non-dimerizing receptors this phenomenon cannot occur. However, we and others have shown previously that after stimulation of a GPCR, BRET increase can be detected between β-arrestin2-Rluc and a plasma-membrane targeted Venus, where the interaction was clearly nonspecific (Donthamsetti, Quejada, Javitch et al., 2015,Gyombolai et al., 2014). This implicates that the reliability of the BRET-HIT approach is weakened at high receptor expression levels because of the high

probability of nonspecific BRET signal. The BRET ratio increase after the costimulation of the Sluc-tagged β_2AR and the β-arrestin binding-deficient AT_1R-Δ319 mutant clearly shows that the β-arrestin2 binding to the β_2AR is elevated, and this signal does not originate from a nonspecific interaction. The results obtained with the C-terminally truncated AT_1R mutant also suggest that AT_1R activation alone cannot induce β-arrestin recruitment to the β_2AR. These results show that the AT_1R-β_2AR heterodimer functions somewhat differently than the AT_1R homodimer or the CXC chemokine receptor 2-α_{1A} adrenergic receptor heterodimer, where activation of one protomer alone results in β-arrestin binding to the other protomer (Mustafa et al., 2012,Szalai, Barkai, Turu et al., 2012) .

Increased BRET signal can originate from increased association or from changes in orientation between the BRET partners. The latter is unlikely to occur here, since we detected similar changes using N- and C-terminally tagged β-arrestin2 variants. In addition, a simple change in orientation could not explain the leftward shift of the dose-response curve of β_2AR-β-arrestin2 binding after coactivation of AT_1R. Increased association of β_2AR with β-arrestin2 after costimulation of the two receptors hypothetically could have three possible mechanisms. After AT_1R activation 1) a higher fraction of β_2ARs could bind β-arrestin; 2) one β_2AR could bind to more β-arrestins concurrently; 3) the interaction between β-arrestin and β_2AR could become more stable, and the elevated BRET ratio reflects this new steady-state. The first possibility (more β_2ARs recruiting β-arrestin) can be ruled out, since we used saturating agonist concentrations. The possibility of one receptor binding more than one β-arrestin molecule simultaneously is contradicted by the recently solved structure of the β_2AR-β-arrestin1 complex (Shukla, Westfield, Xiao et al., 2014). In regard to the third possible mechanism, it is well known that the interaction between β_2AR and β-arrestin is relatively weak and unstable. This interaction can be strengthened by replacement of the C-terminal of β_2AR to the C-terminal of V_2 vasopressin or AT_1 receptor (Anborgh et al., 2000,Oakley et al., 1999). Therefore it is reasonable to assume that the increased stability of the interaction leads to enhanced BRET signal. In fact, the increased stability of the complex was demonstrated in our confocal experiments, which showed that the lifespan of the β_2AR-β-arrestin2 clusters at the plasma membrane is increased after costimulation of AT_1R and β_2AR. The resolution limit of confocal microscopy does not allow us to determine that the clusters whether originate from the plasma membrane only or also

from subplasmalemmal vesicles. However, we did not see β-arrestin2 colocalization with early endosomes, the β-arrestin2 binding was not changed to class B phenotype.

The crystal structure of β_2AR-β-arrestin1 complex shows that there is a large free interface of β-arrestin1 heading toward the plasma membrane (Shukla et al., 2014). It is therefore possible that the protomers bind one β-arrestin molecule concurrently, which would result in a stabilized interaction between β_2AR and β-arrestin. However, the exact nature of the increased stability needs to be addressed in further experiments.

We found that the allosteric modulation of β-arrestin binding is asymmetric between β_2AR and AT_1R, as costimulation of β_2AR could not increase the β-arrestin binding of AT_1R. Based on its β-arrestin binding properties, AT_1R belongs to the family of class B receptors, meaning that β-arrestins stably associate with AT_1R and cotraffic to early endosomes (Oakley et al., 2000). The stability of this interaction might be already near to its maximum, which suggests that a further increase in the binding is unlikely.

We have reported earlier that the conserved DRY motif of the AT_1R is crucial for the allosteric interactions in the AT_1R homodimer pair (Karip, Turu, Supeki et al., 2007,Szalai et al., 2012). Here we found that activation of the DRY/AAY mutant AT_1R was still able to increase the β-arrestin binding properties of β_2AR. This finding suggests that the DRY motif, in contrast to the AT_1R homodimer, is not obligately necessary for the allosteric interaction between AT_1R and β_2AR.

We found that G_q activation is not necessary for the sustained β_2AR-β-arrestin interaction, still we cannot rule out that G_q activation could influence it. It was reported that activation of $G\alpha_q$ subunit targets GRK2 to the plasma membrane, which is important in the regulation of the binding between M_3 muscarinic acetylcholine receptor and β-arrestin2 (Wolters, Krasel, Brockmann et al., 2015).

There is mounting evidence for noncanonical cAMP signaling of several GPCRs, whereas sustained receptor-β-arrestin interaction prolongs the G protein dependent cAMP signaling (Feinstein et al., 2011,Thomsen et al., 2016). We found that coactivation of AT1R with AngII or the biased agonist TRV120023 prolonged the cAMP signaling of β_2AR. It must be noted that in addition to prolonged G_s activation via heterodimer formation, the observed alteration of cAMP signal could also arise from the effect of β-arrestin dependent signaling (e.g. adenylyl cyclase activation or

cAMP-phosphodiesterase inhibition) or competition between AT_1R and β_2AR for the desensitization machinery could also explain the observed effect on cAMP signaling. Nonetheless, our results are in good agreement with a previous study, where the authors have shown that the AT_1R biased agonist [Sar^1,Ile^4,Ile^8]-AngII potentiated the cAMP dependent gene regulation of β_2AR (Christensen, Knudsen, Schneider et al., 2011).

The direct interaction between β_2AR and AT_1R has been reported previously (Barki-Harrington et al., 2003). It was shown that β-blocker drugs inhibit G protein coupling of AT_1R, and the conventional AT_1R antagonist valsartan interferes with the β_2AR-G protein coupling. We investigated whether AT_1R antagonists have similar effects on the β_2AR-β-arrestin interaction. We showed that the conventional AT_1R antagonist candesartan had no effect on the β-arrestin binding of β_2AR, while the β-arrestin-biased agonist TRV120023 could increase this interaction. These results suggest that β-arrestin-biased AT_1R agonists can have very different effects compared to the conventional AT_1R antagonists, not only because they activate the β-arrestin dependent signaling of AT_1R, but also because they could modulate the AT_1R-β_2AR heterodimer. It was reported that the β-arrestin-biased AT_1R agonist [Sar^1,Ile^4,Ile^8]-AngII has different effect on B_2 bradykinin receptor-AT_1R heterodimer function compared to the unbiased AT_1R antagonist valsartan (Wilson, Lee, Appleton et al., 2013). This suggests that β-arrestin-biased AT_1R agonists can have unexpected new effects or side effects, postulating a more careful administration of these drugs in patients in the future. In a recent Phase II clinical trial in heart failure TRV120027 has failed to have the expected positive effects (Trevena, 2016). However, our data show that biased agonists of AT_1R have effects on the arrestin binding of receptor heterodimers, which may have functional relevance during the treatment of patients with inhibitors of AT_1R in other diseases.

