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BRET-monitoring of the dynamic changes of inositol lipid pools in living cells reveals a PKC-dependent PtdIns4*P* increase upon EGF and M3 receptor activation

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ABSTRACT

Deciphering many roles played by inositol lipids in signal transduction and membrane function demands experimental approaches that can detect their dynamic accumulation with subcellular accuracy and exquisite sensitivity. The former criterion is met by imaging of fluorescence biosensors in living cells, whereas the latter is facilitated by biochemical measurements from populations. Here, we introduce BRET-based biosensors able to detect rapid changes in inositol lipids in cell populations with both high sensitivity and subcellular resolution in a single, convenient assay. We demonstrate robust and sensitive measurements of PtdIns4*P*, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 dynamics, as well as changes in cytoplasmic Ins(1,4,5)P_3 levels. Measurements were made during either experimental activation of lipid degradation, or PI 3-kinase and phospholipase C mediated signal transduction. Our results reveal a previously unappreciated synthesis of PtdIns4*P* that accompanies moderate activation of phospholipase C signaling downstream of both EGF and muscarinic M3 receptor activation. This signaling-induced PtdIns4*P* synthesis relies on protein kinase C, and implicates a feedback mechanism in the control of inositol lipid metabolism during signal transduction.

1. 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 (PPIns) [1,2]. In addition, activation of receptor tyrosine kinases (RTK) or G-protein coupled receptors (GPCR) can acutely regulate PPIn levels in response to a variety of external signals. These lipids were first recognized as precursors of the second messengers inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and DAG [3], but in recent years it has become clear that PPIns also have important roles in several cellular functions ranging from the control of ion channels to vesicular trafficking and cell motility [4]. Because of their rapid dynamics and general importance, measuring the changes in the level of these lipids in living cells is paramount to understanding their distinct localizations and functions.

In the past three decades, several techniques were used to determine PPIn levels. One of the first methods was the metabolic labeling of the cells with myo-[^{3}H]inositol or ^{32}P-phosphate followed by lipid extraction and separation by thin-layer chromatography [5]. 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 PPIns. 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 [6]. Other groups used fluorescently labeled lipids, which had the advantage of following spatial and compartmentalized changes in living cells [7], but their major limitation was that the probes did not have the same hydrophobic

Abbreviations: DAG, diacylglycerol; PPIns, phosphoinositides; RTK, receptor tyrosine kinase; GPCR, G-protein coupled receptor; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PM, plasma membrane; BRET, bioluminescence resonance energy transfer; PLC, phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4*P*, phosphatidylinositol 4-phosphate; PI4K, phosphatidylinositol 4-kinase; EGFR, epidermal growth factor receptor; M_3R, type-3 muscarinic receptor; PKC, protein kinase C; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; L10, the first 10 amino acids of Lck; S15, the first 15 amino acids of c-Src; FKBP, FK506 binding protein 12; FRB, fragment of mTOR that binds rapamycin; 5-ptase, 5-phosphatase; MβCD, methyl-beta-cyclodextrin; PI3K, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; Cch, carbachol; BIM, bisindolylmaleimide; PJ, Pseudojanin

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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 [8] however it needed fixed cells, thus following the dynamic changes of the PIs was impracticable. With the introduction of GFP-fused protein domains that recognize PPIns 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 PPIns [9].

Although these methods are not without limitations [10], they are still enormously useful in exploring the PPIn 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. A good example of fine-tuning these methods was recently published when demonstrating orthogonal lipid sensors, capable of in situ quantification of lipid pools in live single cells [11,12]. As another approach, to overcome the difficulties of quantification we developed a new molecular tool-set to monitor various inositol lipid species in the plasma membrane (PM) of stimulated living cells. Here we show that balanced expression of luciferase-fused PPIn-recognizing protein domains and a Venus protein targeted to the PM, allowed us to perform bioluminescence resonance energy transfer (BRET) measurements reflecting PPIn changes in populations of transiently transfected HEK 293T cells.

As PPIn level can quickly change upon hormonal stimulation and this can alter several cellular mechanisms, it is very important for the cells to be able to quickly replace the PPIns and thus to regain their responsiveness to external stimuli. There are several papers, which confirm experimentally and mathematically that following phospholipase C (PLC) activation the resulting Ins(1,4,5)P_3 kinetics are more rapid compared with the decrease and subsequent recovery in PM phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] [13–15]. Therefore, in parallel to degradative phospholipase C, PPIn resynthetic pathways have to be activated upon strong hormonal stimulation. It was also demonstrated that the resynthesis of PM phosphatidylinositol 4-phosphate (PtdIns4*P*) and PtdIns(4,5)P_2 requires a wortmannin-sensitive phosphatidylinositol 4-kinase (PI4K) enzyme [16], now identified as PI4KA [17]. Activation of PI4KA is indispensable in the quick resynthesis of PtdIns(4,5)P_2 because phosphatidylinositol-4-phosphate 5-kinase is considered to be fast and limited primarily by steps that replenish the PM pool of its substrate, PtdIns4*P* [18]. However the exact mechanisms by which this resynthetic process is initiated and regulated remains unclear.

