The role of cross-talk mechanisms between plasma membrane receptors in the regulation of β-arrestin activation

Doctoral theses

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Introduction

G protein-coupled receptors (GPCRs) consist the largest family of plasma membrane receptors. They transmit the intracellular effects of several chemical (such as neurotransmitters, hormones, paracrine mediators) and physical stimuli. Their exceptional biomedical significance is illustrated by the fact that more than one third of the prescribed drugs target GPCRs.

GPCRs transduce their signal through activation of intracellular effectors. Among the several hundred interacting proteins, β-arrestins play prominent role in their signal transduction in addition to G proteins. The binding of β-arrestins to agonist-activated receptor is a two-step process. First, they bind to the GPCR kinase (GRK)-phosphorylated receptor C-tail, then bind to the helix bundle of the activated receptor. The binding between GPCRs and β-arrestins can be transient (class A, occurs only at the plasma membrane) or stable (class B, takes place in endosomes as well). β-arrestins play several roles in the regulation of receptor function. They can inhibit the G protein activation of the receptor, i.e. they

induce receptor desensitization, and they determine the number of receptors in the plasma membrane by promoting receptor endocytosis. Both mechanisms contribute to a decreased drug response to repeated drug administration in GPCR-targeted therapies. Moreover, β-arrestins not only attenuate the signal of GPCRs, but they can qualitatively modify it, thus they also act as effector proteins in signal transduction. The most known example for that is the β-arrestin-dependent regulation of mitogen activated protein kinases (MAPK). So far it has been assumed that β-arrestins are mainly involved in the homologous regulation of the receptors, whereas heterologous mechanisms triggered by other receptors (such as heterologous phosphorylation-induced desensitization) are independent of β-arrestins.

It is widely accepted that GPCRs have not only an active and an inactive conformation, but they can adopt multiple active states, which can differ in their signaling properties. Some GPCR ligands (called biased agonists) stabilize distinct receptor conformations, which can couple only with particular effectors. These findings provided possible strategies for signaling pathway-

specific therapeutic interventions, from which some are already under clinical trials.

Signaling of GPCRs is regulated by multiplex mechanisms. It is known that GPCRs may form higher order complexes (dimers or oligomers), which can dramatically influence the function of the protomers. The intradimeric allosteric interaction can determine the receptor conformation as well as their ability to activate effectors. Notably, investigation of dimerization is highly complicated by the fact that the reliability of the available methods for dimerization detection is limited, hence the existence of some receptor dimers is debated. Growing number of evidence shows that GPCR dimers participate in the fine regulation of physiological processes, in addition, altered functions of receptor dimers are believed to play a role in the pathogenesis of several diseases. However, it is less known about the role of dimerization in the activation of β-arrestins or how can drug molecules, such as biased agonists, affect the function of receptor dimers.

Objectives

During my Ph.D. studies, I investigated the role of cross-talk mechanism between AT_1 angiotensin and other receptors in the regulation of β-arrestin activation.

Our questions in the first part of my research were:

- Can we verify the existence of the AT_1 angiotensin receptor (AT_1R)-β_2 adrenergic receptor (β_2AR) heterodimer with an improved, more reliable approach for detection of dimerization?
- Has AT_1R-β_2AR heterodimerization role in the binding of β-arrestins?
- How do biased AT_1R agonists affect the AT_1R-β_2AR heterodimer?

In the second part of my work, we searched the answers for the following questions:

- Does AT_1R bind β-arrestins upon its heterologous phosphorylation?
- If so, do heterologous and homologous activated β-arrestins differ in their conformation and function?

Methods

Cell culture and transfection

HEK 293T and COS-7 cell lines were cultured in DMEM supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum at 37 °C and 5% CO_2. Transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions. Measurements were made 24- (HEK 293T cells) or 48-hour (COS-7 cells) after transfection.

Bioluminescence resonance energy transfer (BRET) measurements

Expression of fluorescent proteins was determined by fluorescence measurements (excitation at 510 nm, emission was measured at 535 nm). BRET was followed after addition of luciferase substrate coelenterazine *h* (5 μM) by measuring light intensity at donor and acceptor emission maxima with 530 nm and 480 nm filters, respectively. Total luminescene was also determined without filter in the case of BRET titration experiments and to study the expression of mutant donor-tagged constructs. Fluorescence and luminescence values were

measured with Thermo Scientific Varioskan Flash multimode plate reader. All experiments were performed in duplicate or triplicate. BRET was analyzed by BRET ratio calculation (I_{530nm}/I_{480nm}), ΔBRET reflects the agonist-induced change in the BRET signal compared to vehicle-treated controls.

