Chapter 1

Analysis of Phosphatidylinositol Transfer at ER-PM Junctions in Receptor-Stimulated Live Cells

Chi-Lun Chang and Jen Liou

Abstract

Phosphatidylinositol (PI) is an inositol-containing phospholipid synthesized in the endoplasmic reticulum (ER). PI is a precursor lipid for PI 4,5-bisphosphate ($PI(4,5)P_2$) in the plasma membrane (PM) important for Ca^{2+} signaling in response to extracellular stimuli. Thus, ER-to-PM PI transfer becomes essential for cells to maintain $PI(4,5)P_2$ homeostasis during receptor stimulation. In this chapter, we discuss two live-cell imaging protocols to analyze ER-to-PM PI transfer at ER-PM junctions, where the two membrane compartments make close appositions accommodating PI transfer. First, we describe how to monitor PI $(4,5)P_2$ replenishment following receptor stimulation, as a readout of PI transfer, using a $PI(4,5)P_2$ biosensor and total internal reflection fluorescence microscopy. The second protocol directly visualizes PI transfer proteins that accumulate at ER-PM junctions and mediate $PI(4,5)P_2$ replenishment with PI in the ER in stimulated cells. These methods provide spatial and temporal analysis of ER-to-PM PI transfer during receptor stimulation and can be adapted to other research questions related to this topic.

Key words PI, $PI(4,5)P_2$ replenishment, $PI(4,5)P_2$ biosensor, PI transfer protein, ER-PM junctions

1 Introduction

Phosphatidylinositol (PI) 4,5-bisphosphate ($PI(4,5)P_2$) is an inositol-containing phospholipid primarily present in the inner leaflet of the plasma membrane (PM) [1, 2]. $PI(4,5)P_2$ regulates a myriad of cellular functions at the PM, including membrane trafficking, ion channel activity, cytoskeleton dynamics, and store-operated Ca^{2+} entry [1–3]. In addition, $PI(4,5)P_2$ is essential for mediating Ca^{2+} signaling in response to extracellular stimuli. Receptor-activated phospholipase C (PLC) hydrolyzes $PI(4,5)P_2$ in the PM to generate inositol 3,4,5-triphosphate (IP_3), a cytosolic second messenger leading to Ca^{2+} release from the endoplasmic reticulum (ER) and activation of Ca^{2+} signaling events [4]. Animal cells have developed elaborate mechanisms to achieve rapid $PI(4,5)P_2$ replenishment following receptor-induced consumption to maintain $PI(4,5)P_2$-mediated functions and homeostasis. The

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ability of cells to replenish $PI(4,5)P_2$ during receptor stimulation is dependent on the level of its precursor lipid, PI in the ER [5–8]. Phosphatic acid in the PM generated following $PI(4,5)P_2$ hydrolysis activates Nir2 [5, 9], a cytosolic PI transfer protein (PITP), to mediate $PI(4,5)P_2$ replenishment dependent on PI in the ER, suggesting ER-to-PM PI transfer [5]. Nir2 and its homolog, Nir3, function at ER-PM junctions, where the ER and PM form close appositions within 20 nm [10]. At ER-PM junctions, Nir2 and Nir3 contact both membrane compartments simultaneously and mediate ER-to-PM PI transfer in exchange of PM-to-ER phosphatidic acid transfer [5, 9, 11]. Once delivered to the PM, PI is phosphorylated by PI 4-kinase and PI 4-phosphate (PI4P) 5-kinase to generate $PI(4,5)P_2$ [1]. Disrupting the connection at ER-PM junctions reduces the capability of $PI(4,5)P_2$ replenishment following receptor stimulation [3, 11]. Direct visualization of PI in live cells would be ideal to understand the dynamic regulation of ER-to-PM PI transfer at ER-PM junctions. Nonetheless, this is not feasible because there is no PI biosensor available and PI in the PM is likely to be short-lived before its conversion to PI4P and $PI(4,5)P_2$. Thus, measuring $PI(4,5)P_2$ replenishment using PI $(4,5)P_2$ biosensor and PITP accumulation at ER-PM junctions during receptor stimulation are suitable readouts for ER-to-PM PI transfer.

