Investigating the dimerization of G Protein-coupled Receptors in eukaryotic cell lines

Doctoral theses

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Introduction

G Protein-coupled Receptors (GPCRs) are one of the largest family of plasmamembrane receptors. Their importance is shown by the fact that they can be found in almost all eukaryotes and the human genome contains more than 1000 GPCR coding genes. A wide range of physiological processes like sensory perception, neurotransmission, hormonal regulation and immune defense mechanisms are mediated by GPCRs. Among their ligands molecules with different chemical structure can be found, like ions, lipids, nucleotides, biogenic amines, peptides and glycoproteins, but the light-sensing receptors are also GPCRs. Their medical importance is indicated by the fact that almost half of the drugs used in clinical practice modify the function of GPCRs.

Results of the last two decade have shown that GPCRs can form dimers or higher ordered oligomers. A dimer can be formed between the same receptors (homodimerization) or between different receptors (heterodimerization). Dimerization can influence ligand binding, active conformation, interactions with effector molecules (heterotrimeric G Proteins, arrestins), thereby the signaling and internalization of GPCRs. The effects of dimerization on receptor signaling are proposed to have important physiological and pathophysiological consequences, while tissue specific GPCR heterodimers are important future drug targets.

Literature of the dimerization of GPCRs can be classified into two main groups: studies showing directly the dimerization as a protein-protein interaction and studies investigating the functional consequences of dimerization. It is important to note that it can be problematic to reliable show the dimerization with both methods. Distinguishing true dimerization

from aspecific protein-protein interactions is not a straightforward process in the case of plasmamembrane molecules. Functional interactions between GPCRs can be a consequence of dimerization but also the result of crosstalk between the signaling of these receptors.

Although the concept of GPCR dimerization is nowadays widely accepted, there are still many open questions on the field. Neither the structural background of dimerization nor the mechanisms of intradimeric interactions are clearly understood. Besides these topics the reliable demonstration of GPCR heterodimers can be viewed as one of the most important themes of the field, since GPCR heterodimers can be important drug targets in the future.

Objectives

During my PhD studies I investigated the dimerization of the different GPCRs studied in our laboratory and the functional consequences of the dimerization. The main objectives of my experimental work were as follows:

- Verifying the homodimerization of type I angiotensin receptor (AT_1R) with quantitative BRET method
- Creating a conformation sensitive BRET based AT_1R biosensor to directly monitor the conformation changes of AT_1R
- Investigating the effects of AT_1R homodimerization on β-arrestin2 binding, receptor conformation and ligand binding
- Investigating the molecular mechanisms behind the intradimeric interactions in AT_1R homodimer
- Developing a new quantitative BRET based assay to detect the dimerization of GPCRs with higher specificity

Methods

Plasmid constructs

To the C terminal tail region of the GPCRs used in our BRET experiments *Renilla* luciferase or different fluorescent proteins (yellow fluorescent protein – YFP or Venus) were fused. To create the conformation sensitive AT_1R-BS sensor YFP was inserted into the third intracellular loop of AT_1R and *Renilla* luciferase was fused to the C terminal. Mutant AT_1 receptors (DRY/AAY and TSTS/A) were created by site directed mutagenesis.

Arrestin binding of receptors was investigated with C terminal *Renilla* luciferase tagged β-arrestin2. For plasmamembrane targeted fluorescent and luminescent proteins we used the N terminal target sequence of Lyn kinase or the CAAX domain of kRas protein as target sequence. In the inducible heterodimerization system FRB and FKBP protein domains were fused to the plasmamembrane targeted fluorescent of luminescent proteins.

Cell lines, transient transfection

We performed our experiments on CHO (chines hamster ovary) and HEK293 (human embryonic kidney) cells. Cell were cultured in Ham's F12 (for CHO cells) or DMEM (for HEK293 cells) medium supplemented with 10% fetal bovine serum, penicillin and streptomycin.

CHO cells were plated 24 hours prior to transfection on 6-well plates at a density of $5x10^5$ cells/well. Transient transfection was performed with Lipofectamine2000 according to the manufacturer's protocol. HEK293 cells were transfected with Lipofectamine2000 in OptiMem medium and

plated on 96-well plates at a cell density of 75000 cells/well. 6 hours after transfection the medium was always changed for complete medium.