To assess the intramolecular BRET signal of Rluc8-β-arrestin2-FlAsH sensors, cells were labeled with 500 nM FlAsH-EDT_2 in the presence of 12,5 μM ethane-dithiol (EDT) and 0,1% DMSO in modified Ringer solution for 1 hour at room temperature. The excess and the non-specifically bound fraction of FlAsH dye was removed with a solution containing 250 μM EDT. Thereafter, the same protocol as in the ordinary BRET experiments was followed.

Confocal microscopy and image analysis

To determine the lifetime of β-arrestin clusters in the plasma membrane, time-series images were taken from the bottom of cells every 10 seconds for 190 seconds with Zeiss LSM 710 confocal laser-scanning microscope using a 63× objective at 37 °C. Images were analyzed using

ImageJ software, then a machine learning algorithm was used to follow β-arrestin puncta of the 10th frame in the former and latter frames. Based on their lifetime, the puncta were divided into two subgroups.

To assess the class type of β-arrestin binding, images were taken from middle cross-section of the cells 20 to 40 minutes after stimulation at 37 °C.

Co-precipitation experiments

Cells were transfected with plasmids encoding NES-BirA (biotin ligase), β-arrestin2-Cerulean and/or AT_1R-YFP-BAP (BAP: biotin acceptor peptide). After 24 hours, 150 μM biotin was added for 20-24 hours to obtain high extent biotinylation of AT_1R-YFP-BAP. Next, the medium was changed to serum- and biotin-free DMEM containing 1 % bovine serum albumin for 2-4 hours, then the cells were stimulated for 20 minutes at 37 °C. The reactions were stopped by placing on ice and ice-cold phosphate buffered saline (PBS) solution, then the samples were lysed. Biotin-labeled proteins were pulled down using NeutrAvidin agarose resin. The amount of fluorescently-tagged proteins was determined by YFP and

Cerulean fluorescence measurements. Fluorescent images were also taken from the NeutrAvidin beads with a confocal laser-scanning microscope using a 20× objective.

Western blot

Cells were scraped in SDS sample buffer, sonicated briefly, boiled for 15 minutes at 95 °C, and centrifuged for 10 minutes at 4 °C at 20800 g. Proteins in the supernatant were separated by SDS-polyacrylamide gel electrophoresis and were blotted onto polyvinylidene fluoride membranes. Membranes were blocked in PBS containing 0.05% Tween 20 (PBST) and 5% skim milk for 1 hour at room temperature, then were incubated overnight with primary antibodies (mouse anti-phospho-p44/42 MAPK or rabbit anti-p44/42 MAPK diluted 1:1000 in PBST with 5% skim milk) at 4 °C. Membranes were washed 3 times with PBST for 10 minutes, then were incubated with horseradish peroxidase-linked secondary antibody (goat anti-mouse or goat anti-rabbit diluted 1:5000 in PBST containing 5% skim milk) for 1 hour at room temperature, thereafter the washing procedure was repeated three times. The antibodies were visualized by

chemiluminescence. Before incubation with another primary antibody, antibodies were stripped using a guanidine HCl-based solution.

Statistical analysis

GraphPad Prism software was used for graph construction, curve fitting and statistical analysis. For analyzation paired two-sample t-test, one-sample t-test, ordinary or repeated-measurements one-way ANOVA with Bonferroni post-hoc test, two-way ANOVA with Bonferroni post-hoc test or covariance analysis were performed.

