

Investigating the role of plasma membrane phosphoinositides with newly developed biosensors

Ph.D. thesis

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Budapest
2016

Introduction

Inositol lipids are a uniquely important class of phospholipids built from a diacylglycerol (DAG) backbone linked to an inositol ring via a phosphodiester linkage. Reversible phosphorylation of the inositol ring at positions 3, 4 and 5 by a plethora of lipid kinases and phosphatases results in the dynamic formation of seven different phosphoinositides (PPIIn). These lipids were first recognized as precursors of the second messengers inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)P_3$] and DAG, but in recent years it has become clear that PPIIns also have important roles in several cellular functions ranging from the control of ion channels to vesicular trafficking and cell motility. Their levels in the plasma membrane (PM), endomembranes and in the cytoplasm can dynamically change upon hormonal stimulation which can influence these cellular processes thus measuring the level of PPIIns can help us to better understand their distinct functions.

There are several difficulties to face when investigating the role of PPIIns. However they are present in every organelles, their concentration is still very low, sometimes undetectable with the available methods, moreover their level can quickly change upon several external stimuli. There are dozens of enzymes which take part in the synthesis and degradation of PPIIns. Sometimes the same synthetic step can be made by more than one enzyme, thus it is complicated to investigate one's specific role. Different PPIIns are present in the different organelles which helps the proteins to recognize their target compartment. Thus the physiological or artificial change of the PPIIn pools will influence the function of all these proteins which makes it very problematic to specifically investigate distinct cellular functions.

In the past three decades, several techniques were used to determine PPIIn levels. One of the first methods was the metabolic labeling of the cells with myo- ^3H inositol or ^{32}P -phosphate followed by lipid extraction and separation by thin-layer chromatography. These studies were the first steps of the inositol lipid research field but they required millions of cells and they could not give any information about the subcellular localizations of the PPIIns. Measurements of the total lipid mass by mass spectrometry have been achieved with great sensitivity, however it still suffered from the lack of spatial resolution and it could not allow resolution of different regio-isomers. Other groups used fluorescently labeled lipids, which had the advantage of following spatial and compartmentalized changes in living cells, but their major limitation was that the probes did not have the same hydrophobic character and greatly altered the distribution of endogenous lipids inside the cell. Another approach can be the application of antibodies raised against specific isomers of inositol lipids however it

needed fixed cells, thus following the dynamic changes of the PPIs was impracticable. With the introduction of GFP-fused protein domains that recognize PPIs in living single cells, our ability to follow localized changes has been significantly improved and a plethora of knowledge has accumulated regarding the spatial distribution and dynamics of PPIs. Although these methods are not without limitations, they are still enormously useful in exploring the PPI landscape. One difficulty that is increasingly obvious with these methods is the quantification of the changes and the generation of data from a significant number of cells.

The major aim of my Ph.D. work was to develop a method which enables us to follow the dynamic change of various inositol lipid species in the plasma membrane and the cytoplasmic $\text{Ins}(1,4,5)P_3$ in live cells with both high sensitivity and subcellular resolution in a single, convenient assay.

Objectives

However several PPIIn detection methods exist, none of them is perfect. Sometimes they are not sensitive enough, the procedures are difficult, inaccurate and they can not follow the small dynamic changes of PPIIn levels when stimulating the cells with hormones in a concentration close to the physiological range.

The major aims of my Ph.D. work were the following:

- to create intramolecular FRET and BRET sensors which makes us capable to follow reliably the cytoplasmic $\text{Ins}(1,4,5)P_3$ level.
- to create and characterize intermolecular BRET sensors which are highly sensitive, specific and capable of semiquantitative characterization of PM $\text{PtdIns}4P$, $\text{PtdIns}(4,5)P_2$ and $\text{PtdIns}(3,4,5)P_3$ changes with high temporal resolution.

With the help of the newly developed molecular tools we wanted to investigate:

- the inositol lipid changes upon stimulation of cells with agonists of RTK and GPCR and its molecular background.
- the effects of receptor activation induced inositol lipid changes in different cell physiological functions (such as $\text{Ins}(1,4,5)P_3$ signal, receptor internalization).