Here, we show that our newly developed BRET-based approach is highly sensitive and capable of semiquantitative characterization of inositol lipid changes upon stimulation of cells with agonists of RTK and GPCR. Using this method we found that activation of the RTK epidermal growth factor receptor (EGFR) or the GPCR type-3 muscarinic receptor (M_3R) at a low level not only resulted in the already known PLC-mediated hydrolysis of PtdIns(4,5)P_2, but also increased the level of PtdIns4*P*. Using several subtype-specific PI4K inhibitors we show that activation of PI4KA is responsible for this phenomenon, and found to be mediated by the activation of protein kinase C (PKC). Our data thus introduce a robust, sensitive assay for real time readout of PPIn dynamics at the PM, and theoretically also at other individual organelle membranes in live cell populations. This assay reveals a significant increase in the PM PtdIns4*P* level upon both RTK and GPCR activation, and indicates the role for PKC in the regulation of PPIn re-synthesis at the level of PtdIns4*P* generation under low level of agonist stimulation, which is probably close to the physiological condition.

2. Material and methods

2.1. Material

Molecular biology reagents were obtained from Fermentas (Vilnius, Lithuania). Cell culture dishes and plates were purchased from Greiner (Kremsmunster, Austria). Coelenterazine h was purchased from Regis Technologies (Morton Grove, IL). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Rapamycin was obtained from Selleckchem. GeneCellin transfection reagent was from BioCellChallenge (Toulon, France). Atropine was purchased from EGIS (Budapest, Hungary). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma (St. Louis, MO).

2.2. DNA constructs

Wild type human M3 cholinergic receptor (N-terminal 3×-hemagglutinin tagged) was purchased from S&T cDNA Resource Center (Rolla, MO). The human EGF receptor was described earlier [19].

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 [20], the binding-defective PLCδ1(R40L)-PH-GFP [20], Btk-PH-GFP [21] and GFP-OSH2-2xPH [17]. 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 [22] with a protein linker of SSRE between them, and cloned into the C1 vector using XhoI and EcoRI. Next, similar to other constructs [23] 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).

The low affinity intramolecular Ins(1,4,5)P_3 biosensor was described recently [24].

The PM targeted FRB-mRFP and mRFP-FKBP-5ptase constructs used for rapamycin-induced PtdIns(4,5)P_2 depletion were described earlier [25], with the difference that for PM targeting of the FRB protein we used the N-terminal targeting sequence of mouse Lck protein (MGCVCSSNPENNNN), or the N-terminal targeting sequence of human c-Src protein (MGSSKSKPKDPSQRRNNNN) [26,27] instead of GAP43 (MLCCMRRTKQVEKNDDDQKI).

mRFP-FKBP-Sac1 was created from mRFP-FKBP-Pseudojanin (PJ) [28] by removing the 5-phosphatase (5-ptase) domain from the construct. For this, it was first digested with KpnI and BamHI, and then the overhanging ends were filled up with Klenow fragment. The final construct resulted in the expression of mRFP-FKBP-tagged yeast Sac1 phosphatase (GenBank accession number: NM_001179777, residues 2–517) with a small peptide on its C-terminal end (GGSTGSR). In some experiments the mRFP-FKBP-PJ without 5-ptase activity (mRFP-FKBP-PJ-Sac1) was used as 4-phosphatase enzyme [28]. 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.

2.3. Cell culture

HEK 293T and COS-7 cells (ATCC, Manassas, VA) 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 CO_2 incubator at 37 °C in 10 cm tissue culture plastic dishes.

2.4. Bioluminescence resonance energy transfer (BRET) measurements

For BRET measurements HEK 293T cells were trypsinized and plated on poly-lysine-pretreated (0.001%, 1 h) white 96-well plates at a density of 10^5 cells/well together with the indicated DNA constructs (0.12–0.3 μg total DNA/well) and the cell transfection reagent (0.5 μl/well Lipofectamine 2000 or 1.5 μl/well GeneCellin). After 6 h 100 μl/well DMEM