# Investigating the role of phosphoinositides in the endocytosis of G protein-coupled receptors

Doctoral theses

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### **INTRODUCTION**

Phosphatidylinositol (PtdIns) and its phosphorylated derivates known as phosphoinositides are core components of membranes in mammalian cells. Although they constitute only a little fraction of cell phospholipids they have aroused the interest of various research fields in life sciences during the last decades. Attachment of phosphate groups to the inositol ring of PtdIns in various combinations give rise to seven different phosphinositide molecules that naturally occur in mammalian cells and show a typical distribution among different cellular membranes. Phosphoinositides where initially discovered as precursors of intracellular signalling molecules but soon they proved to have a broader role: they themselves can act as regulators of several cellular functions. For example they play a crucial role in the organization of intracellular organelles and the regulation of vesicular transport processes between them. The importance of phosphoinositides is highlighted also by the fact that nearly 50 different kinases and phosphatases take part in their synthesis and degradation, several of which have been linked to common human diseases such as obesity, diabetes and cancer.

The most abundant of the seven phosphoinositides is phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) $P_2$ ]. This lipid occurs mainly in the plasma membrane where it regulates diverse cellular processes. As a substrate for phospholipase C $\beta$  (PLC $\beta$ ) it is necessary for the formation of inositol 1,4,5-trisphosphate and diacylglycerol and thus the Ca<sup>2+</sup> signal and protein kinase C pathway initiated by them. In addition, PtdIns(4,5) $P_2$  also serves as a precursor for phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5) $P_3$ ] that is typically generated as a result of tyrosine kinase receptor activation and can trigger diverse signalling cascades regulating core cell functions such as cell survival and migration. Another important and well-known effect of plasma membrane PtdIns(4,5) $P_2$  is the regulation of ion channels and other membrane transporters: a significant proportion of transient receptor potential channels, as well as many

voltage-gated and leak  $K^+$  channels and other transmembrane transport proteins operate in a PtdIns(4,5) $P_2$ -dependent manner. Plasma membrane PtdIns(4,5) $P_2$ also plays an essential role in the dynamic organization of the cytoskeletal system and the regulation of membrane-cytoskeleton association implemented mainly by its binding to cytoskeletal adaptor proteins.

A major determinant of plasma membrane receptor function is the number of receptors found on the cell surface accessible for agonist ligands, which in turn is determined by the balance between the delivery of receptors to the plasma membrane and their removal by endocytosis. The most studied and best characterized form of endocytosis is the clathrin-mediated pathway which is used as a means of transport to the cell interior by G protein-coupled receptors, receptor tyrosine kinases and several other cell surface proteins.

Clathrin-mediated endocytosis is initiated by a membrane curvature oriented towards the cytoplasm that is followed by the collection of cargo molecules. Cargo proteins are directed to the location of endocytosis by various adaptor molecules: in the case of G protein-coupled receptors this function is fulfilled by  $\beta$ -arrestin molecules that bind to activated receptors. In parallel with the gathering of cargo a clathrin coat is assembled around the membrane invagination resulting in a structure referred to in the literature as the clathrin-coated pit. The neck of the deep, mature clathrin-coated pit is then constricted and gets pinched off the plasma membrane with the help of the GTPase protein dynamin. The coat around the newly formed vesicle disassembles rapidly and cargo molecules are then delivered by the vesicle to the early endosomal compartment where they are sorted for different pathways: they can be routed back to the plasma membrane via the recycling endosome or directed to the late endosome – multivesicular body – lysosome pathway for degradation.

An ever growing amount of evidence suggests that plasma membrane phosphoinositides, and PtdIns(4,5) $P_2$  in particular, play a prominent role in clathrin-mediated endocytosis. Several endocytic proteins have been shown to contain a domain or segment that can bind PtdIns(4,5) $P_2$ . In addition, it has been

confirmed in various cellular systems that (mainly long-term) lowering of plasma membrane PtdIns $(4,5)P_2$  levels by different methods negatively affects the process of clathrin-mediated endocytosis or, more precisely, the endocytosis of a model receptor (transferrin receptor in most cases). Although clathrin-mediated endocytosis has general features common for all types of cargo proteins, significant differences might also exist between various receptors in the adaptor proteins used and other details of the process. This raises the possibility that the endocytosis of distinct receptor families or even receptors can be regulated separately. At the time I started my studies no data similar to the findings with transferrin receptor were available concerning the endocytosis of G protein-coupled receptors.