Methods

DNA constructs

Wild type human M₃ cholinergic receptor was purchased from S&T cDNA Resource Center. The human EGF receptor, the rat type-I angiotensin receptor (AT₁R), the non-internalizing rat type-I angiotensin receptor (AT₁R-Δ319), the β₂AR-luc, Venus-Rab5 and β-arrestin2-mRFP constructs were described earlier by our group.

To create the various phosphoinositide biosensors, first we created a set of lipid binding domains tagged with either Cerulean (for confocal measurements) or with super *Renilla* luciferase (for BRET measurements). For this, we used previously characterized domains including PLCδ1-PH-GFP, the binding-defective PLCδ1(R40L)-PH-GFP, Btk-PH-GFP and GFP-OSH2-2xPH. In addition, we also created the Cerulean- or Luciferase-tagged SidM-2xP4M construct by amplifying the sequences of the P4M domain from the GFP-SidM-P4M construct. Next, similar to other constructs the coding sequence of the PM-targeted Venus in frame with the sequence of the viral T2A peptide was subcloned to 5' end of the tagged lipid binding domain sequences resulting in the transcription of a single mRNA, which will subsequently lead to the expression of two separate proteins in mammalian cells. For PM targeting of Venus the same sequences were used, what we described in case of FRB (see above).

To create low affinity intramolecular Ins(1,4,5)P₃ biosensor the R265K, R269K, R568K, R504K and R265,269K mutations were introduced by site-directed mutagenesis (Agilent Technologies) in the previously created mRFP-InsP₃R-LBD (residues 224-605 of human type-1 InsP₃ receptor). To create the InsP₃ sensors first we made a FRET plasmid backbone by cloning the monomeric Venus into the pEYFP-C1 plasmid, in which YFP was already replaced by Cerulean. The wild type or mutant InsP₃R-LBDs were then inserted between the two fluorophores. From these FRET sensors the BRET sensors were prepared by replacing Cerulean with super *Renilla* luciferase. To improve the optical parameters, another set of BRET sensors were created by replacing Venus with the Venus cp173-Venus tandem used in other sensors like the Epac cAMP sensor. The Ca²⁺ sensor used in the BRET measurements was created by replacing the InsP₃R-LBD with the appropriate sequence derived from Cameleon D3.

The mRFP-FKBP-Pseudojanin, mRFP-FKBP-Sac1dead-5ptase and mRFP-FKBP-Sac1-5ptasedead constructs were a kind gift of Gerald R.V. Hammond. The PM targeted FRB-

mRFP and mRFP-FKBP-5ptase constructs used for rapamycin-induced PtdIns(4,5) P_2 depletion were described earlier, with the difference that for PM targeting of the FRB protein we used the N-terminal targeting sequence of mouse Lck protein, or the N-terminal targeting sequence of human c-Src protein instead of GAP43. The mRFP-FKBP-Sac1 was created from mRFP-FKBP-Pseudojanin (PJ) by removing the 5-phosphatase (5-ptase) domain from the construct.. The 5-ptase enzyme of the previously created PM-FRB-mRFP-T2A-mRFP-FKBP-5ptase [23] was replaced with Sac1, PJ and PJ-Sac1 with the difference that L₁₀ was used as PM target instead of Lyn and only the enzymes were fluorescently tagged.

Cell culture, transfection protocol

HEK 293T and COS-7 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza 12-604) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin in a 5% humidified CO₂ incubator at 37°C in 10 cm tissue culture plastic dishes.

For BRET measurements HEK 293T cells were trypsinized and plated on poly-lysine-pretreated (0.001%, 1 hour) white 96-well plates in 150 µl Opti-MEM (Gibco) medium at a density of 10⁵ cells/well together with the indicated DNA constructs (0.24–0.3 µg total DNA/well) and the cell transfection reagent (1.5 µl/well GeneCellin). After 6 hours 100 µl/well DMEM containing serum and antibiotics was added. Measurements were performed 25 hours after transfection.

For microscopic measurements and Western blot analysis HEK 293T or COS-7 cells were trypsinized and plated on poly-lysine-pretreated (0.001%, 1 hour) No 1.5 glass coverslips in 35 mm plastic dishes at 2-3x10⁵ cells/dish density. After one day the culture medium was changed to 1 ml Opti-MEM medium, and then 200 µl transfection solution containing the indicated DNA constructs (0,5-2 µg total DNA/dish) and 2 µl/dish Lipofectamine 2000 was added. After 6 hours 1 ml DMEM containing serum and antibiotics was added. Measurements were started 24 hours after the transfection.

