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Studying the regulation of endosomal cAMP production in GPCR signaling

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Abstract

We describe methods based on live cell fluorescent microscopy and mass spectrometry to characterize the mechanism of endosomal cAMP production and its regulation using the parathyroid hormone (PTH) type 1 receptor as a prime example. These methods permit to measure rapid changes of cAMP levels in response to PTH, kinetics of endosomal ligand–receptor interaction, pH changes associated with receptor trafficking, and to identify the endosomal receptor interactome.

INTRODUCTION

GPCR constitutes the largest family of cell surface transmembrane receptors that transmit extracellular signals into cells. They are involved in multiple aspects of human health and diseases and are prime pharmacological targets for drug discoveries. Many GPCRs transduce their signals by stimulating the production of cAMP, which is needed to regulate major physiological functions. Once believed to be exclusively generated at the plasma membrane (Pierce, Premont, & Lefkowitz, 2002), cAMP can also be produced from endosomes after receptor internalization (Vilardaga, Jean-Alphonse, & Gardella, 2014). The mechanism and biological relevance of this new mode of cAMP signaling become to be understood for some receptors such as the parathyroid hormone (PTH) receptor (PTHr) and the vasopressin V2 receptor (Cheloha, Gellman, Vilardaga, & Gardella, 2015; Feinstein et al., 2013; Gardella & Vilardaga, 2015). The regulation of these altered modes of cAMP signaling (plasma vs endosomal membranes) has been, at least in part, recently uncovered for the PTHr (Figure 1). In brief, ligand–receptor (L-R) signaling complexes localized at the plasma membrane induce transient cAMP responses that are mainly terminated by the action of cAMP-specific phosphodiesterases (PDE), whereas prolonged cAMP responses are derived from complexes associated within endosomes. Unexpectedly, the internalized L-R signaling complexes contain β -arrestin1 or β -arrestin2, which promotes, rather than

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terminates, cAMP signaling by activating ERK1/2, leading to the inhibition of PDE4 activity and sustained cAMP signaling (Ferrandon et al., 2009). Termination of endosomal signaling is initiated by a negative feedback loop where PTH-mediated PKA activation leads to v-ATPase phosphorylation and subsequent endosomal acidification, resulting in the disassembly of signaling L-R-arrestin complexes and assembly of inactive receptor-retromer complexes (Gidon et al., 2014), which sort the receptor to retrograde trafficking domains (Feinstein et al., 2013). This chapter describes several methods that permit to investigate intracellular endosomes-associated GPCR signaling.

1. MATERIALS

1.1 REAGENTS

1. 6-well plates and 24 mm glass coverslips,
2. Attotfluor cell Chamber (Life Technologies),
3. Fugene 6 (Roche) for cell transfection,
4. Dulbecco's Modified Eagle Media (D5976) supplemented with 10% fetal bovine serum with or without 100 IU penicillin/0.1 mg streptomycin for maintenance of cells and transfections, respectively (all Sigma–Aldrich), and OptiMEM (Invitrogen),
5. Förster resonance energy transfer (FRET) buffer: 150 mM NaCl, 10 mM Hepes, 2.5 mM KCl and 0.2 mM CaCl₂, 0.1% BSA, pH 7.4 for live cells imaging,
6. Ligands: PTH(1–34), and PTH(1–34)^{TMR} and PTH(1–34)^{FITC}, which are labeled with tetramethyl-rhodamine (TMR) or fluorescein isothiocyanate (FITC), respectively,
7. Bafilomycin A1, H89 (all Sigma–Aldrich).

1.2 FÖRSTER RESONANCE ENERGY TRANSFER PRINCIPLE—The principle of FRET and its use as a tool to study kinetics along the individual biochemical events of the GPCR-signaling cascade in live cells has been previously reviewed (Villardaga et al., 2009). Here we used FRET to study receptor–ligand interactions or changes in second messenger (cAMP) production in live cells. We describe methods to record FRET in live cells using either wide-field, total internal reflection fluorescence (TIRF) or confocal fluorescence microscopes.

1.3 WIDE-FIELD FRET

1.3.1 Microscopic system

1. Nikon Ti-PFS inverted microscope.
2. FRET signals are recorded using an inverted wide-field microscope equipped with an oil immersion 40 × N.A. 1.30 Plan Apo objective and a moving stage.
3. Cyan fluorescent proteins (CFP) and yellow fluorescent proteins (YFP) are excited using a mercury lamp. Fluorescence emissions are filtered using a 480 ± 20 nm (CFP) and 535 ± 15 nm (YFP) (values represent center wave-length ± bandwidth)

filter set and collected simultaneously with an ultrasensitive EMCCD camera using a DualView 2 (Photometrics) with a beam splitter dichroic long pass of 505 nm. Fluorescence data are recorded from a single cell.