Live-cell fluorescence microscopy is a powerful tool in understanding dynamic information of cellular functions. This chapter first describes a protocol to measure consumption and replenishment of $PI(4,5)P_2$ during receptor stimulation by a green fluorescence protein-tagged PLCδ-pleckstrin homology domain (GFP-PLCδ-PH) as a $PI(4,5)P_2$ biosensor in live cells [12, 13]. In combination with total internal reflection fluorescence (TIRF) microscopy, which selectively illuminates fluorescence within 100 nm of the PM [14], our approach provides a straightforward pipeline of measurement and analysis of $PI(4,5)P_2$ levels in the PM, indicative of ER-to-PM PI transfer, during receptor stimulation in live cells. $PI(4,5)P_2$ replenishment occurs within seconds following receptor-induced hydrolysis. At endogenous receptor level, addition of high concentration (100 μM) of histamine to HeLa cells leads to a shallow consumption phase showing only ~10% reduction of $PI(4,5)P_2$ in the PM before a rapid replenishment phase that restores $PI(4,5)P_2$ back to basal level within 30 s (Fig. 1a). It is difficult to visualize and analyze such a small change and a short-lived process in live cells. To overcome this difficulty, we apply histamine H1 receptor (H1R) overexpression to augment PI $(4,5)P_2$ consumption. HeLa cells with H1R overexpression exhibit a steep consumption phase with up to ~70% reduction of $PI(4,5)P_2$ in the PM immediately after stimulation (Fig. 1b). This enhanced consumption phase is followed by a prolonged replenishment phase manifesting an initial partial recovery and a subsequent steady state.

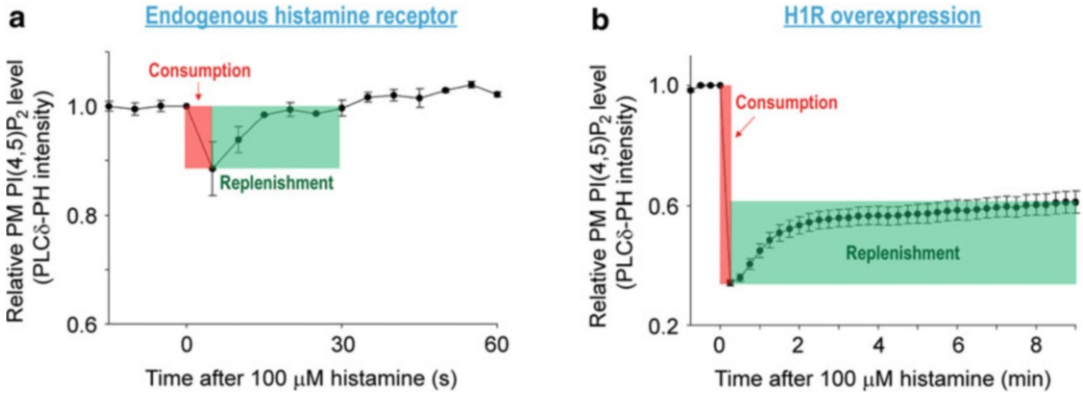

Fig. 1 Monitoring PI(4,5)P_2 levels in the PM during receptor stimulation. Dynamic changes of PI(4,5)P_2 levels induced by 100 μM histamine monitored by TIRF microscopy in HeLa cells transfected with (**a**) GFP-PLCδ-PH or (**b**) H1R and GFP-PLCδ-PH. Consumption and replenishment phases are labeled with red and green, respectively. Mean ± SEM (Standard Error of the Mean) is shown. Figure in (**b**) was originally published in Cell Reports, 5 (2013) 813–825. © Chang and Cell Reports, 2013

At the steady state, only ~60%, instead of 100% of PI(4,5)P_2 in the PM was restored, reflecting the equilibrium between the enhanced consumption and the endogenous replenishment mechanism. Thus, receptor overexpression enables a bigger dynamic range and temporal window for monitoring PI(4,5)P_2 levels in the PM in live cells during receptor stimulation.

