Chapter 3

Development of Nonspecific BRET-Based Biosensors to Monitor Plasma Membrane Inositol Lipids in Living Cells

József T. Tóth, Gergő Gulyás, László Hunyady, and Péter Várnai

Abstract

There are several difficulties to face when investigating the role of phosphoinositides. Although they are present in most organelles, their concentration is very low, sometimes undetectable with the available methods; moreover, their level can quickly change upon several external stimuli. Here we introduce a newly improved lipid sensor tool-set based on the balanced expression of luciferase-fused phosphoinositide recognizing protein domains and a Venus protein targeted to the plasma membrane, allowing us to perform Bioluminescence Resonance Energy Transfer (BRET) measurements that reflect phosphoinositide changes in a population of transiently transfected cells. This method is highly sensitive, specific, and capable of semiquantitative characterization of plasma membrane phosphoinositide changes with high temporal resolution.

Key words Phosphoinositide, BRET, Lipid-binding domain, Plasma membrane, Biosensor

1 Introduction

Detecting intracellular lipids and among them phosphoinositides (PPIn) in particular is a challenging task as they are present in small amounts and show rapid changes and high metabolic turnover [1]. During the last four decades several techniques have been developed that aimed to detect these small changes with high sensitivity and good temporal and spatial resolution [2]. Isotopic labeling of cells with myo-[^{3}H]-inositol or ^{32}P-phosphate followed by lipid separation with different types of chromatography (e.g., paper chromatography, high performance liquid chromatography) were the first experiments able to detect PPIns [3–6]. The major limitations of these methods were that only with the use of complicated membrane separation procedures was it possible to detect PPIns with subcellular resolution, and these techniques lacked the ability to distinguish between different PPIn isomers. The latter remains a problem even for more novel mass spectrometry detection methods [7–11].

Guillaume Drin (ed.), *Intracellular Lipid Transport: Methods and Protocols*, Methods in Molecular Biology, vol. 1949, https://doi.org/10.1007/978-1-4939-9136-5_3,

Using microscopy and fluorescently tagged lipids or labeled PPIn specific antibodies can overcome these problems, but these techniques also have their limitations. When using fluorescently labeled lipids, their modified structure and hydrophobicity can influence their role in the enzymatic routes and their incorporation into membrane bilayers, and makes it very difficult to interpret the results regarding endogenous lipids [12, 13]. When applying antibodies it is always doubtful whether they can distinguish between regio-isomers, and their use is limited to fixed cells [14, 15].

Another method to make lipid changes visible is the application of labeled specific lipid-binding proteins or lipid-binding domains (LBD) [16–19]. Specific LBDs are already available for all seven PPIns [20, 21]. When the level of a certain PPIn in an organelle membrane increases, these LBDs will also be enriched there and this can be followed easily by epifluorescence or confocal microscopy. These experiments enable the detection of massive lipid changes with good temporal and subcellular resolution but the quantification of results is complicated and limited only to enormous, in some cases nonphysiological changes. These LBDs, however, can also be used for more sensitive and semiquantitative resonance energy transfer methods, e.g., Förster resonance energy transfer (FRET) [22] or BRET [23]. An energy transfer signal occurs when there is molecular proximity (approximately 100 Å) between two fluorescently labeled proteins (in the case of FRET) or between a fluorescently tagged (usually with yellow fluorescent protein [YFP] or its improved version such as Venus) and a bioluminescent luciferase-bound protein [24]. When creating PPIn recognizing biosensors we preferred BRET over FRET as BRET measurements do not involve donor excitation and therefore the analysis of BRET data does not require corrections for bleed-through of fluorescent proteins, a major complication for FRET data analysis (Fig. 1a) [16].

The energy transfer signal that we get can be either specific when there is a specific connection between the tagged molecules, or nonspecific when the signal is a result of their colocalization in the same membrane region [23]. Fusing a specific membrane-targeting sequence to the energy transfer acceptor Venus and a LBD to the donor luciferase enables the measurement of lipid changes in one particular membrane compartment. When the tagged membrane contains the target PPIn in relatively high amounts, LBD-luciferase constructs will accumulate there which generates a (nonspecific) energy transfer signal between the LBD-luciferase and membrane-Venus. On the contrary, a reduction in PPIn levels causes the LBD-luciferase to dissociate from the membrane and will result in a reduced signal (Fig. 1b) [25, 26].