1.4 CONFOCAL FRET

1.4.1 Microscopic system

1. Nikon A1R high-speed confocal microscope for time, spatial, and spectral resolutions.
2. FRET signals are recorded using this inverted microscope equipped with a Z-driven piezo motor. Images are acquired using confocal unit, through a 60× N.A. = 1.45 objective.
3. Emission fluorescence signals are acquired using a spectral detection mode and collected by a 32-channel photomultiplier tubes. Typically, a Z-stack of four images (Z step = 500 nm) is acquired every 1–5 min. Fluorescent proteins or peptides containing either CFP, GFP, FITC, YFP, Tomato or mCherry are excited with 440 nm (CFP), 488 nm (GFP or FITC), 514 nm (YFP), or 560 nm (Tomato or mCherry) lasers.

1.5 TIRF FRET

1.5.1 Microscopic system

1. Nikon Ti-PFS inverted microscope.
2. FRET signals are recorded using an inverted wide-field microscope equipped with an oil immersion 60× N.A. 1.45 Plan Apo objective and a moving stage.
3. CFP and YFP are excited using a 440 nm (CFP) and 514 nm (YFP) lasers. Fluorescence emissions are filtered using a 480 ± 20 nm (CFP) and 535 ± 15 nm (YFP) (values represent center wavelength $\pm$ bandwidth) filter set and collected simultaneously with an ultrasensitive EMCCD camera using a DualView 2 (Photometrics) with a beam splitter dichroic long pass of 505 nm. Fluorescence data are recorded from a single cell.

1.6 FRET ANALYSIS—The FRET ratio for single cell experiments is calculated according to Eqn (1)

$$\text{Ratio} \left[\frac{F_{YFP}}{F_{CFP}} \right] = \frac{F_{YFP}^{ex436/em535} - a \times F_{CFP}^{ex436/em480} - b \times F_{YFP}^{ex500/em535}}{F_{CFP}^{ex436/em480}} \quad (1)$$

where F_{YFP} (ex436/em535) and F_{CFP} (ex436/em480) represent the emission intensities of YFP (recorded at 535 nm) and CFP (recorded at 480 nm), respectively, upon excitation at 436 nm; a and b represent correction factors for the bleed-through of CFP into the 535 nm channel ($a = 0.35$) and the cross talk due to the direct YFP excitation by light at 436 nm ($b = 0.06$). F_{YFP} (ex500/em535) represents the emission intensity of YFP (recorded at 535 nm) upon direct excitation at 500 nm and was recorded at the beginning of each experiment. Note that the bleed-through of YFP into the 480 nm channel is negligible in our recording

system. For each measurement, changes in fluorescence emissions due to photobleaching are subtracted. To ensure that CFP- and YFP-labeled molecule expressions are similar in examined cells; experiments are performed in cells displaying comparable fluorescence levels. FRET data are normalized for different expression levels of CFP and YFP molecules according to Eqn (2),

$$N_{FRET} = \frac{F_{YFP}^{ex436/em535} - a \times F_{CFP}^{ex436/em480} - b \times F_{YFP}^{ex500/em535}}{\sqrt{F_{CFP}^{ex436/em480} \times F_{YFP}^{ex500/em535}}} \quad (2)$$

2. EXPERIMENTAL PROCEDURES

2.1 CYCLIC AMP—Generation of cAMP in cells stably expressing PTHR is analyzed in real time using the FRET-based sensor EPAC-CFP/YFP (10) both in wide-field and in TIRF (Figure 2(A) and (B)).

1. Day 1, plate cells on 24-mm coverslip coated with poly-D-Lysine to reach 70% confluency on day 2.
2. Day 2, transfect cells with 1 µg of EPAC-CFP/YFP DNA with a transfection reagent (e.g., Fugene 6 from Roche) according to manufacturer protocol.
3. Day 3, place coverslip in a prewarmed Attolfluor chamber using a forceps and add 1 mL of FRET buffer. Pretreat with Bafilomycin A1 50 nM or H89 100 nM for 30 min when needed.
4. Prior to activation, select 12–18 different XY positions that will be recorded using the moving stage of the microscope.
5. Record baseline FRET (before adding ligands) for 5 min.
6. Add the ligand using a computer-assisted solenoid valve-controlled superfusion device (e.g., ALA-VM8 Scientific Instruments) or manually (usually a 10–30 s pulse).
7. Wash the ligand with FRET buffer with or without Bafilomycin or H89.
8. FRET signals are acquired for 60 min and analyzed as described in Sections 1.3, 1.5, and 1.6.