Next, we describe an alternative approach for analyzing ER-to-PM PI transfer via visualizing Nir2 translocation to ER-PM junctions during receptor stimulation, a necessary step for Nir2 to mediate PI(4,5)P_2 replenishment in stimulated cells [5, 8]. Nir2 tagged with a red fluorescence protein (Nir2-mCherry) localizes in the cytosol in resting cells and displays punctate distribution following receptor stimulation when monitored by confocal microscopy (Fig. 2a). This process, namely Nir2 translocation to ER-PM junctions, is better visualized with TIRF microscopy as Nir2-mCherry shows little signal at the TIRF focal plane before stimulation (Fig. 2b). Following histamine treatment, Nir2 translocates to ER-PM junctions as labeled by MAPPER, a synthetic marker for visualization of ER-PM junctions in live cells [11]. This approach provides spatial and temporal analysis of Nir2 activation and function during receptor stimulation.

In this chapter, we describe live-cell imaging protocols to visualize and to quantitatively analyze PI(4,5)P_2 level in the PM and Nir2 translocation to ER-PM junctions during receptor stimulation, as readouts for ER-to-PM PI transfer. We provide the basic principles and discuss several technical notes as we want to make these protocols straightforward and reproducible. Importantly, these protocols require few fluorescence channels and minimal cell perturbations; therefore, they can be adapted to study ER-to-PM PI transfer in a variety of cellular processes.

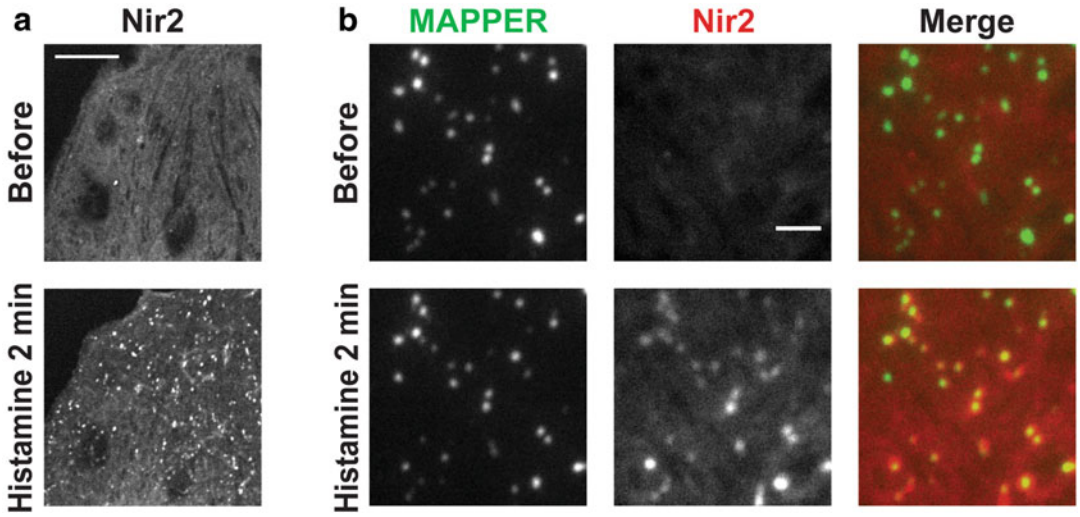

Fig. 2 Nir2 Translocation to ER-PM Junctions. (**a**) Nir2-mCherry translocation induced by 100 μM histamine monitored by confocal microscopy in HeLa cells transfected with H1R. Scale bar, 10 μm. (**b**) Nir2-mCherry translocation to ER-PM junctions induced by 100 μM histamine monitored by TIRF microscopy in MAPPER-expressing HeLa cells transfected with H1R. Scale bar: 2 μm. This figure was originally published in Cell Reports, 5 (2013) 813–825. © Chang and Cell Reports, 2013

2 Materials

Prepare all solutions with molecular biology and cell biology grade reagents. All materials in contact with cells should be sterile. In this protocol, we use HeLa cells with H1R overexpression as a model system. Other cell types, including HEK 293 cells and mouse embryonic fibroblasts, can also be used.