The main difficulty of the method is that it is necessary that cells express both energy donor and energy acceptor molecules, and to make experiments more comprehensive it is required to express

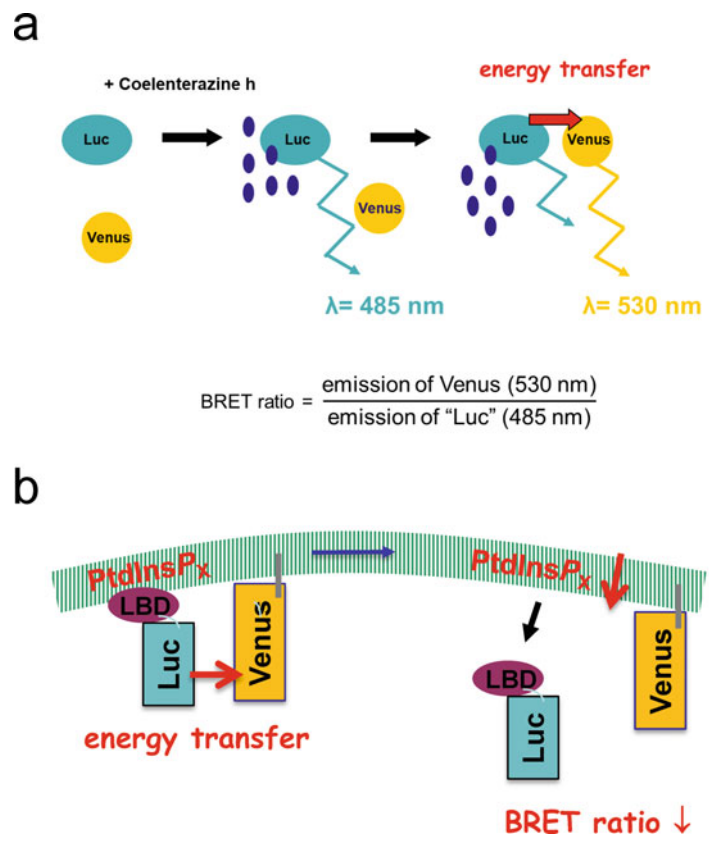

Fig. 1 Schematic representation of bioluminescence resonance energy transfer (BRET) and its application in the quantitative measurement of inositol lipid changes. (**a**) The BRET approach is based on the measurement of emitted light at the wavelength of the donor and acceptor. The ratio of these values correlates with the efficiency of energy transfer and represents the molecular proximity of the luciferase enzyme (Luc), the donor that generates the light by oxidizing its substrate, and the acceptor fluorescent protein (Venus) that is excited by the energy transfer and emits at a longer wavelength. (**b**) Membrane localization of a lipid-binding domain (LBD) can be measured in cells by calculating the energy transfer between the luciferase fused to the LBD and Venus targeted to the membrane. Membrane recruitment of the probe increases the BRET ratio values, while cytoplasmic translocation of the probe results in a decrease. Since there is no direct interaction between the donor and the acceptor fusion proteins, this is considered a nonspecific energy transfer and depends on the expression level of the proteins

them in a constant ratio. To achieve this we designed these sensor sets in a single plasmid configuration where the two fusion proteins are separated by the T2A viral peptide interrupting the polypeptide chains during translation [27]. Figure 2 shows the general design of the plasmids. This method allows a single mRNA molecule to code for two separate proteins with a theoretical ratio of 1:1, and thus