Results

Investigation the cross-talk mechanisms between AT_1R and β_2AR

Previous studies showed that AT_1 angiotensin and β_2 adrenergic receptors are able to physically interact with each other, which may alter their function. First, we wanted to verify the existence of the heterodimer using modified quantitative BRET measurements, a more reliable approach for dimerization detection developed by our group. Next, we examined whether heterodimerization could influence the β-arrestin binding properties of the dimer partners with a BRET-based approach. Donor (Sluc)-tagged β_2AR and acceptor (Venus)-tagged β-arrestin2 and untagged AT_1R were co-expressed. Thus, we were able to selectively follow the β-arrestin2 recruitment of β_2AR, and concomitantly follow the effects of AT_1R stimulation on this interaction. The AT_1R agonist angiotensin II (AngII) alone only slightly increased the BRET signal. Strikingly, simultaneous activation of both receptors potentiated the association between β_2AR and β-arrestin2 in both HEK 293T and COS-7 cell lines. Interestingly, activation of β_2AR did not alter the AT_1R-β-

arrestin2 binding significantly, demonstrating the asymmetric nature of the intradimeric regulation. Using inhibitors and signaling-deficient receptor mutants, we demonstrated that neither G protein nor β-arrestin activation are essential for the observed phenomenon, although its amplitude is determined by the extent of AT_1R expression. These results support the conclusion on the causative role of dimerization. The effects of different AT_1R ligands on the heterodimer functions were also assessed. Co-stimulation of isoproterenol (ISO) with the biased AT_1R agonist TRV120023 elevated the β_2AR-β-arrestin2 interaction, similar to the AngII effect. In contrast, treatment with the conventional AT_1R inverse agonist candesartan had no effect. To further investigate the underlying mechanism of increased β-arrestin2 binding, we determined the lifetime of β-arrestin2 clusters in the plasma membrane by confocal microscopy. Co-stimulation with AngII and ISO significantly increased the lifetime of β-arrestin2 clusters compared to ISO alone. These results suggest that the elevated β-arrestin2 binding of the heterodimerized β_2AR is due to the more stable interaction between the two proteins. We tested whether

heterodimerization influences the β_2AR-induced cAMP signal. Co-treatment of ISO and AngII or TRV120023 induced sustained cAMP signal, showing that AT_1R activation influences β_2AR not only in its β-arrestin binding, but also in its cAMP signaling.

Heterologous regulation of inactive AT_1R through β-arrestins

Although it is generally believed that the active receptor state is necessary for β-arrestin binding, we hypothesized that β-arrestins may also interact with inactive receptors if the receptor is phosphorylated at its C-terminus at proper localization. GRKs mainly phosphorylate the active receptors, unlike other kinases, such as protein kinase C (PKC), which are able to phosphorylate the receptor regardless its activation state. Since the PKC and the GRK phosphorylation sites overlap in AT_1R, we tested whether PKC phosphorylation could trigger AT_1R-β-arrestin interaction in the absence of receptor stimulation. Remarkably, we were able to co-precipitate biotinylated AT_1R-YFP-BAP with β-arrestin2-Cerulean using NeutrAvidin beads upon treatment with

the specific PKC-activator phorbol-ester (PMA). We followed the heterologous β-arrestin2 binding in real time with BRET measurements between Rluc8-tagged AT_1R and β-arrestin2-Venus, which interaction was slower than that of AngII. We observed similar effects when we induced PKC activation through stimulation of α_{1A}-adrenergic (α_{1A}AR) or epidermal growth factor receptor. We proved the role of PKC in the process using its inhibitors (GF109203X or staurosporine), whereas the role of constitutive activity of AT_1R was ruled out with inverse agonist (candesartan) pretreatment. Using receptor and arrestin mutants, we found in BRET and confocal microscopy measurements that both S-T cluster in receptor C-terminus (T332, S335, T336, S338) and two conserved lysine in β-arrestin2 N-domain (K10,11) are essential for the stable β-arrestin binding. These regions directly interact with each other and form a stability lock. In these experiments we also found that PMA induces class B-type β-arrestin2 binding, similar to AngII, but this interaction completely depends on the formation of the stability lock, because mutation of these regions diminished the PMA effect. We also examined the

conformation of β-arrestin2 using intramolecular β-arrestin2 FlAsH BRET biosensors. We found that β-arrestin2 binds to AT_1R with different conformation and dynamics after AngII or PMA treatment. Furthermore, selective disruption of the stability lock induced another distinct active β-arrestin conformation. Thus, at least 3 different active β-arrestin2 conformations can be distinguished.