Western blot

Twenty-four hours after transfection the HEK 293T cells were scraped into SDS sample buffer containing protease and phosphatase inhibitors, briefly sonicated, boiled at 95°C for 5 minutes, and separated on SDS-polyacrilamide gel. Then the proteins were transferred to PVDF membranes and incubated with the appropriate primary and secondary antibodies. The

antibodies were visualized by enhanced chemiluminescence, using Immobilon Western HRP substrate reagents (Millipore).

Confocal microscopy

Before the measurements the coverslips were placed into Attofluor cell chambers (Invitrogen) and the medium was changed to 800 μ l of a modified Krebs–Ringer buffer containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl₂, 0.7 mM MgSO₄, 10 mM glucose, and 10 mM Na-HEPES, pH 7.4. Confocal measurements were performed at 35°C using a Zeiss LSM 710 scanning confocal microscope and a 63x/1.4 oil-immersion objective. The indicated reagents were also dissolved in modified Krebs–Ringer buffer and were added manually in 200 μ l, and mixed three times. Post-acquisition picture analysis was performed using the Photoshop (Adobe) software to expand to the full dynamic range but only linear changes were allowed.

FRET measurements

Before the measurements the coverslips were placed into Attofluor cell chambers (Invitrogen) and the medium was changed to 800 μ l of a modified Krebs–Ringer buffer. Measurements were performed at room temperature using an inverted microscope (Axio Observer D1) equipped with a 40x/1.3 oil-immersion objective (Plan-APO) and a Cascade II camera (Photometrics). Excitation wavelengths (435 nm and 500 nm) were set by a monochromator connected to a 75 W Xenon lamp (DeltaRAM). The emitted light was separated by a dichroic beamsplitter (Chroma 69008bs), and then detected through the appropriate emission filters for Cerulean (470/24 nm) and Venus (535/30 nm). Images were acquired every 5 s. The indicated reagents were also dissolved in modified Krebs–Ringer buffer and were added manually in 200 μ l, and mixed three times. The MetaFluor (Molecular Devices) software was used for data acquisition, whereas for further data analysis including background subtraction, bleed through correction and 535/470 emission ratio calculation the MetaMorph (Molecular Devices) software was applied.

BRET measurements

Before measurements the medium of cells was changed to modified Krebs–Ringer buffer (50 μ l. Measurements were performed at 37°C using a Mithras LB 940 multilabel reader (Berthold). The measurements started with the addition of the cell permeable luciferase substrate, coelenterazine h (40 μ l, final concentration of 5 μ M), and counts were recorded

using 485 and 530 nm emission filters. Detection time was 500 ms for each wavelength. The indicated reagents were also dissolved in modified Krebs–Ringer buffer and were added manually in 10 μ l. BRET ratios were calculated by dividing the 530 nm and 485 nm intensities, and normalized to the baseline. In case of the intramolecular Ins(1,4,5) P_3 sensor values are given as I_0/I ratios therefore the elevation of cytoplasmic Ins P_3 level corresponds to an increased value on the graphs. Since the absolute initial ratio values depended on the expression of the sensors in case of the intermolecular inositol lipid sensors the resting levels were considered as 100%, whereas the 0% was determined from values of those experiments where cytoplasmic *Renilla* luciferase construct was expressed alone.

Quantification of proteins by SDS-PAGE

After performing BRET measurements, cells from the 96-well plates were incubated overnight at -85°C in SDS sample buffer containing protease and phosphatase inhibitors. On the following day cells were scraped, briefly sonicated, boiled at 95°C for 5 minutes, and separated on SDS-polyacrilamide gel. For the detection of fluorescent proteins Amersham Typhoon Phosphor Imager (Molecular Dynamics) was used. For the excitation we used an Argon laser (532 nm wavelength). The emitted light was detected by a 610 nm BP filter.

Statistical analyses

Data analyses and graphs were made by Sigmaplot 10.0 (Systat Software). Statistical analyses were performed by one-way ANOVA (analysis of variance) followed by Bonferroni t-test or by two-way ANOVA followed by Holm-Sidak test using SigmaStat 3.5 program (Systat Software Inc). To calculate the half-time values (τ) of the Ins(1,4,5) P_3 sensors' decay phase, a curve fitting procedure was applied to the values in each individual experiment using the 3 parametric exponential decay equation of ($y=y_0+ae^{-bx}$). τ values were then averaged and subjected to a t-test.