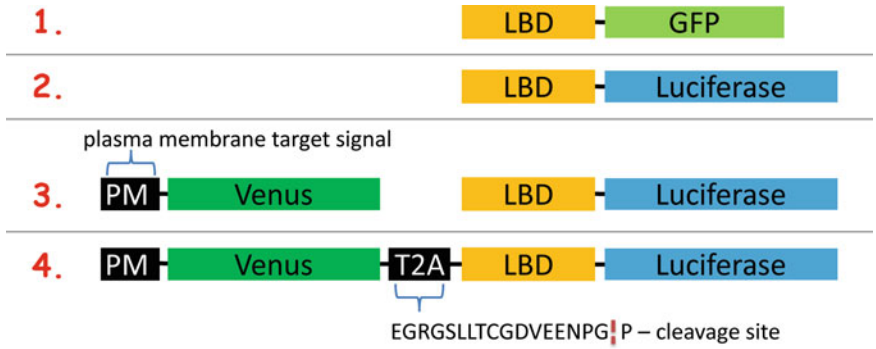

Fig. 2 Generation of the inositol lipid biosensors. The first step is to select the lipid-binding domain. Since the method is based on the specific interaction between the various inositol lipids and their lipid-binding proteins, first and foremost, well characterized and validated lipid-binding domains need to be selected that are generally accepted and used, e.g., in confocal measurements as fluorescently tagged inositol lipid sensors. A list of these sensors can be found in recent reviews [16]. Our choices were the P4M domain (546–647) of the SidM protein (Q29ST3) (in tandem) for PtdIns(4)*P*, the PH domain (1–170) of PLCδ1 (P51178) for PtdIns(4,5)P_2, the PH domain (266–399) of GRP1 (O08967) with a nuclear export signal (NES, ALQKKLEELELDE) for PtdIns (3,4,5)P_3 and the PH domain (109–334) of TAPP1 (Q9HB21) (also in tandem) for PtdIns(3,4)P_2. Other domains can be selected for additional sensors, and the method also allows the screening of domains as potential biosensors in the future. We used the GFP-tagged form of each sensor as a starting point. The second step is to replace the fluorescent protein with luciferase. Although many different versions of luciferase enzymes are available, we chose to replace the fluorescent protein with a mutant version (S87R, M185 V, K189 V, V267I) of *Renilla* luciferase (P27652) [28] which we have been using for many years in our laboratory. Although there are smaller and more stable versions of this enzyme, a great advantage of *Renilla* luciferase is that the inexpensive and easily available Coelenterazine h can be used as substrate. Most probably, other versions can also be applied with an appropriate substrate and acceptor fluorophore. Keep the original linker sequence of the sensors. For control experiments (to check the localization and the degradation of the fusion proteins) we also generated constructs which contain Cerulean in place of the luciferase. The third step consists of creating a construct that drives the expression of the fluorescent protein Venus on the cytoplasmic surface of the membrane on which the inositol lipid is supposed to be measured. Until now only the plasma membrane-targeted Venus has been used, in which the N-terminal 10 amino acid residues of Lck (MGCVCSSNPE) provide the stable recruitment of the protein mostly to the membrane [29].In the last step, the coding sequence of the T2A peptide (EGRGSLLTCGDVEENPGP) is inserted in frame between the two other sequences without a STOP codon. The advantage of this viral T2A peptide is that during translation a molecular cleavage occurs within it, leading to the expression of two separate proteins in equimolar amounts [27]

only transfection of the cells with one plasmid is required for the experiments [16, 25].

Here, we describe a BRET-based methodology, as opposed to directly monitoring translocation of a fluorescently tagged biosensor with microscopic techniques, which has the advantage of simpler quantification, higher sensitivity, and compared to biochemical assays with the application of organelle-selective biosensors, we can achieve PPIn region-isomer detection signal with good temporal and compartment-specific spatial resolution. Nevertheless, BRET is a relatively easy-to-use technique and its popularity will only increase because of its statistical robustness, lower equipment costs, and time commitment.

2 Materials

2.1 Cell Transfection

1. Cultured cells of interest: HEK293T, HEK293, COS-7, Neuro 2A, CHO, HeLa cell lines were tested. For one 96-well plate, about 8–10 million cells grown on a 10 cm Petri dish are required.
2. Opti-MEM medium (Gibco).
3. DMEM (Lonza) supplemented with 10% (v/v) of fetal bovine serum (FBS, Euro Clone).
4. 0.25% trypsin solution.
5. Plasmid DNA (mammalian expression vectors with CMV promoter and kanamycin resistance) coding for the inositol lipid sensors or only the cytoplasmic luciferase enzyme.
6. Transfection reagent: GeneCellin (BioCellChallenge) for HEK293T or Lipofectamine 2000 (Thermo Fisher Scientific) for other cell types.
7. Cell culture microplate, 96-well, flat bottom, white (Greiner Bio-One), coated with poly-lysine or not depending on the cell type that is used.