2.1 Cell Culture and Transfection

1. HeLa cells (CCL-2 line) from American Type Culture Collection.
2. Culturing medium: Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum and 1× penicillin and streptomycin solution.
3. Trypsin-EDTA solution (0.25%).
4. Ca^{2+}/Mg^{2+}-free PBS: 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4.
5. Hemocytometer.
6. Lab-Tek chambered cover glasses or MatTek dishes.
7. TransIT-LT1 transfection reagent (Mirus). TransIT-LT1 has good transfection efficiency but low cytotoxicity to HeLa cells.
8. Opti-MEM.

2.2 Plasmids

1. H1R. Other plasmids containing PLC-activating receptors, such as M1 muscarinic acetylcholine receptor, are also suitable (cDNA Resource Center and Addgene).
2. GFP-PLCδ-PH as PI(4,5)P_2 biosensor (Addgene).
3. MAPPER for labeling ER-PM junctions (please send request to Dr. Jen Liou).
4. Nir2-mCherry (or Nir3-mCherry, please send request to Dr. Jen Liou).
5. Plasmids with a protein of interest.

2.3 TIRF Microscopy

1. Extracellular buffer (ECB): 125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 20 mM HEPES-NaOH, pH 7.4, 10 mM glucose, and 1.5 mM $CaCl_2$.
2. Histamine stock solution (1000×): Histamine (Sigma Aldrich) is dissolved at 100 mM in sterile water. Store the stock solution at 4 °C.
3. A TIRF microscope with live-cell and time-lapse imaging capabilities.

3 Methods

3.1 Measurement of PI(4,5)P_2 Replenishment During Receptor Stimulation

3.1.1 Cell Culture and Transfection

Performed all these steps at 37 °C or with pre-warmed reagents.

1. Culture HeLa cells in cell culture dishes or flasks in an incubator supplemented with humidified air and 5% CO_2. Change media routinely every 2–3 days. When the cells reach ~90% confluency, subculture the cells. Do not overgrow HeLa cells; this may lead to poor performance in subsequent experiments.
2. Rinse HeLa cells with 1× PBS, add trypsin solution (1 mL per T-25 flask), and incubate for 2–3 min until the cells detach. Add 3 volumes of culture medium to neutralize trypsin solution and pipet the cell suspension a few times.
3. Collect the cell suspension in a conical tube and pellet the cells by centrifugation at 200 × *g* for 2–5 min. Remove supernatant and resuspend cells in 1 mL of culture medium.
4. Count the cells using a hemocytometer and seed 10,000 cells in 300 μL of medium into each well of an 8-well Lab-Tek chambered cover glass. This seeding density will result in ~50% confluency. Other types of imaging cover glasses may also be used. Because HeLa cells attach well to imaging cover glasses, no prior coating is required.
5. Perform transfection 24 h after plating the cells. Mix 0.3 μL of TransIT-LT1 with 50 ng of H1R plasmid and 25 ng of GFP-PLCδ-PH plasmid in 30 μL of Opti-MEM and incubate

the mixture at room temperature for 15 min. Remove the medium from the chambered cover glass and replace with 270 μL of fresh medium with 30 μL of transfection mixture in each well. Incubate the transfected cells for 16–20 h before imaging.

3.1.2 Live-Cell Imaging

Experiments are performed at room temperature.