Results

Development of intramolecular Ins(1,4,5) P_3 sensor for single-cell and cell population measurements

Structural studies showed that Ins P_3 binding leads to a conformational change of the human type-I Ins(1,4,5) P_3 receptor LBD, which can be translated to a change in FRET signal between two appropriate fluorophores (e.g. Cerulean and Venu) placed at the two ends of the LBD. For BRET measurements we replaced Cerulean to luciferase and to enhance the efficiency of energy transfer we also created constructs with tandem yellow fluorescent tag (cp173-Venus) on their C-termini.

It has been show earlier that deletion of the N-terminal 223 amino acids increases the affinity of the LBD, so the 224-605 LBD has a higher affinity than the native Ins(1,4,5) P_3 receptor. Therefore, we decided to engineer slightly lower affinity mutants in order to improve its off-rate upon decrease in Ins(1,4,5) P_3 but still keep their abilities to detect the increase of Ins(1,4,5) P_3 level. We decided to perform a moderate modification by mutating the Ins(1,4,5) P_3 binding site. Based on the crystal structure of the binding domain, we selected and replaced specific arginine residues (R265, R269, R504 and R568) to lysines.

To investigate the Ins P_3 binding properties of these proteins, in vitro binding assays were performed using [3 H]-Ins(1,4,5) P_3 as the tracer. The binding of R265K, R269K and R568K were weaker compared to the wild type protein, while the R504K and the double mutant R265,269K were very poor Ins(1,4,5) P_3 binders. Based on these results, the R265K were selected for further analysis, and we created R265K mutant FRET and BRET sensors similar to the wild type sensor.

Development of intermolecular PPIIn sensors

To follow changes of lipid pools in the PM we decided to use non-specific BRET principles and created intermolecular sensors. The lipid recognition specificity of them is based on well-known and widely-used lipid-binding domains. For PtdIns4 P measurements we compared two different peptides previously used as PtdIns4 P recognizing domains; the tandem PH domain of OSH2 protein and the P4M domain of SidM protein. To increase the PM PtdIns4 P detection sensitivity, similar to the OSH2 PH domains, P4M domains were also used as tandems. For PtdIns(4,5) P_2 measurement the PH domain of PLC δ 1 was used, while

PtdIns(3,4,5) P_3 we used the PH domain of the Btk. These PPIIn-binding domains were linked to the *Renilla* luciferase enzyme required for BRET measurements. We also fused these domains to Cerulean for microscopy detection. In order to measure the PM fraction of the various PPIIn pools, the energy acceptor Venus was targeted to the PM, using either the first 10 amino acids of Lck (L10) or the first 15 of c-Src (S15), known as PM target sequences.

For optimal measurements of intermolecular BRET, cells have to express both components (the PM-targeted Venus and the lipid binding domain-fused luciferase) in a constant stoichiometry. To ensure the perfect ratio of co-expression, we created a single plasmid, which contained the coding sequences of both of the proteins separated by the sequence of the viral T2A peptide. During the translation, a molecular cleavage occurs within the T2A peptide, leading to the expression of two separate proteins in equimolar amounts. To test whether cleavage really does occur, HEK 293T cells transiently transfected with the fluorescent sensors were subjected to SDS-PAGE, and Western Blot analysis to detect the fragments with an anti-GFP antibody. We found that the cells expressed mainly the cleaved form of our intermolecular sensors and only a very small fraction of the sensors was present in an uncut form.

To visualize the intracellular localization of our sensors we performed confocal microscopy in COS-7 cells expressing the fluorescent sensor. The PLC δ 1-PH and the OSH2-2xPH showed clear PM localization in unstimulated cells, while the SidM-2xP4M sensors were located to the PM and the Golgi, where PtdIns4 P levels are considered to be high. As in resting cells the level of PtdIns(3,4,5) P_3 is low, the Btk-PH were found in the cytosol. As expected, both PM-targeting peptides (L₁₀ and S₁₅) were localized mostly to the PM.