## **OBJECTIVES**

During my PhD studies I investigated the cellular effects of phosphoinositides, primarily their role in the endocytosis of plasma membrane receptors. The main objectives of my experimental work were as follows:

- Development of a method for monitoring the post-activation movement and the endocytic process of plasma membrane receptors as a sequence of molecular interactions, based on bioluminescence resonance energy transfer (BRET)
- Comparison of the intramembrane movement and endocytosis of the wild type and a G protein activation-defected, as well as a desensitizationresistant mutant form of type 1 angiotensin receptor (AT<sub>1</sub>R) applying the above mentioned method
- Assessment of the impact of artificially induced acute reduction in plasma membrane  $PtdIns(4,5)P_2$ , as well as PtdIns(4)P levels on the endocytic process of  $AT_1R$  and other plasma membrane receptors
- Comparison of the impact of 5-phosphatase-mediated and hormoneinduced, phospholipase Cβ-mediated PtdIns(4,5)P<sub>2</sub>-depletion on receptor endocytosis

#### METHODS

#### **Plasmid constructs**

In our experiments we used either the untagged or the humanized *Renilla* luciferase-labeled version of  $AT_1R$  ( $AT_1R$ -luc). The other receptors ( $5HT_{2C}R$ ,  $\beta_2AR$ , EGFR) were fused with a modified *Renilla* luciferase optimized by point mutations ("Super luciferase"), with the luciferase always attached to the C-terminal, cytoplasmic end of the receptor. The plasmids coding the G protein activation-defected DRY-AAY mutant and the internalization-incompetent TSTS-AAAA mutant  $AT_1Rs$  were created with the QuikChange<sup>®</sup> Site-Directed Mutagenesis Kit from the sequence of the wild type  $AT_1R$ .

The endocytic marker molecules were labelled with various fluorescent proteins (yellow fluorescent protein – YFP, Venus and Cerulean). Fluorescent molecules were fused to the C-terminus of  $\beta$ -arrestin 2 and the N-terminus of the endosomal marker Rab5 molecule. For labelling of the plasma membrane we fused the fluorophores to several different sequences that targeted them to distinct specialized microdomains of the plasma membrane. The ordered (lipid raft) regions of the plasma membrane were labelled with the help of the N-terminal target sequence of either the Lyn or the Lck kinase proteins which get myristoylated and palmitoylated. The C-terminal CAAX motif of the K-Ras protein was used as a marker for the disordered (non-raft) structures of the plasma membrane.

The PtdIns(4,5) $P_2$ -depletion system that we used consisted of two protein constructs: the FRB domain targeted to the plasma membrane by the above mentioned Lyn or Lck sequence and the catalytic domain of type IV inositol polyphosphate 5-phosphatase (5-phosphatase) fused to the FKBP domain. In certain experiments these two proteins were expressed from a single plasmid using the viral T2A connecting sequence which enabled the production of two separate protein molecules in equal quantities by interrupting the translation of the single mRNA molecule. The FRB domain fused to wild type or TSTS-AAAA mutant  $AT_1Rs$  were used as a plasma membrane anchor for the 5phosphatase in some experiments. For depletion of PtdIns(4)*P* the catalytic domain of the Sac1 enzyme with a 4-phosphatase activity was used as described above, instead of the 5-phosphatase.

PtdIns(4,5) $P_2$ -depletion was verified with the help of the pleckstrin homology domain of phospholipase C $\delta$ 1 (PLC $\delta$ 1PH) that binds this phosphoinositide specifically. The domain was labelled with either *Renilla* luciferase, Venus or YFP on the C-terminus.

## **Cell lines, transient transfection**

We carried out our experiments on either of two human embryonic kidney fibroblast cell lines: the original HEK293 cell line or one of its variants which contained the T antigen of the SV40 virus (HEK293T). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as well as penicillin and streptomycin.