In summary, we propose a model in which activation of the AT_1R stabilizes the β-arrestin binding of β_2AR in the heterodimer of AT_1R and β_2AR (Fig. 8). The unbiased or biased activation of the AT_1R affects the dimer partner β_2AR directly, which alters the β-arrestin binding to the β_2AR.

Acknowledgements

L.H. and P.V. were supported by National Research, Development and Innovation Fund (NKFI K116954 and K105006, respectively). The excellent technical assistance of Ilona Oláh and Eszter Halász is greatly appreciated.

Declaration of interest

The authors declare no conflict of interest.

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734

Highlights:

Heterodimerization between AT_1R and β_2AR enhances β-arrestin coupling of β_2AR.

Heterodimerization increases the lifespan of β-arrestin2 clusters after β_2AR stimulation.

Biased AT_1R ligands alter the function of heterodimerized β_2AR.

Figure

Figure 1

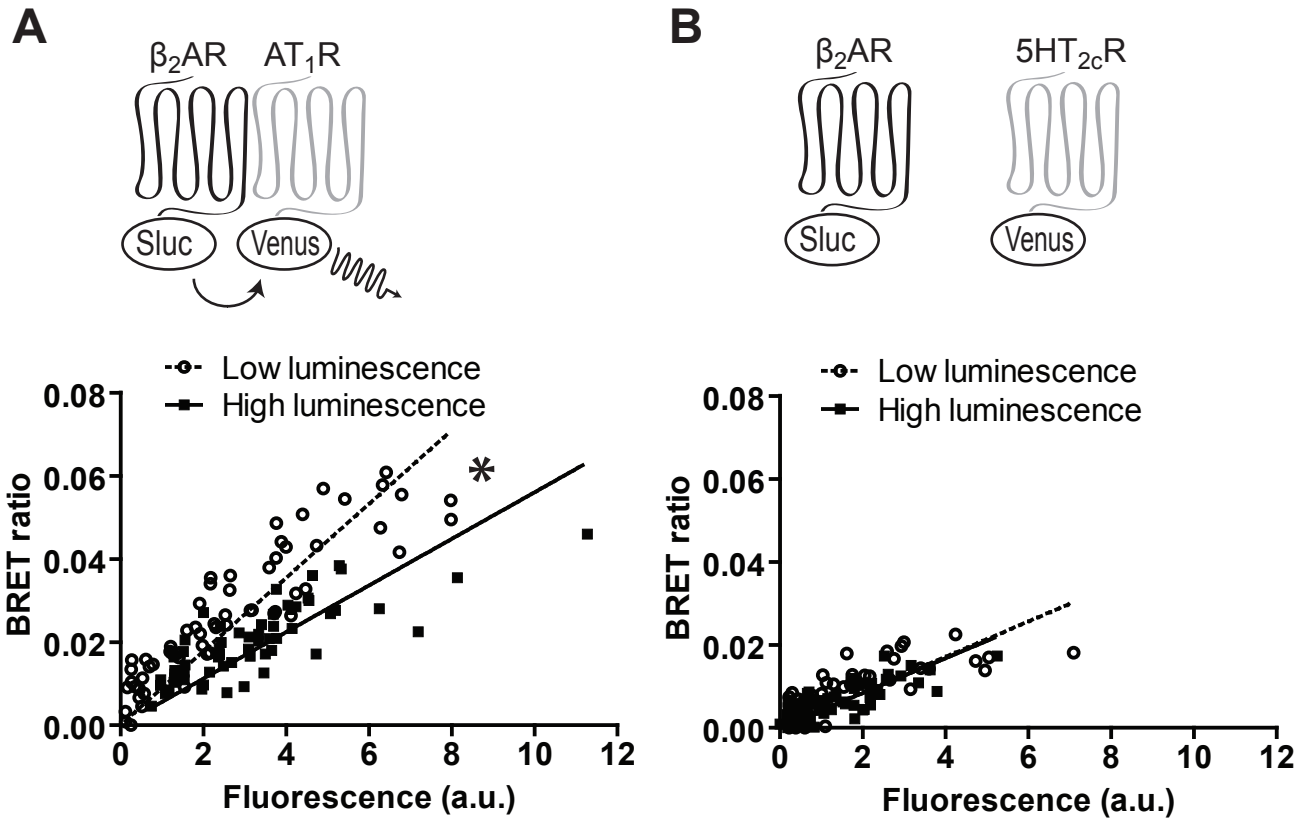


Figure 2

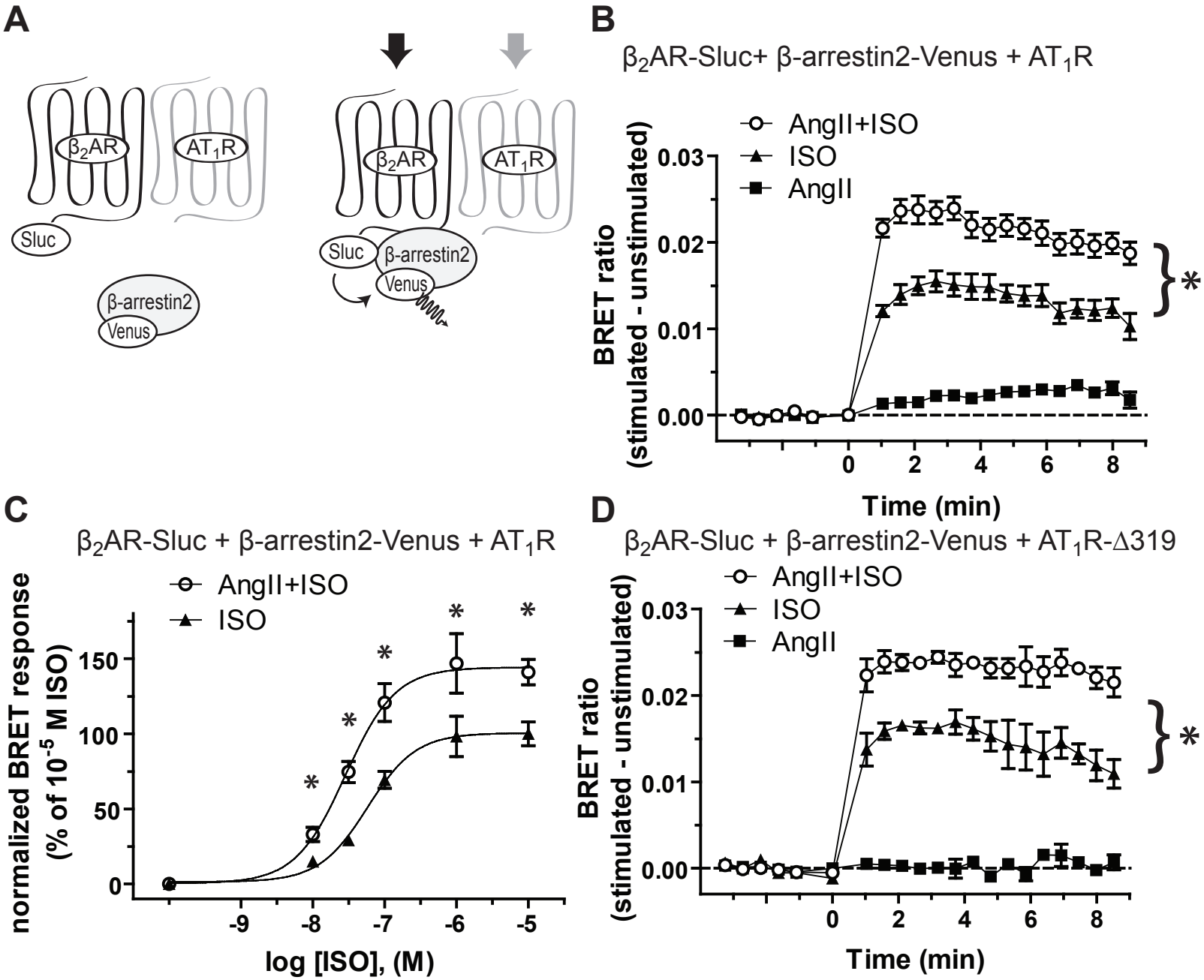
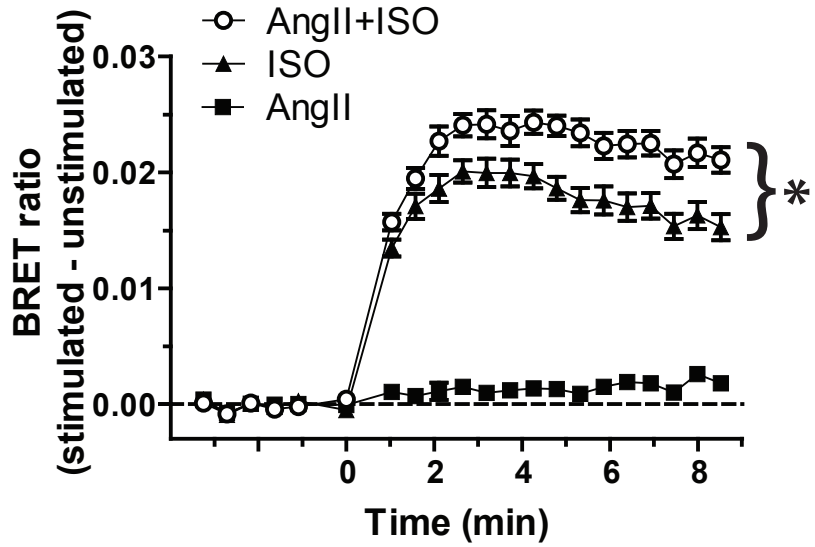

A
β2AR
AT1R
Sluc
β-arrestin2
Venus
B
β2AR-Sluc+ β-arrestin2-Venus + AT1R
AngII+ISO
ISO
AngII
BRET ratio (stimulated - unstimulated)
Time (min)
C
β2AR-Sluc + β-arrestin2-Venus + AT1R
normalized BRET response (% of 10-5 M ISO)
log [ISO], (M)
D
β2AR-Sluc + β-arrestin2-Venus + AT1R-Δ319