To test the newly developed biosensors under the circumstances of BRET measurement, HEK 293T cells were transfected with different amount of plasmids (0.03 – 0.12 μ g/well on a 96 well plate) encoding the sequence of L₁₀-Venus-T2A-PLC δ 1-PH-Luc, or the binding defective R40L mutant PLC δ 1-PH containing construct. To cause complete depletion of PM PPIIn, cells were treated with 10 μ M ionomycin and 10 μ M wortmannin at the same time. We could detect a robust decrease of the BRET signal, and its minimum value (which was close to the one detected with the non-binding mutant sensor) was the same regardless of the expression level, which indicates the sensor's high sensitivity even in case of low lipid levels.

Comparison of the wild type and mutant Ins(1,4,5) P_3 sensors' dynamic range and reversibility

To compare the Ins(1,4,5) P_3 -induced signals of the wild type and low affinity sensors, HEK 293T cells were transfected with the cDNAs of both the human AT₁ receptor and the Sluc/Venus version of the appropriate Ins(1,4,5) P_3 sensor. BRET changes were then recorded following angiotensin II (Ang II) stimulation. Increasing concentrations of Ang II from 10⁻¹² to 10⁻⁷ M resulted in an increasing BRET signal in case of both the wild type and the mutant sensors. The signals also showed a kinetic difference as the concentration of Ang II was increased. While the maximal response was the same, a moderate shift to the right occurred on the dose-response curve in case of the sensor with the R265K mutation reflecting the lower affinity of this mutant sensor

To investigate the reversibility of the Ins(1,4,5) P_3 binding of the sensors, HEK 293T cells transiently expressing the M₃ cholinergic receptor were stimulated first with carbachol (10 μ M), which activated the Gq signaling pathway and elevated the cytoplasmic Ins(1,4,5) P_3 and Ca²⁺ levels. These responses can be quickly terminated by adding the competitive antagonist atropine (10 μ M). We found that the rising phases of the signals, between the wild-type and mutant Ins(1,4,5) P_3 sensors. In contrast, the mutant sensor showed a significantly enhanced off-rate ($\tau=23.0\pm 2.3$ s) compared to the wild type sensor ($\tau=44.9\pm 7.5$ s) (Mean \pm S.E.M. n=5, p=0.024), and a complete return to the baseline. Parallel measurements of cytoplasmic Ca²⁺ concentration, performed under the same experimental condition in cells that expressed the BRET version of Cameleon D3, a previously described low K_D Ca²⁺ sensor, revealed the termination of the Ca²⁺ signal upon atropine treatment with a kinetic, which was highly similar to the one showed by the mutant Ins(1,4,5) P_3 sensor. Thus we decided to use the R265K mutant sensor for further experiments.

Determination of the newly developed sensors' lipid selectivity

Next we compared the OSH2-2xPH and the SidM-2xP4M probes, both used previously as PtdIns4P reporters, for their specificity and suitability in our system. For this, we selectively changed the level of PtdIns4P using a previously described rapidly inducible PPI_n depletion system that is based on the heterodimerization of FKBP and FRB. In this approach, the phosphatase is fused to the FKBP protein, and upon addition of rapamycin the enzyme rapidly translocates to the membrane, where its binding partner, the FRB domain, is targeted.

As shown before, PtdIns(4,5) P_2 levels, reported by the PLC δ 1-PH domain, decreased rapidly when either the INPP5E 5-phosphatase enzyme alone or in combination with the

Sac1-phosphatase (which has 4-phosphatase activity) was recruited to the PM. Recruitment of the Sac1-phosphatase alone to the PM did not cause a decrease in the PLC δ 1-PH-generated BRET signal. In cells expressing the SidM-2xP4M based biosensor, we detected a decrease in the normalized BRET signal upon the recruitment of the Sac1 enzyme. When the 5ptase was recruited to the PM, the BRET signal from the SidM-2xP4M reporter showed a slight increase consistent with the conversion of PtdIns(4,5) P_2 to PtdIns4*P*. Combination of the Sac1 and 5-ptase enzymes again caused a decrease in the SidM-2xP4M-derived BRET signal. When the same experiments were carried out in cells expressing the OSH2-2xPH containing sensor instead of the SidM-2xP4M, we obtained different results. Depletion of either PtdIns4*P* or PtdIns(4,5) P_2 failed to cause any change in the BRET signal and it only decreased when both lipids were eliminated from the PM. We also performed confocal microscopy in HEK 293T cells transfected with the T2A version of our lipid-depleting system and the fluorescently-tagged lipid-binding domains and we obtained similar results.