1. Remove medium from the transfected chambered cover glass, rinse once with 300 μL of ECB and add 200 μL of ECB. Incubate the chambered cover glass at room temperature for another 10–15 min avoiding light exposure before experiment. This step allows the cells to reach equilibrium with the ECB and room temperature.
2. Prepare 5× histamine solution by adding 1 μL of 1000× histamine stock solution to 200 μL of ECB. Vortex briefly and spin down.
3. Image cells using TIRF microscopy. Find the desired cells and set up time-lapse imaging process. In general, acquiring an image every 10–15 s is fast enough to monitor the dynamic change in $PI(4,5)P_2$ levels in the PM.
4. Start the imaging process. After taking images of resting cells for 30 s–1 min, add 50 μL of 5× histamine solution. A marked reduction in GFP-PLCδ-PH intensity is expected immediately after histamine addition (Fig. 3a, middle panel). Add the treatment in between frames so the imaging process will proceed uninterrupted. To rapidly generate a homogenous histamine solution of 100 μM, carefully pipet up and down a few times without touching any parts of the imaging cover glasses.
5. Continue imaging for another 5–10 min until no obvious change in GFP-PLCδ-PH intensity was observed. A partial recovery in GFP-PLCδ-PH intensity can be observed within 2 min (*see* Fig. 3a, right panel).

3.1.3 Image Analysis

1. Open acquired images using Fiji (https://fiji.sc/).
2. Open the plugin Time Series Analyzer V3 (the plugin can be downloaded here: https://imagej.nih.gov/ij/plugins/time-series.html) and two windows (*Time Series V3_0* and *ROI Manager*) will appear.
3. Use the *polygon selection tool* to draw a region of interest (ROI) covering the PM area stably attached throughout the imaging process. Once done, click on "Add" (hotkey "t") in the *ROI Manager* window. Repeat this step to select more ROIs and a region avoiding cells as a background measurement.