2.2 BRET Measurement

1. Plate reader with the capacity of measuring luminescent light with high sensitivity (at least 7 amol ATP/well) and with the appropriate filters (480/20 nm filter for Renilla luciferase and 530/20 nm filters for Venus). Fluorescence measurement capability with the appropriate filters or monochromators is highly recommended, because it enables monitoring the level of expression by measuring the protein fluorescence (excitation wavelength at 510 nm and emission wavelength at 535 nm). Temperature control, mixing function, and low dead space injectors (as many as possible) are also recommended (*see* **Note 1**).
2. Acquisition software: Time lapse protocol is required that includes the change of fluorescence and luminescence modes, as well as the control of injectors.
3. Stock solution of 0.5 mM coelenterazine h (Regis Technologies) in 100% ethanol. Prepare 1 mL stocks that can be stored at −20 °C for months. Dark, screw-cap micro tubes should be used to decrease light-induced degradation and evaporation. It is diluted freshly just before the application in the BRET measurement.
4. Measuring solution (Krebs-Ringer buffer): 10 mM HEPES-HCl, pH 7.4, 120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM glucose.
5. Computer with any data analysis software.

3 Methods

3.1 Planning of the BRET Experiment

Considering that measurements are carried out in duplicate or triplicate and those non-stimulated controls must also be included, a high number of the wells is required to allow the simultaneous measurement of several conditions as well as cells transfected with different sensors. Using only the central 6 × 10 wells of a 96-well plate (we have found that the wells on the edge are not very reliable and therefore are used only for non-transfected cells and for preliminary experiments) and applying only one stimulus (which requires typically two non-stimulated and three stimulated wells distributed randomly), twelve biosensors can be measured (2 in one row, 12 in 6 rows). Obviously, application of various stimuli or pretreatment reduces the number of the biosensors, but still, careful planning is required prior to the experiment to determine both the transfection and the measurement protocol.

3.2 Cell Transfection

Transfection is carried out in Opti-MEM medium, according to the manufacturer's protocol,

1. For one well of the 96-well plate, mix 25 μL of Opti-MEM containing 1.5 μL of GeneCellin or 0.5 μL of Lipofectamine 2000 with 25 μL of Opti-MEM containing 0.05 μg of plasmid of the biosensor.
2. Incubate at room temperature for 30 min.
3. During that incubation time, cells cultured in a 10 cm tissue culture Petri dish are trypsinized using 5 mL of 0.25% trypsin solution for 5 min at 37 °C. Centrifuge the cells at low speed for 5 min. Resuspend the cell in 3 mL of Opti-MEM. A cell density of 750,000 cells/mL is set.
4. For each well, 100 μL of the cell suspension (containing 75,000 cells) is added to 50 μL of the DNA/transfection reagent slurry, then mixed and pipetted into the well. Note that the wells at the edge of the plate must only contain 100 μL of the cells.
5. After 4–6 h, add 100 μL of FBS-supplemented (10%) DMEM to each well.

3.3 BRET Measurement

1. Measurements are performed 24–26 h after transfection and start with the change of medium to 50 μL/well of the Krebs-Ringer buffer (*see* **Note 2**).
2. Preparation of the equipment. Make sure that the plate reader is switched on and reached the set temperature, which is usually 37 °C. Fill up the injectors (if used) and open the protocol file (*see* **Note 3**).
3. Optional step: measure the fluorescence of Venus prior to the addition of luciferase substrate. Usually the entire plate is measured.