Next we examined the effects of different PI4K inhibitors on the translocation of the aforementioned sensors. For this, we used pretreatment with the recently published PI4KA-specific inhibitor (A1) at 10 nM concentration. For PI4KB inhibition, we used 250 nM PIK-93, and we also used wortmannin (Wm) at 10 μ M and LY294002 at 100 μ M as non-selective type III PI4K inhibitors. For controls we used the BRET ratio that was measured in cells treated with DMSO. 10 min pretreatment with 10 nM A1, 10 μ M Wm and 100 μ M LY294002 caused a robust and significant in the signal from SidM-2xP4M, while those from OSH2-2xPH and PLC δ 1-PH were not affected. Pretreatment with 250 nM PIK-93 did not cause any change in the BRET ratio from any of the sensors. Since this compound mainly inhibits PI4KB, which primarily controls Golgi PtdIns4*P* levels, its impact on the PM PtdIns4*P* level is minimal at best. Taken together, all of these data suggest that SidM-2xP4M is the probe of choice for reliable detection of PM PtdIns4*P* levels and caution should be used when utilizing the OSH2-2xPH for this purpose. Therefore, in the following experiments we used SidM-2xP4M as PtdIns4*P* sensor.

Investigating PtdIns(4,5) P_2 -rich microdomains in the PM

As the structure of the PM is heterogenous, it raised the possibility that using different PM target sequences in our sensors results in different signals. The PM targeting sequence (L₁₀) used in the above studies is considered to provide PM targeting to ordered regions (also called as rafts). It contains a sequence that is both myristoylated and palmitoylated. The N-terminal c-*Src* sequence (S₁₅) is also myristoylated but contains several basic residues instead of

palmitoylation, and has been claimed to be excluded from rafts. Thus we also created a PtdIns(4,5) P_2 sensor in which the acceptor fluorescent protein were targeted to the PM by S_{15} . To compare the sensors HEK 293T cells were transfected with the sensors and either with the wild type or the mutant non-internalizing ($\Delta 319$) AT $_1$ R and then were stimulated with 100 nM Ang II, but we could not detect any differences between the sensors. Next we investigated whether the effect of the 5ptase recruitment on PtdIns(4,5) P_2 depletion depends on the sequence that is used for PM targeting of the enzyme. We compared the use of L_{10} -FRB and S_{15} -FRB on the effects of 5-ptase recruitment on Ins(1,4,5) P_3 -signal evoked by 100 nM Ang II in cells expressing wild type AT $_1$ R, the R265K sensor and one from the two different form of the lipid depletion system. Again, we could not detect any differences no matter in which microdomain we depleted the PtdIns(4,5) P_2 . As a next step, we compared the use of L_{10} -FRB and S_{15} -FRB on the effects of 5-ptase recruitment and PtdIns(4,5) P_2 depletion when monitoring its level by three different PM-targeted Venus constructs (L_{10} , S_{15} or PLC $\delta 1$ -PH) for comparisons. There was no difference between the signals regardless of which targeting sequence was used. Thus, the nBRET ratio changes under our experimental conditions did not depend on where the 5ptase enzymes were targeted, or where we measure the PPIs' level in the PM. As we could not detect any microdomain-related differences between the L_{10} and S_{15} containing anchors and sensors, the L_{10} target sequence was used in subsequent experiments.

Monitoring the agonist-induced changes of the PPIs upon stimulation of EGF and type-3 muscarinic receptors

Next we followed the dynamic changes of the various PM PPIs upon hormonal stimulation. For this, we chose the tyrosine kinase EGFR, known to activate both PI3K and PLC γ , and the G protein-coupled M $_3$ R, which activates PLC β . In HEK 293T cells expressing the biosensors and the EGFR 100 ng/ml EGF caused a rapid increase in PtdIns(3,4,5) P_3 reflecting PI3K activation and activation of PLC was reflected in a small increase in Ins(1,4,5) P_3 levels. As both enzymes uses PtdIns(4,5) P_2 as substrate, we could detect a slight decrease in the level of it. Surprisingly, we found that the PM PtdIns4 P level showed a substantial increase at the same time. When M $_3$ R expressing cells were stimulated with 100 μ M carbachol (Cch), a robust decrease in both the PtdIns4 P and PtdIns(4,5) P_2 was observed with a concomitant increase in Ins(1,4,5) P_3 . Since Cch stimulation causes a much more robust PLC activation than EGF, we wanted to evaluate lipid changes after stimulation by lower concentrations of Cch where the PLC activation is more comparable to those evoked by EGF. Lowering the concentration of Cch to 100 nM, we detected similar Ins(1,4,5) P_3 increases and

PtdIns(4,5) P_2 depletion as with EGF and at this low concentration of Cch, PtdIns4 P also showed an increase.