For BRET experiments HEK293 or HEK293T cells were grown in 10 cm plastic dishes and were trypsinized before transfection. For transient transfection, cells were plated on poly-lysine-pretreated 96-well plates at 5- $10x10^4$  cells/well density together with the intended DNA constructs and the cell transfection reagent (Lipofectamine 2000 or GeneCellin). Measurements were performed 24-26 hours after transfection. For microscopy experiments HEK293T cells were cultured on poly-lysine-pretreated glass coverslips at  $3x10^5$  cells/coverslip density, one day before transfection. Transfection was carried out by applying Lipofectamine 2000 reagent and a total amount of 1-2 µg DNA/coverslip, 24 hours prior to the experiment.

### **BRET** method

To examine the interaction between the investigated receptors and various endocytic marker proteins we used a method based on bioluminescence resonance energy transfer (BRET). The principle underlying this method is the energy transfer without foton emission between a bioluminescent donor and a fluorescent acceptor molecule that occurs when the two molecules are in appropriate proximity (less than 10 nm distance) to each other. In our BRET system we applied *Renilla* luciferase as the donor molecule. This enzyme emits light with a 480 nm peak intensity while oxidating its substrate coelenterasine. If an appropriate acceptor molecule (YFP or Venus in our case) is located close enough, however, the energy transfer occurs and the acceptor fluorophore will emit light instead at its own emission wavelength (with maximal intensity at about 530 nm). In our experiments we fused these donor and acceptor molecules to the interaction partners to be studied and the energy transfer between them was characterized as the ratio of acceptor and donor intensities (BRET ratio) which is dependent on the donor-acceptor distance. BRET measurements were carried out 24-26 hours after transfection at 37°C using a Mithras LB940 multilabel reader (Berthold) or a Varioskan<sup>TM</sup> Flash multilabel reader (Thermo Scientific).

### **Confocal microscopy**

The intracellular localization of AT<sub>1</sub>R and the endocytic markers as well as their colocalization were tested by confocal microscopy. The untagged receptors were visualized by fluorescent ligands (angiotensin II labelled with Rhodamine or Alexa-Fluor488) while the endocytic molecules were fused to different fluorescent proteins for confocal experiments. The measurements were performed at 35°C on HEK293T cells cultured on coverslips and transfected as described earlier. Confocal images were taken with a Zeiss LSM510 scanning confocal microscope and a 63x objective. Post-acquisition picture analysis was performed using Photoshop (Adobe) software.

## Statistical analysis

Data analysis and figures were processed with Sigmaplot 10.0 (Systat Software). Differences between groups were tested by two-way ANOVA and Bonferroni post-hoc tests and a p value less than 0.05 was considered significant.

#### RESULTS

During my doctoral work my primary aim was to study the consequences of the acute decrease of plasma membrane  $PtdIns(4,5)P_2$  levels on the endocytosis of G protein-coupled receptors. To do this we first set out to develop a method with which the endocytic route of receptors from the plasma membrane to the endosome can be detected as a sequence of molecular interactions in live HEK293 cells. This method was based on BRET which enabled detection of molecular proximity with high sensitivity. The movement of receptors was followed as a change in energy transfer between receptors tagged with Renilla luciferase and fluorescent molecules fused to marker molecules that label distinct cell compartments or interact directly with the receptors. Using this approach we managed to detect the stimulus-induced endocytosis of three G protein-coupled receptors,  $AT_1R$ ,  $5HT_{2C}R$  and  $\beta_2AR$ from the aspect of three different interactions. Association of activated receptors with  $\beta$ -arrestin 2 caused a dose-dependent increase in the BRET signal whereas detachment from fluorescently labelled plasma membrane markers was observed as a signal decrease. Arrival of receptors in the early endosomal compartment was indicated by a dose-dependent rise in the BRET signal with Rab5. The connection and colocalization of  $AT_1R$  with the above mentioned partner molecules were confirmed by confocal microscopy. BRET measurements in hyperosmic medium which inhibits clathrin-mediated endocytosis verified that the signal changes corresponded to the endocytosis of receptors.