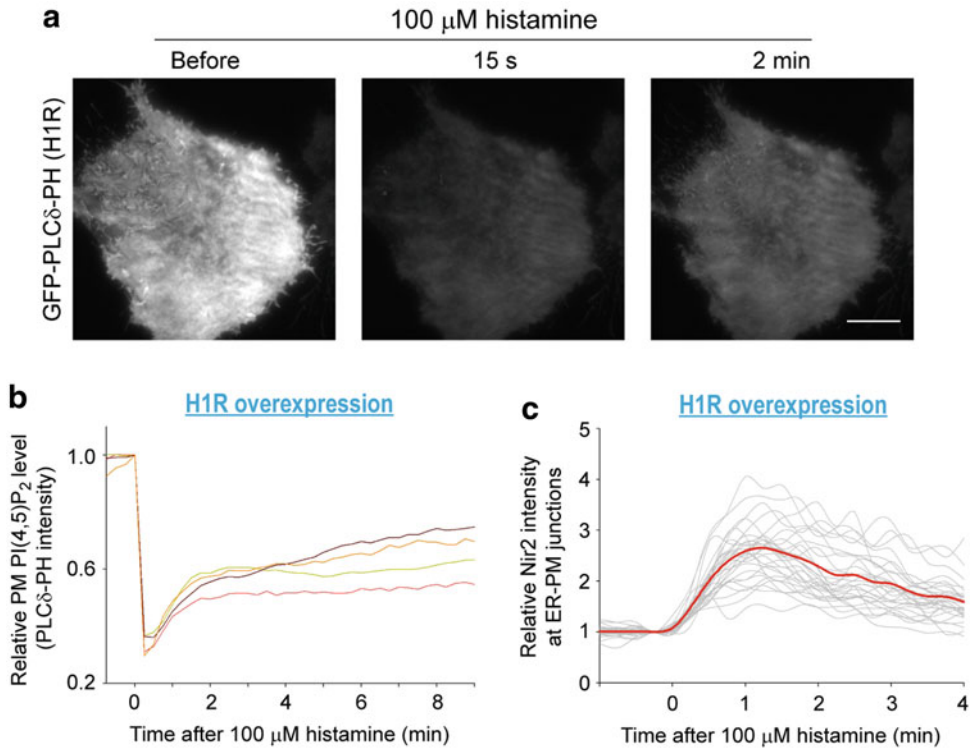

Fig. 3 Analysis of $PI(4,5)P_2$ replenishment and Nir2 translocation to ER-PM junctions during receptor stimulation. (**a**) Changes in intensity of GFP-PLCδ-PH following histamine treatment in HeLa cells cotransfected with H1R monitored by TIRF microscopy. Representative images are shown. Scale bar: 10 μm. (**b**) Example traces of typical $PI(4,5)P_2$ consumption-replenishment during receptor stimulation as described in (**a**). Traces from four individual cells are shown. (**c**) Example traces of Nir2 translocation to ER-PM junctions during receptor stimulation as described in Fig. 2b. Individual traces from 29 ER-PM junctions (gray) and the derived average trace (red) in a cells are shown

4. Go to *Analyze* > *Set Measurements* and check the mean gray value. Once done, click "Get Average" in *Time Series V3_0* window and time trace data for the individual ROI will be generated.
5. Copy the time trace data into a spreadsheet. Subtract the background data from the ROI data and normalize the background-subtracted data to time zero (the frame right before the addition of 5× histamine solution).
6. Plot and analyze the traces. Please refer to examples in Fig. 3b.

3.2 Measurement of PITP Translocation to ER-PM Junctions During Receptor Stimulation

3.2.1 Transfection

1. Transfect HeLa cells on imaging cover glasses with 50 ng of H1R plasmid, 40 ng of MAPPER plasmid, and 60 ng of Nir2-mCherry plasmid (or Nir3-mCherry) following the procedure described in Subheading 3.1.

3.2.2 Live-Cell Imaging

1. Prepare cells and set up imaging process as described in Subheading 3.1. Use MAPPER channel to find an optimal TIRF focal plane for imaging. Nir2 is in the cytosol and shows no concentration at ER-PM junctions in resting cells; thus, the Nir2 channel is not suitable to set up the focal plane for the experiment (Fig. 2b, upper panels).
2. Start the imaging process. After taking images of resting cells for 30 s–1 min, add 50 μL of 5× histamine solution. Nir2 accumulation at ER-PM junctions (colocalization with MAPPER) is detectable within 30 s and reaches a plateau within 1–2 min after histamine addition (Fig. 2b, bottom panels).

3.2.3 Image Analysis

1. Open acquired images using Fiji and open the plugin Time Series Analyzer V3 as described in Subheading 3.1.
2. Use the *oval selection tool* to draw an ROI that covers an ER-PM junction using the MAPPER channel. Once done, click on "Add" (hotkey "t") in the *ROI Manager* window. Repeat this step to select 15–30 representative ER-PM junctions in each cell as well as a background region.
3. Right click on the images to *duplicate* Nir2 channel and check "show all" in the *ROI manager* window. The same ROIs selected in MAPPER channel will appear in the duplicated Nir2 channel. An alternative approach is to directly draw ROIs covering Nir2 puncta in a poststimulation (~2 min) image of Nir2 channel when MAPPER channel is not available.
4. Set *Measurements* to mean gray value. Click "Get Average" in *Time Series V3_0* window and time trace data for the individual ROI will be generated.
5. Copy the results into a spreadsheet. Subtract the background from the ROI data and normalize the background-subtracted data to time zero.
6. Plot and analyze the traces. Please refer to examples in Fig. 3c.