Figure 3

A

β_2AR-Sluc + β-arrestin2-Venus + AT_1R-DRY/AAY



B

β_2AR-Sluc + β-arrestin2-Venus + AT_1R

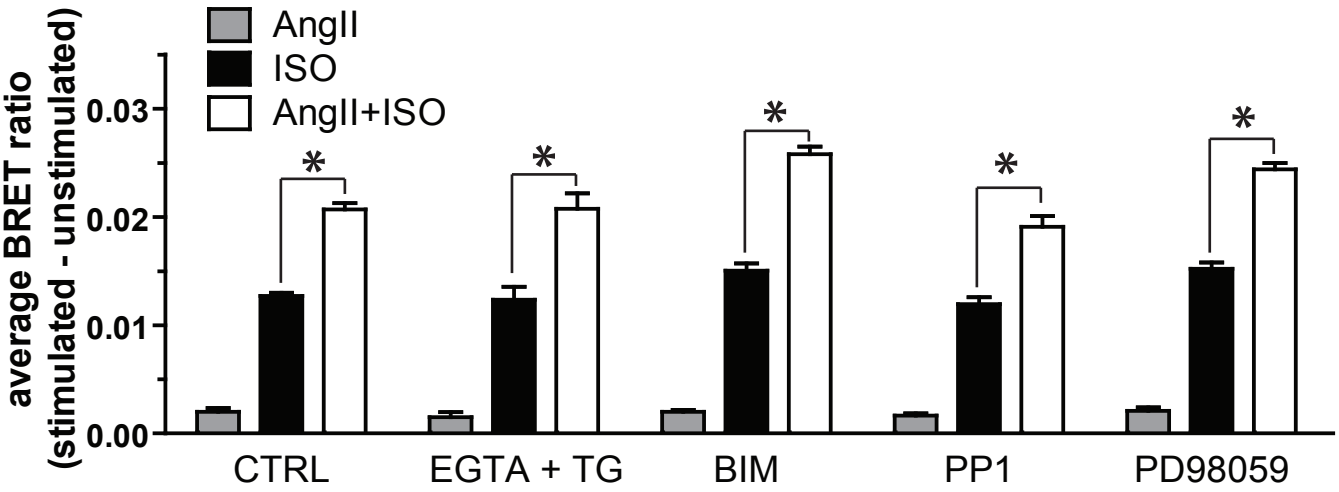


Figure 4

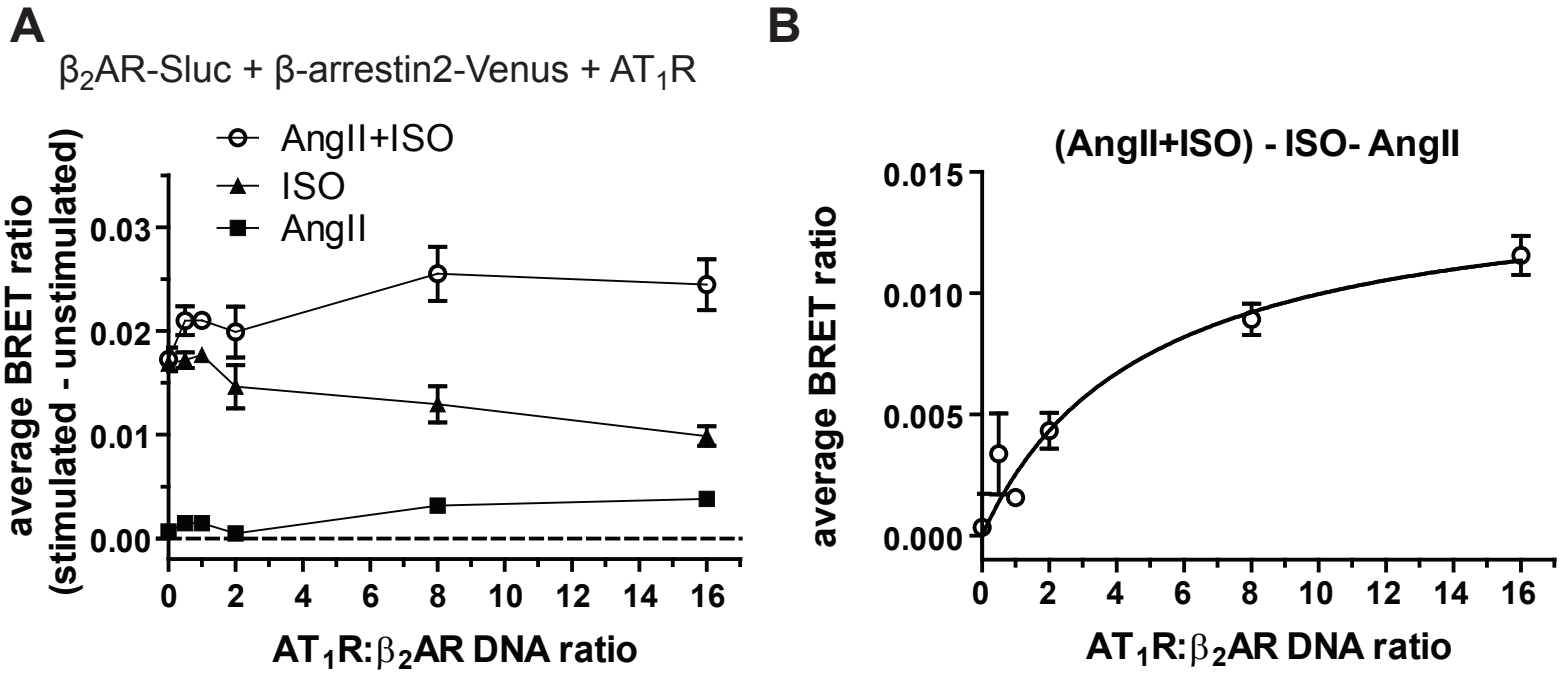

A
β2AR-Sluc + β-arrestin2-Venus + AT1R
AngII+ISO
ISO
AngII
average BRET ratio (stimulated - unstimulated)
0.03
0.02
0.01
0.00
0 2 4 6 8 10 12 14 16
AT1R:β2AR DNA ratio
B
(AngII+ISO) - ISO- AngII
average BRET ratio
0.015
0.010
0.005
0.000
0 2 4 6 8 10 12 14 16
AT1R:β2AR DNA ratio

Figure 5

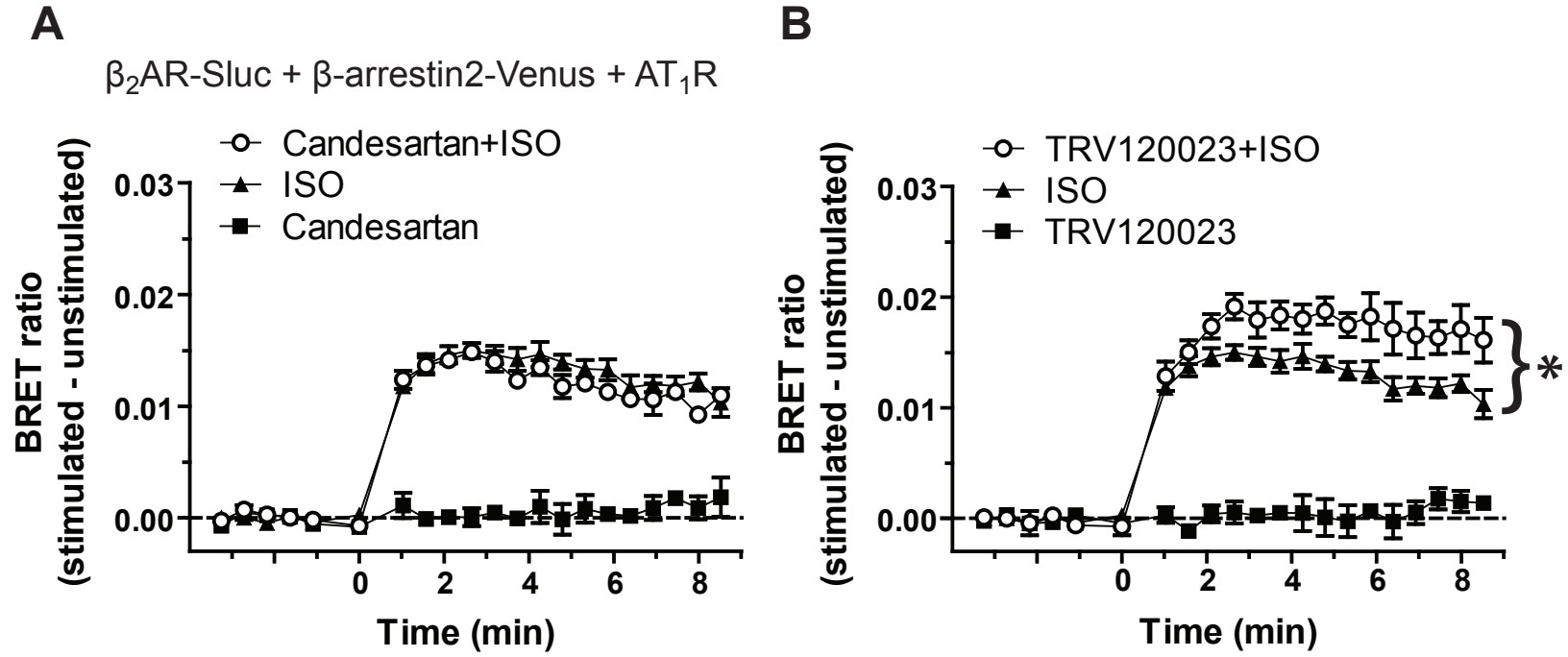

A
β2AR-Sluc + β-arrestin2-Venus + AT1R
Candesartan+ISO
ISO
Candesartan
BRET ratio
(stimulated - unstimulated)
0.03
0.02
0.01
0.00
0
2
4
6
8
Time (min)
B
TRV120023+ISO
ISO
TRV120023
BRET ratio
(stimulated - unstimulated)
0.03
0.02
0.01
0.00
0
2
4
6
8
Time (min)
*