4. Dilute 0.5 mM stock solution of coelenterazine h with pre-heated measuring solution (40× dilution).
5. Add 40 μL to each well included in the run (this additional 2.5× dilution achieves the final concentration of 5 μM). The plate has to be briefly shaken.
6. Luminescent measurement. The measurement is started and interrupted several times if drugs are added manually. Either using the injectors or pipettes, drugs are given in a volume of 10 μL, which results in a 10 times dilution for the first addition, 11 times dilution for the second addition, and so on. The plate has to be shaken manually or by the equipment after the administration of drugs.

3.4 Data Analysis

1. Expression level of the constructs. If **step 3** in Subheading 3.2 was performed (measuring the Venus fluorescence), the calculation of the average of the wells transfected with the same constructs reflects the expression level of proteins. The error is also useful as it reveals how similar the parallels were. The fluorescence of non-transfected cells can be used for autofluorescence correction.
2. Analysis of the luminescence curves—The kinetics and also the values of both emission curves (emission at 480–530 nm) are very similar, because the level of energy transfer is usually small; therefore, it is enough to analyze only one of them (we usually analyze the 480 nm). Emission curves have a characteristic kinetic (*see* Fig. 3, upper middle graph). The initial fast increase reflecting the diffusion of the substrate into the cells is always followed by a slower decrease, which is due to the irreversible degradation of the enzymatic activity. The disappearance of the signal is a limitation regarding the length of the measurement. Usually the average of the three biggest values of the corresponding wells are selected (red circle), and used for the calculation of the average, which also reflects the expression level of the sensor proteins.
3. Calculate the BRET ratio values by dividing the emission intensity measured at 530 nm with the one measured at 480 nm. If the applied stimulus resulted in a change compared to the non-stimulated controls, two separate groups of curves should be seen (*see* Fig. 3, upper right graph).
4. We are always expecting a time-dependent spontaneous change of the BRET ratio values even in the case of the non-stimulated cells. This change is characteristic for the various sensors, and can be greatly reduced by preincubating the cells in the measuring solution at 37 °C (*see* **Note 2**). To maintain the absolute values of the BRET ratio, this spontaneous change is determined by calculating the difference between each data point