4 Notes

1. A PM marker without affinity to $PI(4,5)P_2$ would be ideal to include, at least in initial experiments, to make sure that there is no significant photobleaching and the cells do not move out of the TIRF plane during the experiment.
2. Due to the nature of transient transfections, the expression level of H1R varies from cell to cell. Cells occasionally exhibit overconsumption when H1R level is too high (Fig. 4). In these cells, $PI(4,5)P_2$ consumption is too strong and dominates the endogenous replenishing mechanisms leading to no or little $PI(4,5)P_2$ replenishment. In contrast, cells with low H1R overexpression show underconsumption and a very narrow window of $PI(4,5)P_2$ replenishment, similar to HeLa cells without H1R overexpression. Please be aware of these two types of cells as they should be excluded from data analysis to avoid biased interpretation. It would be useful to optimize transfection condition for suitable H1R expression levels or alternatively have cell lines stably expressing H1R.
3. GFP-PLCδ-PH domain is the most commonly used biosensor for $PI(4,5)P_2$ in live cells. Nonetheless, PLCδ-PH domain also binds to IP_3 generated via $PI(4,5)P_2$ hydrolysis [15], which may cause inaccurate measurements of $PI(4,5)P_2$ in the PM using fluorescence microscopy. Therefore, validation of the results using other approaches is recommended. For example, Tubby-GFP is another well-documented $PI(4,5)P_2$ biosensor for live cells with no significant affinity for IP_3 [16]. $PI(4,5)P_2$ immunostaining is another approach to visualize $PI(4,5)P_2$ in fixed cells [17].

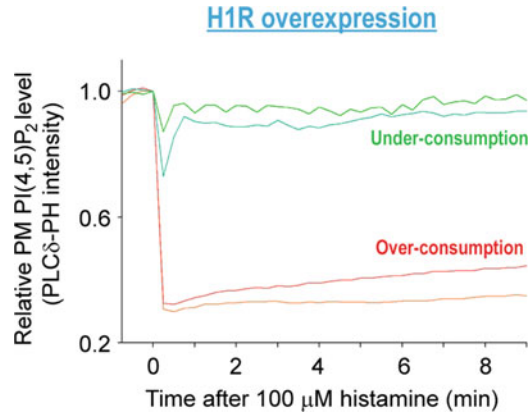

Fig. 4 Example traces of underconsumption (green) and overconsumption (red)

4. Original studies used confocal microscopy to characterize GFP-PLCδ-PH as a $PI(4,5)P_2$ biosensor in live cells [12, 13]. Confocal midsection images were taken to detect GFP-PLCδ-PH signal in the PM. In our experience, the midsection of HeLa cells tends to move following treatment during live-cell imaging. This situation makes quantification more difficult and increases the noise of the results. The adherent surface of HeLa cells usually shows little or no movement throughout the imaging process. Thus, using TIRF microscopy not only reduces phototoxicity but also leads to easier quantification and more reliable and consistent results.
5. MAPPER labels ER-PM junctions by targeting the ER and PM simultaneously, meaning that MAPPER provides tethering at ER-PM junctions. Similar to other tethers at ER-PM junctions [18, 19], MAPPER overexpression at higher levels leads to expansion of ER-PM junctions. The expanded ER-PM junctions manifest patch-like structures, instead of puncta, as visualized by fluorescence microscopy. To avoid experimental artifacts, cells with patch-like ER-PM junctions should be excluded. It would be useful to optimize transfection condition for suitable MAPPER expression. Selecting stable MAPPER-expressing cell lines with puncta-like ER-PM junction is also recommended.
6. MAPPER targets to ER-PM junctions via binding to $PI(4,5)P_2$ in the PM. Thus, a reduction of MAPPER signal can sometimes be observed following receptor stimulation due to reduction of $PI(4,5)P_2$, especially in H1R overexpressing cells. The decrease in MAPPER intensity does not necessarily represent reduced ER-PM junctions since there are other endogenous tethers to maintain ER-PM junctions. Similarly, Nir2 accumulation at ER-PM junctions in stimulated cells may not indicate an increase in ER-PM junctions. It should be noted that many proteins come and go at ER-PM junctions without affecting these subcellular structures.

Acknowledgements

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