Figure 6

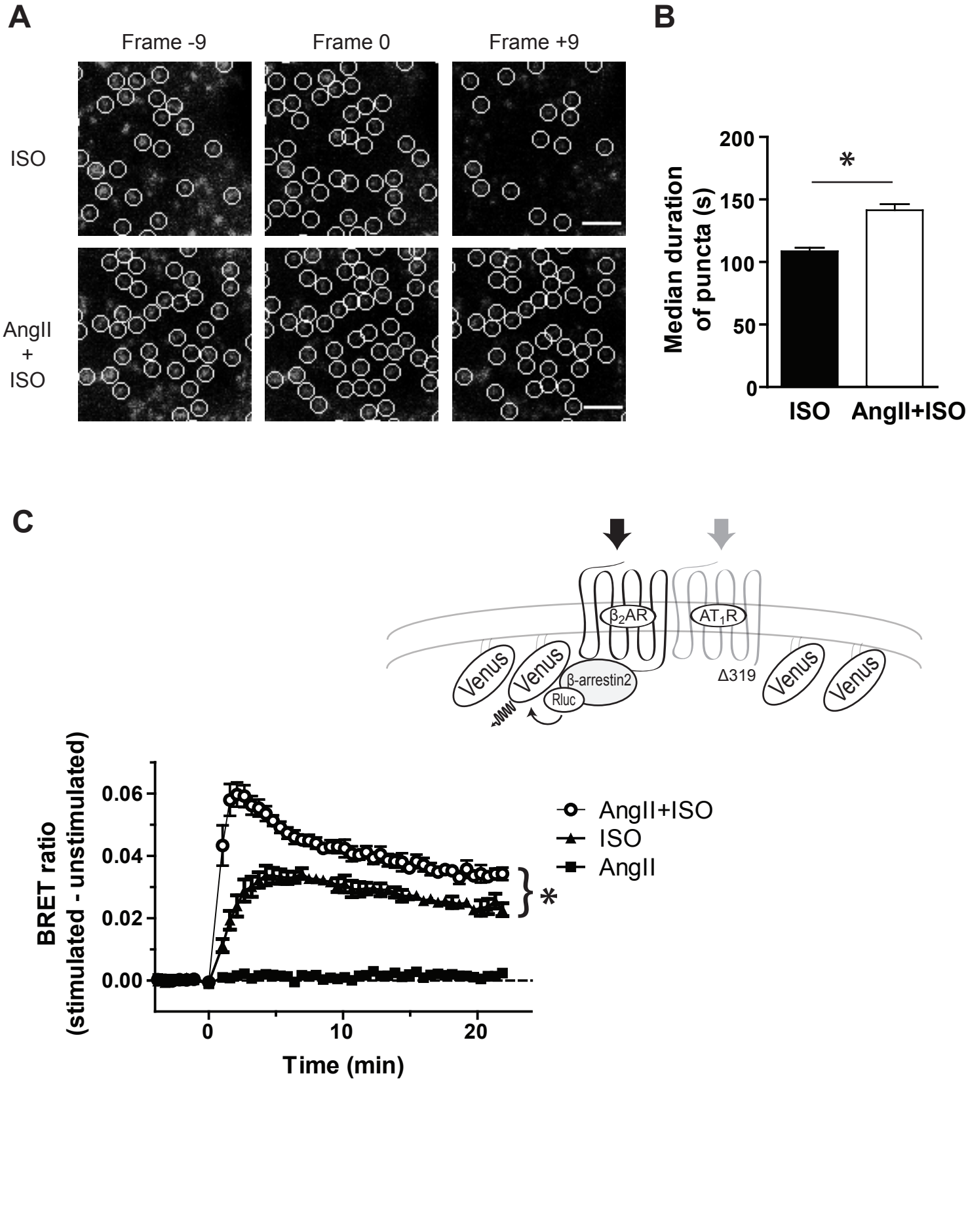
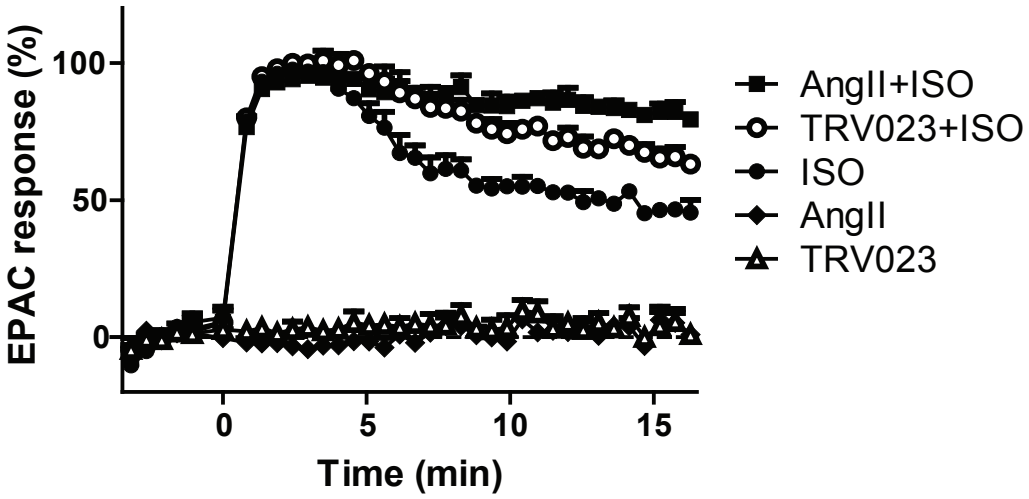
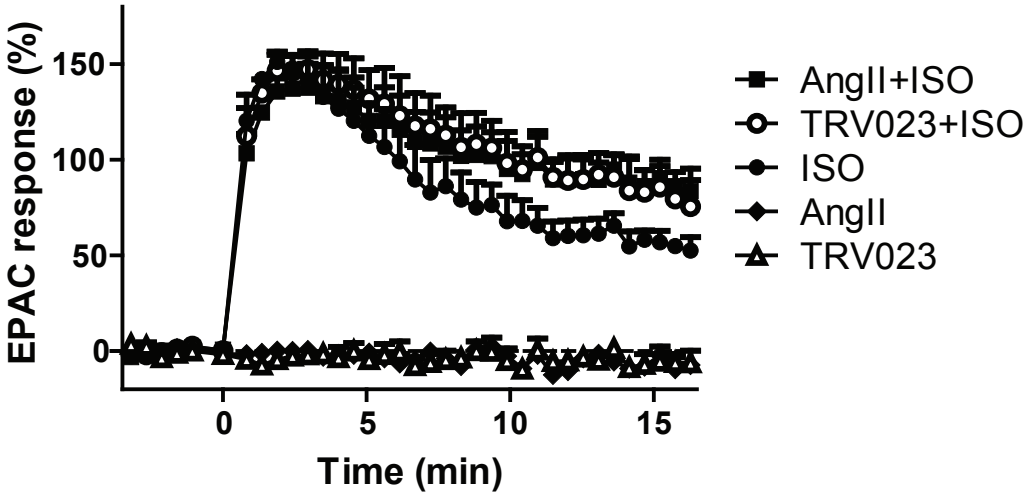