Bioluminescence resonance energy transfer (BRET) measurements

In our experiments changes of receptor conformation, binding of β-arrestin2 to AT_1R and the interactions between different GPCRs was measured by the method of bioluminescence resonance energy transfer (BRET). BRET is an energy transfer method, which is based on the non-radiative energy transfer between a bioluminescent energy donor and a fluorescent energy acceptor. Occurrence of the energy transfer requires the molecular proximity (<10 nm) of the donor and acceptor molecules. *Renilla* luciferase, the energy donor used in our experiments oxides its substrate coelenterazine h, leading to light emission at 485 nm emission maxima. If the acceptor fluorescent protein (YFP or Venus in our experiments) is in molecular proximity, energy transfer leads to excitation of the acceptor and increased emission at 530 nm. BRET ratio, defined as

$$BRET_{ratio} = \frac{Emission_{530\,nm}}{Emission_{485\,nm}}$$

indicates the molecular proximity between donor and acceptor molecules.
BRET measurements were performed 24 hours after transfection at 37°C using a Mithras LB940 (Berthold) or a Variskan Multimode Reader (Thermo Scientific) plate reader.

Ligand dissociation assays

The dissociation of ^{125}I labeled angiotensin II was measured 24 hours 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 hours 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 +/- 1 μM cold angiotensin II. After different time elapsed, cells were washed two times with ice cold PBS, to remove dissociated ^{125}I angiotensin II, and bound radioactivity was measured by γ-spectrometry (Wallac 1470 Wizard) after solubilization with 0.5 M NaOH/ 0.05% SDS.

Monte Carlo simulations

Simulations were performed on a 2 dimensional membrane lattice containing 100x100 hexagons with periodic boundary conditions. Monomers of donors and acceptors were randomly placed on the empty hexagons of lattice. Total number of molecules was varied from 200 to 2000. The molecules were moved in each simulation step. For each monomer molecule a random neighboring hexagon was selected to move to. If the selected hexagon was occupied with another molecule, move was rejected and not repeated. For moving dimers, both protomers had to arrive onto a free hexagon, and falling apart of dimers was not allowed during the move step. Generally, dimers were allowed to move to the same direction and/or rotate around each other. Besides moving, monomers could form dimers and dimers could fall apart into monomers in each simulation step. For each monomer molecule one neighbor was randomly selected (if there was any), and the two molecules could form a dimer with a $p_{association}$ probability depending on the type of molecules. Dimers could dissociate in each time step with a probability $p_{dissociation}$, also depending on the protomers of the dimer. Simulation was performed for 1000 time steps, and simulated BRET values were calculated based on the total number of neighboring donor-acceptor pairs. Specific and non-specific interactions were simulated

by modifying the $p_{association}$ and $pd_{issociation}$ values. Simulations were written in Python 2.7.

Statistical analysis

Data analysis and figures were processed with GraphPad Prism 4.03 (GraphPad Software). Differences between groups were analyzed by 2-way ANOVA and Bonferroni post-hoc test. P values less than 0.05 were considered significant.

Results

During my doctoral work I investigated the dimerization of GPCRs and the functional consequences of dimerization.

In our experiments we investigated the intradimeric interactions and the molecular mechanisms behind them in the type I angiotensin receptor (AT_1R) homodimer, as a model system. Using an antagonist resistant AT_1R we set up a system where we were able to stimulate selectively one protomer of AT_1R homodimer, and follow the activation process of the other protomer. To follow the activation of the non-stimulated protomer we measured the β-arrestin2 binding and the conformational changes of this receptor by BRET based methods.

Our result showed that stimulation of one protomer of AT_1R homodimer leads to different conformation and β-arrestin2 binding of the other protomer. Based on our experiments with mutant AT_1 receptors we have shown that the conserved DRY region of the stimulated protomer is required for these effects.

We also investigated the intradimeric interactions using ligand dissociation experiments. The dissociation of ^{125}I labeled angiotensin II was increased in the presence of high concentrations of unlabeled angiotensin II. This negative cooperative ligand binding is widely accepted as a consequence of allosteric interactions within receptor dimers. We could not detect this negative cooperative ligand binding in DRY/AAY receptor expressing cells, suggesting also the importance of DRY region for intradimeric interactions.