For labelling the plasma membrane we used two types of target sequences: the N-terminal segment of the tyrosine kinase Lyn which is primarily found in the raft microdomains of the plasma membrane; and the C-terminal CAAX motif of the KRas protein which localizes preferably to non-raft regions. AT<sub>1</sub>R produced differential BRET signal changes when tested with these two plasma membrane markers. In contrast to the immediate drop in BRET signal observed with the raft marker, KRas CAAX showed an initial increase upon stimulation which might reflect the post-stimulatory movement of the receptor between plasma membrane microdomains. Using the G protein activationdefected DRY-AAY and the phosphorylation-resistant and internalizationincompetent TSTS-AAAA mutant forms of  $AT_1R$  we demonstrated that this post-stimulatory movement is presumably dependent on G protein activation but does not require receptor phosphorylation.

To acutely reduce plasma membrane  $PtdIns(4,5)P_2$  levels we used a method previously developed by our group that is based on heterodimerization of the protein domains FRB and FKBP. FRB was anchored to the plasma membrane by the previously mentioned N-terminal target sequence, whereas FKBP was fused with the catalytic domain of a 5-phosphatase enzyme capable of dephosphorylating  $PtdIns(4,5)P_2$ . Addition of rapamycin induces heterodimerization of the two domains which in turn causes the translocation of the originally cytoplasmic 5-phosphatase to the plasma membrane where it degrades PtdIns $(4,5)P_2$ . We verified the functionality of our depletion system in confocal microscopy and BRET experiments with the help of a protein domain (PLC $\delta$ 1PH) which binds PtdIns(4,5) $P_2$  specifically and confirmed that the addition of rapamycin was followed by a significant decrease in plasma membrane PtdIns $(4,5)P_2$  levels.

In the next stage we combined our BRET-based method for monitoring receptor endocytosis with the PtdIns(4,5) $P_2$  depletion system. We were able to show that plasma membrane PtdIns(4,5) $P_2$  depletion entirely inhibits the appearance of activated AT<sub>1</sub>R, 5HT<sub>2C</sub>R and  $\beta_2$ AR in the Rab5-positive early endosome compartment. However, binding of  $\beta$ -arrestin 2 to activated receptors was unaffected by the absence of this phosphoinositide. We observed a partial inhibition in the detachment of receptors from plasma membrane markers after degradation of PtdIns(4,5) $P_2$ .

To further investigate the cause underlying this partial inhibition seen with plasma membrane markers we performed confocal microscopy experiments. For these measurements we improved our  $PtdIns(4,5)P_2$  depletion system in order to get reliable lipid degradation on the single-cell level. We expressed the two proteins required for depletion from a single coding plasmid with the help of a connecting viral T2A sequence which resulted in equimolar amounts of the two proteins in each cell. After having confirmed the PtdIns(4,5) $P_2$  degrading ability of this new system we further examined the endocytosis of AT<sub>1</sub>R using fluorescent ligands. We found that in the absence of PtdIns(4,5) $P_2$  activated AT<sub>1</sub>Rs were able to form clusters along the plasma membrane but in contrast to control measurements they did not enter the cells. We showed that AT<sub>1</sub>R colocalized with the light chain of clathrin in these clusters which suggests that these clusters might correspond to clathrin-coated pits of the plasma membrane.

We also tested the role of plasma membrane PtdIns(4)P in receptor endocytosis. To do this, we replaced the 5-phosphatase in our depletion system with a 4-phosphatase that dephosphorylates PtdIns(4)P specifically. As assessed in BRET experiments, selective degradation of PtdIns(4)P did not influence the post-stimulatory delivery of  $\beta_2$ ARs to Rab5-positive endosomes. Combined application of the 4-phosphatase and 5-phosphatase domains, however, was able to exert the inhibitory effect on endocytosis.