A
Frame -9
Frame 0
Frame +9
ISO
AngII
+
ISO
B
Median duration of puncta (s)
200
150
100
50
0
*
ISO
AngII+ISO
C
β2AR
AT1R
Venus
Venus
β-arrestin2
Rluc
Δ319
Venus
Venus
BRET ratio
(stimulated - unstimulated)
0.06
0.04
0.02
0.00
0
10
20
Time (min)
AngII+ISO
ISO
AngII
*

Figure 7

A


EPAC response (%)
100
50
0
0
5
10
15
Time (min)
AngII+ISO
TRV023+ISO
ISO
AngII
TRV023

B


Ca2+-depleted
EPAC response (%)
150
100
50
0
0
5
10
15
Time (min)
AngII+ISO
TRV023+ISO
ISO
AngII
TRV023

C

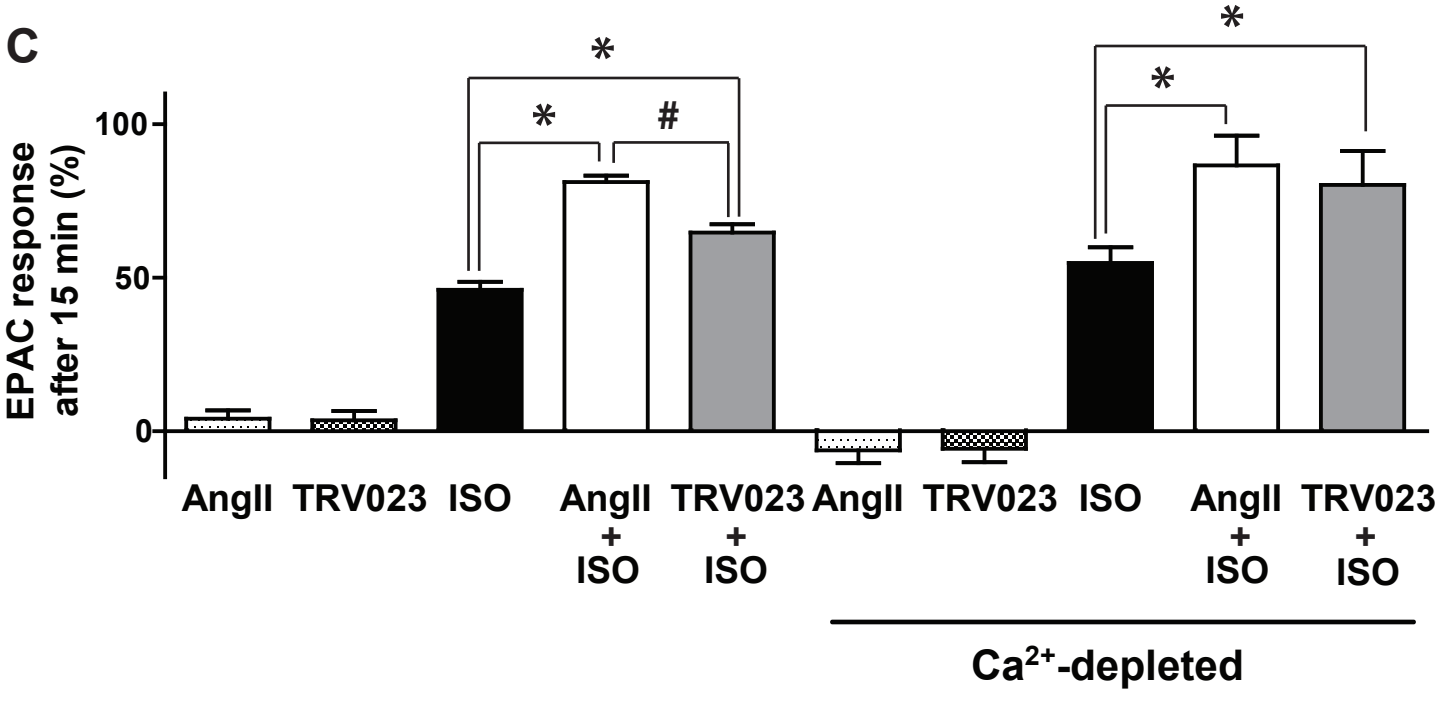
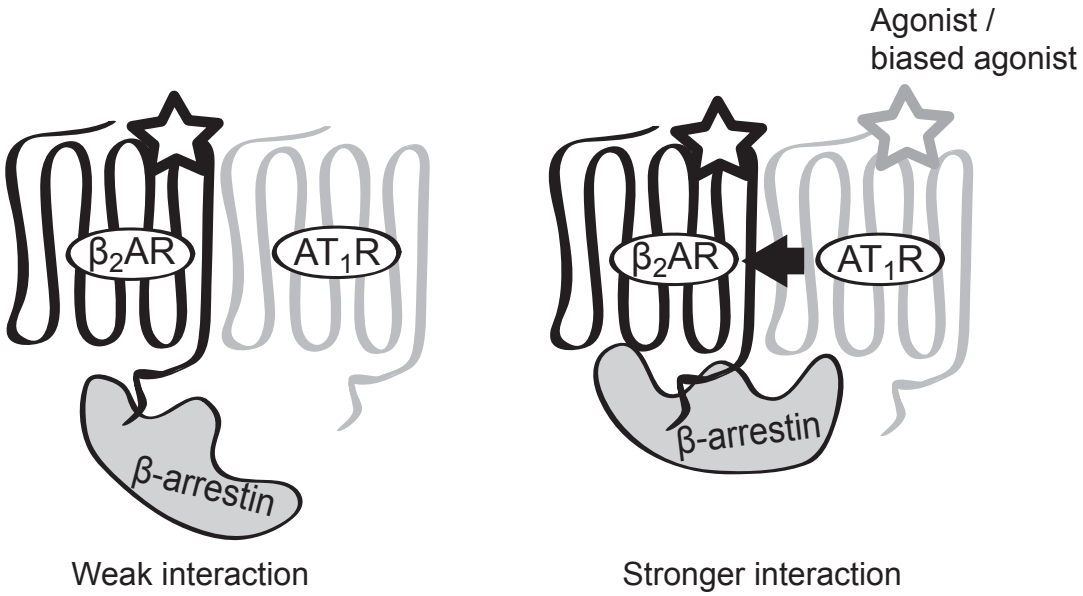