We also wanted to detect the same changes in the PPIIn pools in COS-7 cells with confocal microscopy, using the Cerulean containing PPIIn sensors. When stimulating M₃R with 10⁻⁷ M Cch the movement of the Cerulean-SidM-2xP4M and the Cerulean-PLC δ 1-PH was hard to evaluate, but massive hormonal stimulus (10⁻⁴ M Cch) caused an easily detectable translocation of the PPIIn-binding domains. These examples clearly demonstrated the advantage of the BRET approach as it detected even the small changes in the lipid pools that were hard to evaluate with simple imaging.

Investigating the background of the hormone induced elevation of the plasma membrane PtdIns4 P level

To investigate which PI4K enzyme(s) are important in the agonist-induced increase in PtdIns4 P upon stimulation with EGF or low concentration of carbachol, we pretreated cells expressing either the EGFR or the M₃R together with the PtdIns4 P sensor with various PI4K inhibitors. Inhibition of PI3Ks with low concentration of Wm (100 nM) or inhibition of PI4KB with 250 nM PIK-93 did not prevent the EGF or carbachol induced elevation of PtdIns4 P . In contrast, application of PI4KA specific inhibitor 10 nM A1 or using 10 μ M Wm (10 min), which inhibits all PI3Ks and also type III PI4Ks greatly reduced the PtdIns4 P levels even before stimulation and prevented the elevation of PtdIns4 P upon stimulation. As the application of the agonist caused no increase in PtdIns4 P levels when PI4KA was inhibited, it clearly implicated the role of PI4KA in the hormone-induced elevation of PtdIns4 P .

Next we explored the mechanism(s) by which agonists increase PI4KA activity. One of the common elements between RTK and GPCR stimulation is the activation of PLC and PKC. Pretreatment of the cells with bisindolylmaleimide (BIM), a pan PKC inhibitor (2 μ M 10 min) did not cause any change in the basal level of PM PtdIns4 P , but completely abolished its EGF- or Cch-evoked increase. Conversely, 100 nM PMA, activator of PKC induced a modest elevation in PtdIns4 P levels that was completely prevented by BIM pretreatment. These results suggested that PKC plays an important role in the maintenance of PtdIns4 P pools in the PM upon receptor activation.

The role of PtdIns4P resynthesis in signal transduction

We wanted to investigate the role of PtdIns(4,5) P_2 and PtdIns4P in the signal transduction of EGFR and M₃R. For this purpose we depleted either PtdIns(4,5) P_2 or PtdIns4P at the PM with the rapamycin-induced recruitment of 5ptase or Sac1 enzyme and stimulated the cells with EGF or carbachol. We measured Ins(1,4,5) P_3 levels and looked for the impact of depleting the respective lipids on Ins(1,4,5) P_3 formation. Depletion of PtdIns(4,5) P_2 by a recruited 5ptase measured 5 min after the rapamycin treatment almost completely abolished the agonist-evoked Ins(1,4,5) P_3 production. Reducing the levels of PtdIns4P by Sac1 recruitment had only a moderate effect on the subsequent Ins(1,4,5) P_3 . Pretreatment of the cells with A1, which depletes PM PtdIns4P via inhibiting the resynthesis of PtdIns4P, had a strong inhibitory effect on Ins(1,4,5) P_3 formation. Statistical analysis revealed that depleting the PtdIns(4,5) P_2 pool of the PM or inhibiting the synthesis of PtdIns4P by A1 had a significantly higher impact on the Ins(1,4,5) P_3 signal comparing to Sac1 recruitment. These findings are in good agreement with an earlier report suggesting that maintenance of PtdIns(4,5) P_2 pools during agonist stimulation can be achieved even at reduced PtdIns4P levels as long as the PI4KA enzyme is able to synthesize PtdIns4P at the PM.