β-arrestins participate in the regulation of the intracellular fate of AT_1R. To follow the intracellular trafficking of AT_1R, we measured BRET between Rluc8-tagged AT_1R and Venus-tagged intracellular vesicle markers (Rab4, Rab5, Rab7, Rab11) Both AngII and PMA stimulation elevated the BRET signal in the case of all Rab constructs, reflecting the redistribution of the receptor from the plasma membrane to intracellular vesicles. However, after PMA stimulation the appearance of AT_1R in the degradation pathway (Rab7) was decreased. These results suggest that heterologous activated β-arrestin2 regulates the intracellular fate of the receptor differently. It is known that activated β-arrestins are able to scaffold receptor-β-arrestin-MAPK complexes. We examined

whether the inactive-receptor bound β-arrestin also possesses this property. To test this, we developed a BRET-based approach, in which we measured energy transfer between AT_1R-Rluc8 and Venus-MEK1 or ERK2-Venus in the presence of overexpressed β-arrestin2. We found a significant increase in the BRET signal upon PMA treatment, which was smaller than that of AngII but proportional to the extent of AT_1R-β-arrestin2 binding. We observed similar results when we induced PKC activation by α_{1A}AR stimulation. These results suggest that not only active receptors may participate in signal transduction, but inactive receptors can contribute to signaling through the formation of signaling complexes.

Conclusions

We verified the existence of heterodimerization between β_2 adrenergic receptor and AT_1 angiotensin receptor using more reliable quantitative BRET titration measurements. We characterized the functional properties of the β_2AR-AT_1R heterodimer. We demonstrated that β-arrestin binding of β_2AR is potentiated by the co-activation of AT_1R. We showed that the increased interaction is due to the sustained binding between β_2AR and β-arrestin. We demonstrated that conventional AT_1 receptor antagonists and biased AT_1 receptor agonists alter the function of β_2 adrenerg-AT_1 angiotensin receptor heterodimer differently.

We proved that unliganded and inactive AT_1R is capable to bind β-arrestin2 upon PKC activation. We showed that the structural basis of this interaction is the stability lock, a bond formed by receptor C-terminal serine-threonine clusters and two conserved lysine in N-domain of β-arrestin2. We demonstrated that the stability lock stabilizes the AT_1R-β-arrestin2 interaction and determine the active conformation of β-arrestin2.

We assessed that β-arrestin2 binds to AT_1R with distinct and static conformation upon PKC activation, in contrast to the agonist-induced dynamic conformation.

β-arrestin2 bound to PKC-phosphorylated AT_1R triggers receptor endocytosis but the homologous and heterologous pathways govern the intracellular fate differently. Furthermore, we demonstrated that heterologous activated β-arrestin2 induces the formation of AT_1R-β-arrestin2-MAPK signaling complexes, which results suggest that AT_1R as a scaffold protein may also participate in signaling pathways of other receptors.

List of publications

Publications related directly to the theses:

Tóth AD[1], Prokop S[1], Gyombolai P, Várnai P, Balla A, Gurevich VV, Hunyady L, Turu G. (2018)

Heterologous phosphorylation-induced formation of a stability lock permits regulation of inactive receptors by β-arrestins.

J Biol Chem, 293(3):876-892. **IF: 4.125**

[1] These authors contributed equally to this work.

Tóth AD, Gyombolai P, Szalai B, Várnai P, Turu G, Hunyady L. (2017)

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

Mol Cell Endocrinol, 442:113-124. **IF: 3.754**

Other publications:

Prokop S, Perry NA, Vishnivetskiy SA, **Toth AD**, Inoue A, Milligan G, Iverson TM, Hunyady L, Gurevich VV. Differential manipulation of arrestin-3 binding to basal and agonist-activated G protein-coupled receptors.
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Szakadáti G, **Tóth AD**, Oláh I, Erdélyi LS, Balla T, Várnai P, Hunyady L, Balla A. (2015)
Investigation of the fate of type I angiotensin receptor after biased activation.
Mol Pharmacol, 87(6):972-81. **IF: 3.931**

Gyombolai P, **Tóth AD**, Tímár D, Turu G, Hunyady L. (2015)
Mutations in the 'DRY' motif of the CB1 cannabinoid receptor result in biased receptor variants.
J Mol Endocrinol, 54(1):75-89. **IF: 2.947**