EPAC response after 15 min (%)
100
50
0
*
*
#
*
*
*
AngII
TRV023
ISO
AngII + ISO
TRV023 + ISO
AngII
TRV023
ISO
AngII + ISO
TRV023 + ISO
Ca2+-depleted

Figure 8



Weak interaction

Stronger interaction

Figure 1

β_2AR forms heterodimer with AT_1R

An improved form of BRET titration experiments was performed in HEK 293T cells. Increasing amount of plasmid encoding β_2AR-Sluc and increasing amount of plasmids encoding AT_1R-Venus or $5HT_{2C}R$-Venus were transfected in the cells, while keeping the total DNA amount at the same level by adding pcDNA3.1 (A and B). BRET ratio is plotted as the function of fluorescence, the measured points were divided in two subgroups: cells showing low or high luminescence. In case of AT_1R-Venus expressing cells (A) the BRET ratio was also dependent on the measured luminescence, indicating specific interaction between the two proteins. The BRET ratio in the $5HT_{2C}R$-Venus expressing cells (B) was not dependent on the luminescence, showing that there is no specific interaction between the two molecules. Dependence of BRET ratio on luminescence was determined by covariance analysis (*,$p<0.05$, n=3).

Figure 2

Activation of AT_1R differs the β-arrestin binding properties of β_2AR

A, Schematic representation of our BRET-based system. β_2AR is tagged with Sluc, AT_1R is untagged and β-arrestin2 is labeled with Venus. Upon β_2AR receptor stimulation β-arrestin2 translocates to the receptor, which enables resonance energy transfer to occur. We can also observe the effect of concomitant AT_1R stimulation. B, HEK 293T cells (70000/well) were transfected with 25 ng plasmid encoding β_2AR-Sluc, with 100 ng plasmid encoding AT_1R and with 100 ng plasmid encoding β-arrestin2-Venus pro well. The change of BRET ratio was measured after stimulation with 100 nM angiotensin II (AngII), with 10 µM isoproterenol (ISO) or with both. C, The effect of 100 nM AngII on the ISO dose-response curve. Each point represents the average BRET ratio change. 100% reflects the BRET ratio change after 10 µM ISO treatment. To better observe the AngII effect on the ISO response, the BRET ratio change after AngII+ISO treatment was normalized to the AngII alone treated points. D, The cells were transfected with 25 ng plasmid encoding β_2AR-Sluc, with 100 ng plasmid encoding C-terminal truncated AT_1R mutant (AT_1R-Δ319) and with 100 ng plasmid encoding β-arrestin2-Venus. Data are mean±SEM, n=3-6. All the statistical analysis was made on the raw data, * means significant interaction between the two treatments ($p<0.05$, Two-Way ANOVA).

Figure 3

The AT_1R mediated potentiation of β-arrestin2 binding to β_2AR is not dependent on intracellular signaling

A, HEK 293T cells were transfected with plasmids encoding β_2AR-Sluc, G protein coupling deficient AT_1R mutant (AT_1R-DRY/AAY) and β-arrestin2-Venus (25 ng, 100 ng, 100 ng pro well, respectively), and BRET was measured after ISO (10 µM) or AngII (100 nM) stimulations.

B, The cells were transfected with plasmids encoding β_2AR-Sluc, AT_1R and β-arrestin2-Venus (25 ng, 100 ng, 100 ng pro well, respectively). For calcium depletion the medium was changed to calcium-free modified Kreb's Ringer medium, and the cytoplasmic calcium was chelated with 100 µM EGTA. Thereafter the cells were pretreated with 200 nM thapsigargin (TG) for 5 minutes to deplete the intracellular stores. To block protein kinases, the cells were pretreated with vehicle (DMSO), 2 µM bisindolylmaleimide I (BIM), 1 µM PP1 or 10 µM PD98059 for 30 minutes, as indicated. 100 nM AngII and 10 µM ISO were used as stimuli in BRET measurements. The columns represent the average BRET ratio change in each experiment. Data are mean±SEM, n=3-10. * means significant interaction between ISO and AngII treatments ($p<0.05$, Two-Way ANOVA).

Figure 4

The β-arrestin2 binding of β_2AR is dependent on AT_1R expression

HEK 293T cells were transfected with 25 ng plasmid encoding β_2AR-Sluc, with 100 ng plasmid encoding β-arrestin2-Venus, and with increasing amount of plasmid encoding untagged AT_1R (0, 12.5, 25, 50, 200 and 400 ng) pro well. Empty pcDNA3.1 vector was also added to keep the transfected DNA amount constant. 100 nM AngII and 10 µM ISO were used as stimuli. Data are mean±SEM, n=3. A: The average BRET-change was plotted as the ratio of the transfected DNA encoding β_2AR-Sluc and AT_1R. B: To reveal the AngII mediated potentiation on the ISO effect, the BRET-change after ISO stimulation was subtracted from the BRET change after costimulation with AngII and ISO. Furthermore the BRET-change after AngII treatment was also subtracted, as it reflects the β-arrestin2 binding to AT_1R. Mathematically, the BRET ratio change was calculated as (AngII+ISO) – ISO – AngII. One-site specific binding curve was fitted on the measured points using GraphPad Prism 4 Software (r^2=0.9).

Figure 5

Different effects of an unbiased antagonist and a β-arrestin biased agonist on the function of the AT_1R-β_2AR heterodimer

HEK 293T cells were transfected with plasmids encoding β_2AR-Sluc, AT_1R and β-arrestin2-Venus (25 ng, 100 ng, 100 ng pro well, respectively). BRET ratio was measured after treatments with 10 µM ISO, 10 µM candesartan (A) and 1 µM TRV120023 (B). Data are mean±SEM, n=3. * means significant interaction between the treatments ($p<0.05$, Two-Way ANOVA).