Note 1: Both the pulse with PTH(1–34) and the chase with FRET buffer are done while the acquisition is running. These two steps need to be executed very carefully in order to keep the focus plane stable. This procedure is facilitated by using the Nikon “Perfect Focus System.”

Note 2: Wide-field FRET signals for each position are acquired usually every 30 s; so defining a number of XY positions compatible with the time loop is needed in order to maximize sampling efficiency.

Note 3: TIRF acquisition is extremely sensitive to z variation. Keeping the focal plane stable is facilitated by restricting the number of XY position to 2 and by using the Nikon “Perfect Focus System” *installed in inverted Ti-PFS microscope*.

Note 4: m-Cherry/mTomato/DsRed-tagged proteins can be transfected together with EPAC-CFP/YFP sensor and selected prior FRET acquisition in order to specifically study their effect on cAMP generation.

2.2 ENDOSOMAL RECEPTOR DYNAMICS—FRAP experiments to determine the stability of receptor–arrestin complexes localized on endosomes have been previously described (Vilardaga, Romero, Feinstein, & Wehbi, 2013). Here we described a method to record interactions of PTHR with either β -arrestin1 or a retromer complex subunit using by real-time wide-field and confocal FRET.

1. Day 1, plate cells on 24-mm coverslip coated with poly-D-Lysine to reach 70% confluency on day 2.
2. Day 2, transfect cells with 1 μ g of PTHR C-terminally tagged with CFP (PTHR-CFP) and 0.5 μ g of a β -arrestin-YFP fusion or 0.5 μ g Vps29-YFP fusion along with 0.5 μ g of Vps-26 and Vps-35 cDNA.
3. Day 3, place coverslip in a prewarmed Attofluor chamber using a forceps and add 1 mL of FRET buffer. Pretreat with Bafilomycin A1 50 nM or H89 100 nM for 30 min when needed.
4. Prior to activation, select 12–18 different XY positions that are defined and recorded using the moving stage of the microscope.
5. FRET signals are acquired for 60 min and analyzed as described in Sections 1.4 and 1.6.

Note 1: Both the pulse with PTH(1–34) and the chase with FRET buffer are done while the acquisition is running. These two steps need to be executed very carefully in order to keep the focus plane stable and are quite facilitated by using the Nikon “Perfect Focus System” installed in inverted Ti-PFS microscopes.

Note 2: Wide-field FRET signals for each position are acquired usually every 30 s; so defining a number of XY positions compatible with the time loop is needed in order to maximize sampling efficiency.

2.3 ENDOSOMAL PTH/PTHR LOCALIZATION—Localization of PTHR and PTH monitored by real-time confocal imaging (Figure 3(A) and (B)).

1. Day 1, plate cells on 24-mm coverslip coated with poly-D-Lysine to reach 70% confluency on day 2.
2. Day 2, transfect cells as follow: 0.5 μ g of Rab5-GFP and Rab7-GFP alone or 0.5 μ g Rab5-mCh and Rab7-mCh together with 1 μ g PTHR-GFP cDNA.
3. Day 3, place coverslip in a prewarmed Attofluor chamber using a forceps and add 1 mL of FRET buffer. Pretreat with Bafilomycin A1 50 nM or H89 100 nM for 30 min when needed.
4. Prior to activation, select 12–18 different XY positions that will be recorded using the moving stage of the microscope.

5. Add PTH-TMR or PTH by a computer-assisted solenoid valve-controlled superfusion device or manually (usually a 10–30 s pulse).
6. Wash the ligand with FRET buffer with or without Bafilomycin or H89.

2.4 ENDOSOMAL pH—pH measurement along the endocytic pathway is based on FITC sensitivity to protonation (Lanz, Gregor, Slavík, & Kotyk, 1997).

2.4.1 Establishing a standard curve for pH (Figure 3(C))

1. Dilute 100 nM PTH(1–34)^{FITC} in FRET buffer with pH ranging from 7.0 to 4.
2. Record FITC fluorescence signals for each pH values using the acquisition parameters described below. FITC fluorescence values are normalized to the value obtained for pH 7.2 and plotted over a range of pH values.