The role of PtdIns4P resynthesis in receptor internalization

In our previous work we demonstrated that PtdIns(4,5) P_2 -depletion evoked by the recruitable 5ptase inhibits the internalization of GPCRs. However depleting PtdIns4P by Sac1 does not have any effect on it. We wanted to further investigate this process. Our main question was whether when activating a $G\alpha_q$ receptor the *de novo* PtdIns4P synthesis is necessary for maintaining the internalization [similar to what we have seen in case of the Ins(1,4,5) P_3] signal or not. For that, we transfected HEK 293T cells with β_2 AR-luc and Venus-Rab5 constructs and we measured the BRET signal between them (increasing ratio means the internalization of β_2 AR). To be able to cause a significant PLC β activation the wild type M₃R, and to increase the rate of internalization the β -arrestin2-mRFP constructs were also transfected into the cells.

Our results showed that only intense PLC activation (10^{-4} M Cch) and thus robust PtdIns(4,5) P_2 -depletion inhibited the internalization of β_2 AR (evoked by 1 μ M isoprenaline). 10 nM A1 pretreatment (10 min) alone did not have any effect on the internalization or on the level of PM PtdIns(4,5) P_2 . However in A1 pretreated cells even 10^{-7} M Cch could decrease the extent of β_2 AR's internalization, but it did not influence the level of PtdIns(4,5) P_2 depletion. Two-way ANOVA analysis and Holm-Sidak post hoc test revealed that in A1

pretreated cells Cch stimulation leads to a significantly higher inhibition of the β_2 AR's internalization than in control cells, but there is no difference in the extent of lipid depletion. These data suggest the existence of functionally distinct PPIIn pools in the PM.

Conclusions

According to our aims, we sum up the following conclusions:

- We created an improved $\text{Ins}(1,4,5)P_3$ sensor which can be used to monitor both the increase and decrease of $\text{Ins}(1,4,5)P_3$ levels in live cells. We demonstrated that replacing key arginines within the $\text{Ins}(1,4,5)P_3$ binding site of human type-I $\text{Ins}(1,4,5)P_3$ receptor with lysines results in decreased ligand affinity.
- We presented a BRET-based tool-set for robust and sensitive measurements of PM $\text{PtdIns}4P$, $\text{PtdIns}(4,5)P_2$ and $\text{PtdIns}(3,4,5)P_3$ dynamics with high temporal resolution in live cells. We found that SidM-2xP4M is a better choice for detecting PM $\text{PtdIns}4P$ than the widely used OSH2-2xPH. In our experiments we could not detect any differences in the inositol lipid composition between the PM's ordered and disordered regions.
- Our results reveal a previously unappreciated synthesis of $\text{PtdIns}4P$ that accompanies activation of both EGF and M_3 receptor. This signaling-induced $\text{PtdIns}4P$ synthesis relies on PI4KA and PKC.
- We clearly demonstrated that PI4KA activation is necessary for maintaining $\text{Ins}(1,4,5)P_3$ production and receptor internalization when parallel $\text{PtdIns}(4,5)P_2$ consumption is present. However these processes are not sensitive to artificial $\text{PtdIns}4P$ degradation which implicates the existence of functionally distinct PPIn pools in the PM.

List of publications

The PhD thesis is based on the following publications:

Toth JT, Gulyas G, Toth DJ, Balla A, Hammond G, Hunyady L, Balla T, Varnai P. (2016) BRET-monitoring of the dynamics changes of inositol lipid pools in living cells reveals a PKC-dependent PtdIns4P increase upon EGF and M3 receptor activation. *Biochim Biophys Acta*. 1861(3):177-187. **IF:** 5.162

Gulyas G* & Toth JT*, Toth DJ, Kurucz I, Hunyady L, Balla T, Varnai P. (2015) Measurement of inositol 1,4,5-trisphosphate in living cells using an improved set of resonance energy transfer-based biosensors. *PLoS ONE* 10(5):e0125601. **IF:** 3.234 (*These authors contributed equally)

Other publication:

Toth DJ, Toth JT, Gulyas G, Balla A, Balla T, Hunyady L, Varnai P. (2012) Acute depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate impairs specific steps in endocytosis of the G-protein-coupled receptor. *J Cell Sci*, 125: 2185-97. **IF:** 5.877