Figure 6

Costimulation of AT_1R and β_2AR increases the duration of β-arrestin2 clusters

HEK 293T cells were grown on glass coverslips, and were transfected with plasmids encoding β_2AR-Cerulean, AT_1R-Δ319 and β-arrestin2-Venus (1 µg, 4 µg, 0.5 µg pro

well, respectively). 5 to 15 minutes after stimulation, 20 images were taken at the bottom of the cells every ten seconds by confocal laser-scanning microscope. A, Representative β-arrestin2 clusters at fist (Frame -9), tenth (Frame 0) and nineteenth (Frame +9) frames. The β-arrestin2 clusters were identified on the tenth (Frame 0) frame by the neuronal network algorithm, and followed through all the frames. The circles show the identified puncta on Frame 0, and corresponding clusters on Frame -9 and Frame +9. Only some of the β-arrestin2 puncta remain through all the frames after ISO treatment. After AngII+ISO cotreatment, big fraction of β-arrestin2 puncta is apparent on all the frames, indicating increased lifespan of β-arrestin2 clusters. Scale bar 2 µm. B, Median lifespan of β-arrestin2-Venus puncta upon ISO or AngII+ISO treatment. After ISO and AngII+ISO treatment, the median durations of puncta in each cells (7222 from 40 cells and 6003 puncta from 35 cells, respectively, 3 independent experiments) were determined. The median duration of clusters after ISO and AngII+ISO treatment was significantly different ($p<0.001$, analyzed with Student's t-test). C, HEK 293T cells were transfected with plasma membrane targeted Venus, β-arrestin2-Rluc, untagged β_2AR and β-arrestin binding deficient AT_1R-Δ319. The plasma membrane target sequence was the first 10 amino acids of Lck, which is known to be myristoylated and palmitoylated. The nonspefic bystander BRET was measured, the increase of bystander BRET origins from the enrichment of β-arrestin2 at the plasma membrane after translocation to β_2AR. 10 µM ISO and 100 nM AngII were used as stimuli. Data are mean±SEM, n=3. * means significant interaction between ISO and AngII treatments ($p<0.05$, Two-Way ANOVA).

Figure 7

AT_1R activation prolongs β_2AR mediated cAMP signaling

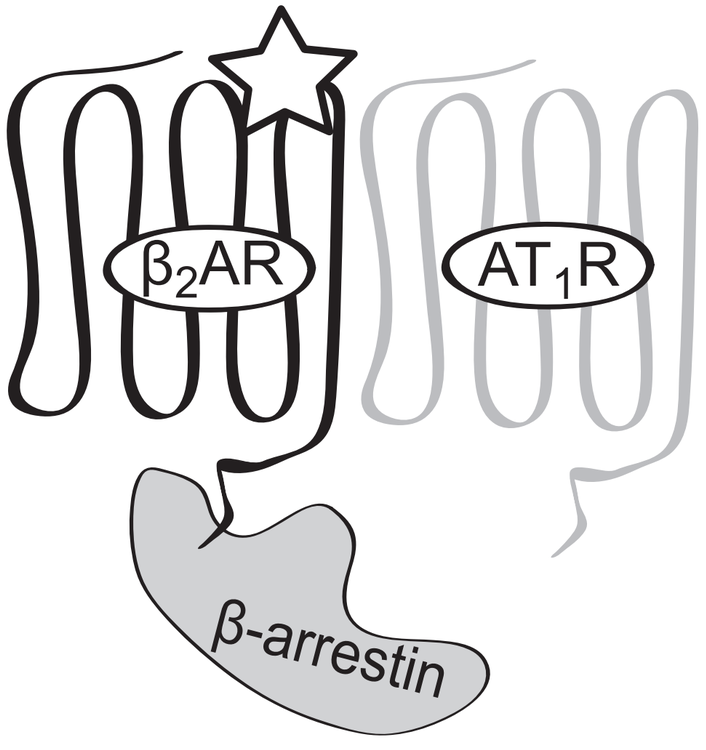
HEK 293T cells were cotransfected with AT_1R and the BRET-based cAMP biosensor EPAC (100-100 ng pro well). BRET ratio was measured after ISO (10 µM), AngII (100 nM), TRV120023 (1 µM) treatments. BRET measurements were made in A, modified Kreb's-Ringer medium or B, calcium-free modified Kreb's-Ringer supplemented with 100 µM EGTA and with 200 nM thapsigargin. BRET ratios are expressed as the percent of the highest ISO induced BRET ratio change in modified Kreb's-Ringer medium (100% EPAC response). Data are mean±SEM, n=3. C, EPAC responses after 15 minutes of stimulation are shown. Data are mean±SEM, n=3. * means significant interaction between ISO and AngII or TRV023 treatments ($p<0.05$, Two-Way ANOVA), # means significant difference between AngII+ISO and TRV023+ISO treatments examined by One-Way ANOVA with Bonferroni post hoc test.

Figure 8

Proposed model of the function of the AT_1R - β_2AR heterodimer

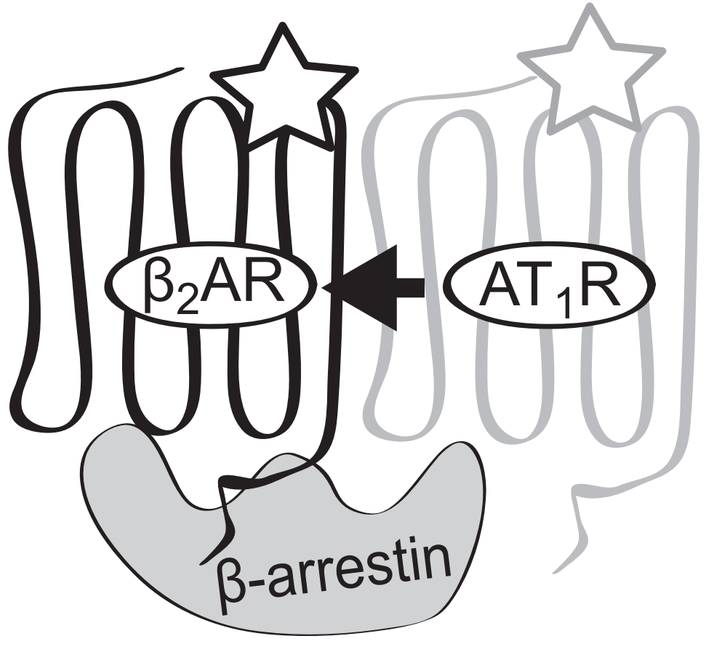
The stimulation of β_2AR leads to a weak interaction between the receptor and β-arrestin. When AT_1R is coactivated, AT_1R allosterically modulates the β_2AR and enhances its β-arrestin binding properties.

Graphical Abstract



Weak interaction

Agonist /
biased agonist



Stronger interaction

Supplementary Material

Click here to download Supplementary Material: B2AR_AT1R_arrestin_supplfigures.pdf

Supplementary Material Fig. legends

Click here to download Supplementary Material: supplfigurelegends_beta2adr_arrestin.doc