order the 5-phosphatase-mediated  $PtdIns(4,5)P_2$ In to compare degradation with the depletion induced by hormones through PLC $\beta$  activation, we created the AT<sub>1</sub>R-FRB domain fusion construct, both with wild type and desensitization-resistant TSTS-AAAA mutant receptors. These fusion proteins could either serve as a plasma membrane anchor for FKBP-5-phosphatase after rapamycin treatment, or induce  $PtdIns(4,5)P_2$  degradation via PLC $\beta$  activation when stimulated by angiotensin II. We confirmed in BRET measurements that both rapamycin and angiotensin II treatment caused  $PtdIns(4,5)P_2$  depletion. As tested by the BRET interaction between  $\beta_2AR$  and Rab5, wild type AT<sub>1</sub>R-FRB was able to prevent endocytosis of  $\beta_2AR$  through both rapamycin-induced lipid depletion and stimulation by angiotensin II. In the case of TSTS-AAAA mutant AT<sub>1</sub>R-FRB, however, while 5-phosphatase-mediated PtdIns $(4,5)P_2$  depletion still caused endocytosis inhibition, lipid degradation after angiotensin II stimulation did not interfere with the appearance of  $\beta_2AR$  in the early endosome.

#### **CONCLUSIONS**

In my doctoral work I studied the post-stimulatory movement and endocytosis of plasma membrane receptors and the role of phosphoinositides in these processes. Our results enable us to draw the following conclusions:

- We established a method for the detection of post-stimulatory intramembrane movement and endocytosis of plasma membrane receptors as a sequence of molecular interactions in live cells.
- Our data raise the possibility that wild type AT<sub>1</sub>R is not uniformly distributed in the plasma membrane but located in specific microdomains. Upon activation (but before internalization) this distribution might change which presumably requires G protein activation. The changes within the plasma membrane observed with AT<sub>1</sub>R do not occur after the activation of 5HT<sub>2C</sub>R and EGFR.
- We showed that during the agonist-induced endocytosis of  $AT_1R$  and two other G protein-coupled receptors ( $\beta_2AR$  and  $5HT_{2C}R$ ), binding of  $\beta$ arrestin 2 to the receptors occured independently from PtdIns(4,5) $P_2$ . However, degradation of PtdIns(4,5) $P_2$  caused a partial inhibition in the detachment of receptors from plasma membrane markers, whereas their delivery to the early endosome was completely abolished. We demonstrated that in the absence of PtdIns(4,5) $P_2$  activated  $AT_1Rs$  appear in clathrin-coated structures of the plasma membrane but their maturation and/or the fission of clathrin-coated vesicles from the plasma membrane is blocked. Our data confirmed that PtdIns(4)P is not required for agonistinduced endocytosis of  $\beta_2AR$  and it cannot compensate the lack of PtdIns(4,5) $P_2$  in the process.

• We found that in contrast with 5-phosphatase-mediated PtdIns(4,5) $P_2$  depletion, lipid degradation achieved by PLC $\beta$  activation through an internalization-incompetent AT<sub>1</sub>R mutant was unable to inhibit the endocytosis of  $\beta_2$ AR. These results are in accordance with the assumption that functionally distinct pools of PtdIns(4,5) $P_2$  might exist in the plasma membrane.

## LIST OF PUBLICATIONS

## My doctoral theses are based on the following publications:

**Toth DJ**, Toth JT, Gulyas G, Balla A, Balla T, Hunyady L,Varnai P. Acute depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate impairs specific steps in endocytosis of the G-protein-coupled receptor. *J Cell Sci*, 2012; 125: 2185-97.

## IF: 5,877

Balla A, **Toth DJ**, Soltesz-Katona E, Szakadati G, Erdelyi LS, Varnai P,Hunyady L. Mapping of the localization of type 1 angiotensin receptor in membrane microdomains using bioluminescence resonance energy transferbased sensors. *J Biol Chem*, 2012; 287: 9090-9.

IF: 4,651

## Additional publication:

Varnai P, Toth B, **Toth DJ**, Hunyady L,Balla T. Visualization and manipulation of plasma membrane-endoplasmic reticulum contact sites indicates the presence of additional molecular components within the STIM1-Orai1 Complex. *J Biol Chem*, 2007; 282: 29678-90

## IF: 5,581