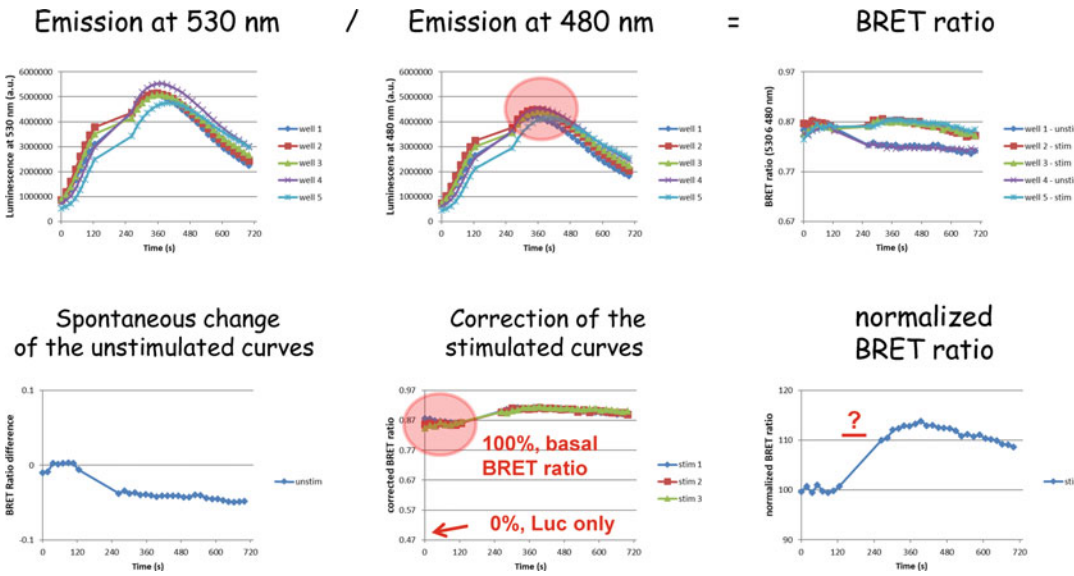

Fig. 3 Workflow of the data analysis. The analysis of the measurement of five wells is shown, in which cells were transfected with the same biosensor, and only one stimulus was applied after the tenth data point in three wells, while two wells got the vehicle only (non-stimulated). BRET ratio values that correlate with the lipid levels are calculated by dividing the emission intensity values measured at 530 nm with the ones measured at 480 nm (upper graphs). Spontaneous change calculated from the average of the non-stimulated curves (lower left graph) is used for the correction of the stimulated curves (lower middle graph). Finally, data are expressed as a percentage value, where 100% is the basal BRET ratio and 0% is the value that can be measured using the same equipment and protocol in cells that express luciferase only (in this experiment 0.468). Red circles show the data that reveals the expression level of the biosensors (upper middle graph) and the basal BRET ratio (lower middle graph). In this experiment stimulus was applied manually, which takes time and leads to a huge time gap on the graph. The exact addition of the stimulus cannot be determined as indicated with question mark (lower right graph). This gap can be greatly reduced by using injectors for stimulations

and the average of the three points prior to stimulation (*see* Fig. 3, lower left graph).

5. Correction of stimulated curves for the spontaneous change of the control. By subtracting the spontaneous change from each of the stimulated curves we get the absolute ratio values that already reflect the membrane localization of the sensor proteins (*see* Fig. 3, lower middle graph). The average of the three data points prior to stimulation is calculated and considered as basal BRET ratio value (red circle). Although this value depends on many factors (filters, sensor expression level), it can be used to evaluate the resting membrane binding level of the lipid-binding domains.
6. Finally, the normalized BRET ratios, which reflect the change of the inositol lipids, are expressed as a percentage value where 100% is the basal BRET ratio value and 0% is set to the absolute

minimum of the BRET ratio, determined by wells where cells express the luciferase protein only (Fig. 3, lower middle and right graphs) (*see* **Note 4**).

4 Notes

1. There are many plate readers on the market capable of BRET measurements. In our laboratory, the following readers have been used: Mithras LB940 (Berthold Technologies), Varioskan Flash (Thermo Fisher Scientific), CLARIOstar (BMG Labtech). When purchasing a new plate reader, the following issues should be considered: (1) Both fluorescence and luminescence detection. Fluorescence measurement is needed to follow the expression of the fluorescence protein. (2) Wave selection by monochromators or filters. While monochromators are excellent for the measurement of the Venus fluorescence, for luminescence (BRET), the application of filters may result in greater signals. (3) Top reading is required when white plates, which give better signals, are used. (4) The number of injectors determines how versatile a measurement can be. (5) The acquisition software and support availability are also important issues.
2. We noticed that after changing the media to the Krebs-Ringer solution, incubation of the cells at 37 °C for 30–60 min greatly reduces the spontaneous change of the BRET ratio values, most probably by stabilizing the fluorescence of the Venus protein. Pretreatment of the cells can be performed during this time.
3. The following points should be considered for the creation of the right measurement protocol. First, BRET measurements are typically carried out in several runs. The number of wells included in one run depends on many different factors, such as time resolution, signal-to-noise ratio, and exposure time. Measurement of the two emission intensities (at emission wavelength of 480–530 nm) cannot happen exactly at the same time (which would be ideal), but at least has to occur sequentially for each well (in the so-called "by well" configuration, as opposed to "by plate"). The usual exposure time is a few hundred milliseconds (250 ms in our experiments) for each wavelength, which means that together with the mechanical movement of the plate, the measurement of one well requires 0.75–1.5 s. For example, when two rows (20 wells) are measured in a run, roughly 3–4 measurement points can be achieved in every minute. This number can be increased by decreasing the exposure time, but that in turn will increase the signal-to-noise ratio. Better time resolution can be achieved by

including fewer wells in each run, which in turn increases the number of runs and the length of the entire experiment. Based on our experience, the total length of the experiment (with preincubation) should be limited to 120–150 min.