In an other set of experiments we investigated the dimerization of V_2 vasopressin receptor (V_2R) with various other GPCRs by quantitative

BRET (qBRET) experiments. This method is used to distinguish between specific dimerization and non-specific interactions resulting only from overexpression and random collisions of labeled plasmamembrane proteins. In qBRET experiments constant amount of energy donor labeled receptor is coexpressed with increasing amount of acceptor labeled receptors, and BRET ratio is plotted as a function of acceptor/donor expression ratio. In the case of specific interaction a saturation curve is observed, while non-specific interaction leads to lineal relationship.

Investigating the dimerization of V_2R with AT_1R, β_2 adrenergic receptor (β_2AdR), CB1 cannabinoid receptor (CB_1R) and V_2R we observed saturation qBRET curves, suggesting the dimerization of these receptors, but also found that donor amount was not constant in our experimental setup.

To analyze the effects of non-constant donor expression on qBRET curves we performed Monte Carlo simulations and experiments with an inducible dimerization system, and found that decreases of donor expression can lead to saturation qBRET curves also in the case of non-specific interactions. We set up a modified qBRET method and with this new method we were able to distinguish between specific and non-specific interactions also when donor expression is not constant. With this new method we confirmed the homodimeriztation of V_2R and the homodimerization of CaSR calcium sensing receptor, and also the heterodimerization between V_2R and V_{1a} vasopressin receptor. We also showed that V_2R and CaSR do not form heterodimers with each other and with the investigated AT_1R, type II angiotensin receptor (AT_2R), β_2AdR and CB_1R. These results suggest that dimerization is a specific phenomenon, as only 3 of the investigated 13 receptor-receptor interactions were true dimerization.

Conclusions

During my doctoral work I investigated the dimerization of GPCRs and the functional consequences of dimerization. Our results enable us to draw the following conclusions:

- With quantitative BRET experiments we have verified the homodimerization of AT_1 angiotensin receptors.
- We created a BRET based, conformation sensitive biosensor to directly detect the changes of the AT_1R conformation.
- We have shown that stimulation of one protomer of AT_1R homodimer results the altered agonist binding affinity, altered conformation and β-arrestin2 binding of the other protomer.
- Our results indicate that the intradimeric interactions within the AT_1 receptor homodimer require the conserved DRY sequence of the stimulated protomer.
- With Monte Carlo simulations and experiments in an inducible dimerization system we have shown that classic qBRET experiments can lead to false positive results when the expression of energy donor labeled receptor is not strictly constant
- We developed a modified qBRET method to differentiate between specific and non-specific interactions also when donor expression is not constant.
- With this new method we have confirmed the homodimerization of V_2 vasopressin and CaSR calcium sensing receptors and the heterodimerization of V_2 and V_{1a} vasopressin receptors. Our results suggest that dimerization of GPCRs is a specific phenomenon, as

the V_2R and CaSR receptors did not form dimers with the investigated AT_1R, AT_2R, β_2AdR and CB_1R receptors.

List of publications:

My doctoral theses are based on the following publications:

Szalai B, Barkai L, Turu G, Szidonya L, Várnai P, Hunyady L. Allosteric interactions within the AT1 angiotensin receptor homodimer: role of the conserved DRY motif. Biochemical Pharmacology, 2012, 84(4): 477-485
IF: 4,576

Szalai B, Hoffmann P, Prokop S, Erdélyi L, Várnai P, Hunyady L. Improved methodical approach for quantitative BRET analysis of G protein coupled receptor dimerization. PLoS ONE, 2014, 9(10): e109503
IF: 3,234
IF: 4,576

Szalai B, Hoffmann P, Prokop S, Erdélyi L, Várnai P, Hunyady L. Correction: Improved methodical approach for quantitative BRET analysis of G protein coupled receptor dimerization. PLoS ONE, 2016, 11(5): e156824

My other publications:

Szekeres M, Turu G, Orient A, Szalai B, Süpeki K, Cserző M, Várnai P, Hunyady L. Mechanisms of angitensin II-mediated regulation of aldosterone synthase expression in H295R human adrenocortical and rat adrenal glomerulosa cell. Molecular and Cellular Endocrinology, 2009, 302(2): 244-253
IF: 3,503