4. To get the ratio value corresponding to 0% energy transfer, additional experiments are required in which cells express the cytoplasmic luciferase enzyme, but no forms of the Venus protein. Depending on the optical parameters of the equipment (e.g., filters) this number can be quite different. The advantage of this normalization is that experiments carried out using different equipment will be comparable, plus the effect of different expression levels of the biosensors achieved in the various experiments is also reduced. Figure 4 reveals an

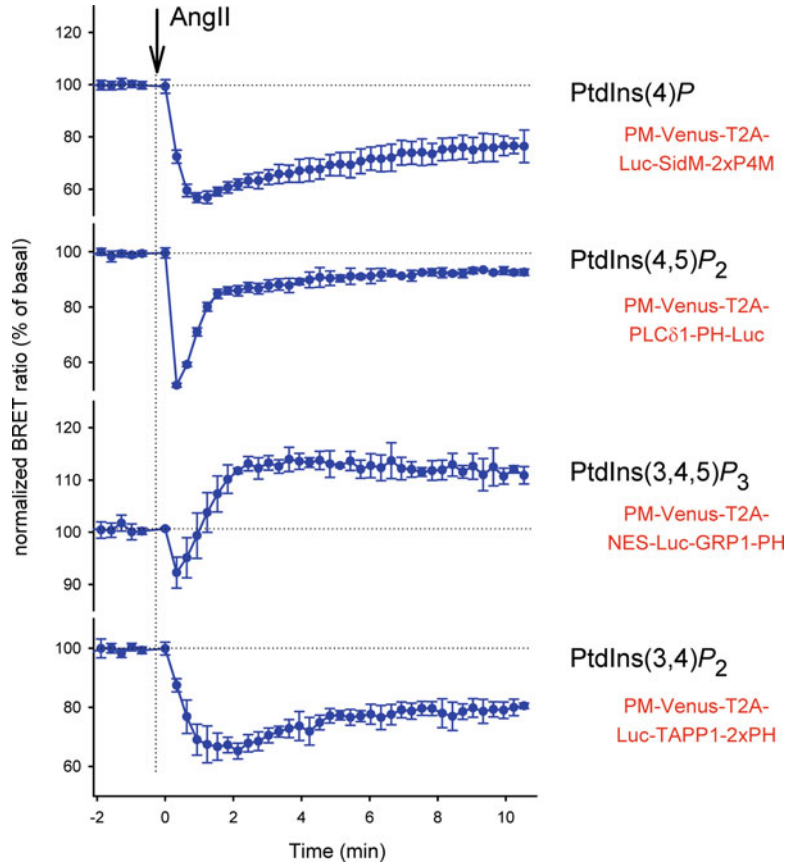

Fig. 4 Effects of the activation of a Gq-coupled receptor on plasma membrane inositol lipids. HEK293 cells expressing rat AT1a receptor were stimulated with submaximal concentration of angiotensin II (AngII). Cells were transiently transfected with the biosensors indicated on the figure. For these experiments, only two rows of a 96-well plate were used without the first and last wells of each row. Means ± S.D., $n = 4$

example of an experiment in which four different sensors were used.

Acknowledgments

This work was supported by the Hungarian National Research, Development and Innovation Fund Grants NKFIH K105006 (to PV), NVKP 16-1-2016-0039 (to LH). The technical assistance of Kata Szabolcsi and Dániel Nagy is highly appreciated.

References

1. Balla T (2013) Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol Rev 93:1019–1137
2. Idevall-Hagren O, De Camilli P (2015) Detection and manipulation of phosphoinositides. Biochim Biophys Acta 1851:736–745
3. Hokin LE, Hokin MR (1958) Phosphoinositides and protein secretion in pancreas slices. J Biol Chem 233:805–810
4. Hokin MR, Hokin LE (1953) Enzyme secretion and the incorporation of P32 into phospholipides of pancreas slices. J Biol Chem 203:967–977
5. Nakanishi S, Catt KJ, Balla T (1995) A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. Proc Natl Acad Sci U S A 92:5317–5321
6. Traynor-Kaplan AE, Harris AL, Thompson BL et al (1988) An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. Nature 334:353–356
7. Hsu FF, Turk J (2000) Characterization of phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate by electrospray ionization tandem mass spectrometry: a mechanistic study. J Am Soc Mass Spectrom 11:986–999
8. Kielkowska A, Niewczas I, Anderson KE et al (2014) A new approach to measuring phosphoinositides in cells by mass spectrometry. Adv Biol Regul 54:131–141
9. Wenk MR, Lucast L, Di Paolo G et al (2003) Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. Nat Biotechnol 21:813–817
10. Kraft ML, Klitzing HA (2014) Imaging lipids with secondary ion mass spectrometry. Biochim Biophys Acta 1841:1108–1119
11. Wakelam MJ (2014) The uses and limitations of the analysis of cellular phosphoinositides by lipidomic and imaging methodologies. Biochim Biophys Acta 1841:1102–1107
12. Golebiewska U, Kay JG, Masters T et al (2011) Evidence for a fence that impedes the diffusion of phosphatidylinositol 4,5-bisphosphate out of the forming phagosomes of macrophages. Mol Biol Cell 22:3498–3507
13. Lipsky NG, Pagano RE (1983) Sphingolipid metabolism in cultured fibroblasts: microscopic and biochemical studies employing a fluorescent ceramide analogue. Proc Natl Acad Sci U S A 80:2608–2612
14. Hammond GR, Dove SK, Nicol A et al (2006) Elimination of plasma membrane phosphatidylinositol (4,5)-bisphosphate is required for exocytosis from mast cells. J Cell Sci 119:2084–2094
15. Hammond GR, Schiavo G, Irvine RF (2009) Immunocytochemical techniques reveal multiple, distinct cellular pools of PtdIns4P and PtdIns(4,5)P(2). Biochem J 422:23–35
16. Varnai P, Gulyas G, Toth DJ et al (2017) Quantifying lipid changes in various membrane compartments using lipid binding protein domains. Cell Calcium 64:72–82
17. Varnai P, Balla T (2008) Live cell imaging of phosphoinositides with expressed inositide binding protein domains. Methods 46:167–176
18. Balla T, Varnai P (2009) Visualization of cellular phosphoinositide pools with GFP-fused protein-domains. Curr Protoc Cell Biol 24 (24):24
19. Stauffer TP, Ahn S, Meyer T (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. Curr Biol 8:343–346
20. Hammond GR, Balla T (2015) Polyphosphoinositide binding domains: key to inositol lipid biology. Biochim Biophys Acta 1851:746–758

21. Stahelin RV, Scott JL, Frick CT (2014) Cellular and molecular interactions of phosphoinositides and peripheral proteins. Chem Phys Lipids 182:3–18
22. van der Wal J, Habets R, Varnai P et al (2001) Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. J Biol Chem 276:15337–15344
23. Toth DJ, Toth JT, Gulyas G et al (2012) Acute depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate impairs specific steps in endocytosis of the G-protein-coupled receptor. J Cell Sci 125:2185–2197
24. Hamdan FF, Percherancier Y, Breton B et al (2006) Monitoring protein-protein interactions in living cells by bioluminescence resonance energy transfer (BRET). Curr Protoc Neurosci 5(5):23
25. Toth JT, Gulyas G, Toth DJ et al (2016) BRET-monitoring of the dynamic changes of inositol lipid pools in living cells reveals a PKC-dependent PtdIns4P increase upon EGF and M3 receptor activation. Biochim Biophys Acta 1861:177–187
26. Kuo MS, Auriau J, Pierre-Eugene C et al (2014) Development of a human breast-cancer derived cell line stably expressing a bioluminescence resonance energy transfer (BRET)-based phosphatidyl inositol-3 phosphate (PIP3) biosensor. PLoS One 9:e92737
27. Szymczak AL, Workman CJ, Wang Y et al (2004) Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol 22:589–594
28. Woo J, von Arnim AG (2008) Mutational optimization of the coelenterazine-dependent luciferase from Renilla. Plant Methods 4:23
29. Gulyas G, Radvanszki G, Matuska R et al (2017) Plasma membrane phosphatidylinositol 4-phosphate and 4,5-bisphosphate determine the distribution and function of K-Ras4B but not H-Ras proteins. J Biol Chem 292:18862–18877