2.4.2 Measuring pH variation along the endocytic pathway of PTHR (Figure 3(D))

1. Day 1, plate cells on 24-mm coverslip coated with poly-D-Lysine to reach 70% confluency on day 2.
2. Day 2, transfect cells with 1 mg of PTHR-CFP DNA.
3. Day 3, place coverslip in a prewarmed Attotfluor chamber using a forceps and add 1 mL of FRET buffer. Pretreat with Bafilomycin A1 50 nM or H89 100 nM for 30 min when needed.
4. Prior to activation, select 12–18 different XY positions that will be recorded using the moving stage of the microscope.
5. Fluorescence emissions are recorded in 3D using spectral detection mode in Nikon A1.
6. Add PTH-FITC by a computer-assisted solenoid valve-controlled superfusion device or manually (usually a 10–30 s pulse).
7. Wash the ligand with FRET buffer with or without Bafilomycin or H89.

2.4.3 Analyzing pH variation: Data obtained after spectral deconvolution are analyzed as follows: (1) a region of interest is drawn around each single cell; (2) respective FITC and CFP fluorescence levels are recorded; (3) the ratio $F_{\text{FITC}}/F_{\text{CFP}}$ is plotted over time; (4) the ratio $F_{\text{FITC}}/F_{\text{CFP}}$ is normalized to the values for $t = 1$; (5) pH is estimated by comparison with the linear relationship between FITC fluorescence and pH obtained in Section 2.4.1.

Note 1: Both the pulse with PTH(1–34) and the chase with FRET buffer are done while the acquisition is running. These two steps need to be executed carefully in order to keep the focus plane.

Note 2: CFP emission allows to normalize the variation of FITC due to both variation in total amount of available fluorophores and variation due to focus changes induce by endosomes movements.

2.5 ENDOSOMAL DOMAIN TRAFFICKING OF PTHR—Measuring the internalization of PTHR and transfer from endosomal domains labeled with β -arrestin1 to domains labeled with Vps35 retromer subunit. These experiments are performed in live cells to avoid the loss of endosomal domain structure that normally happens upon fixation.

2.5.1 Transfect HEK293 cells

1. Day 1, plate cells on MatTek glass-bottom dishes coated with poly-D-Lysine to reach 70% confluency on day 2.
2. Day 2, transfect cells with 1 μ g each of plasmid DNA encoding PTHR-GFP, β -arrestin1-dTomato, YFP-Vps29, and unlabeled Vps27 and Vps35. For ultrastable PTHR-arrestin interaction, replace WT β -arrestin1-dTomato with β -arrestin1 (IVF-AAF)-dTomato (Burtey et al., 2007).

2.5.2 Image trafficking of PTHR-GFP, day 4 or 5 (Figure 4)

1. Using a confocal microscope in spectral mode and a 37 °C stage heater, identify suitably expressing cells.
2. Induce PTHR internalization using 100 nM PTH(1–34).
3. Using a high-power objective and optimal resolution and pinhole settings for deconvolution, begin imaging endosomes at 10 min following challenge. At this time point, PTHR-GFP should first become visible on Vps29-labeled structures.
4. 3D image stacks are helpful for deconvolution, but a 2D image centered on each endosome of interest is sufficient when fast acquisition and minimal photobleaching are important.
5. Continue imaging endosomes in preidentified cells for 30 min. Note the time of each capture.
6. For optimal sharpness and accuracy deconvolve endosomal images using, e.g., Huygens software from SVI, Inc.

2.5.3 Analysis of endosomal colocalization

1. Using ImageJ, split three-color images into three two-channel images that represent each pairwise combination of fluorophores.
2. Using the JACoP plugin for ImageJ, calculate the Pearson's coefficient for the three fluorophore pairings in each endosome. Colocalization between β -arrestin1 and Vps29 should be low and represents an important negative control for this experiment. If domains labeled with β -arrestin1 and retromer cannot be clearly distinguished, then the experiment is unlikely to succeed.
3. Organize Pearson's results into "bins" of time, representing, for example, 5 min increments from the ligand challenge.
4. Use ANOVA to test for significant changes in colocalization over time. Use appropriate pairwise comparisons to compare PTHR- β -arrestin1 with PTHR-Vps29 at each time point. In some colocalization assays, a threshold of Pearson's =

0.5 is used to make a binary distinction between “colocalized” and “not colocalized.”

Note 1: The soluble retromer complex (Vps27/29/35) is only stable as a heterotrimeric complex, so transgenic expression of one subunit can require transfecting all three. It is useful to test whether this is necessary in a given experiment.

Note 2: Excessive overexpression produces artifactual results, so make sure to verify that each cell expresses all three constructs at modest levels. PTHR should label the membrane, Vps29 should label punctate endosomes, and β -arrestin1 should label diffusely in the cytoplasm. Ignore cells in which one label is excessively bright or aggresomes are visible. Further, separating close fluorophores such as GFP/YFP using spectral imaging requires comparable levels of fluorescent signal. Avoid cells in which one fluorophore is significantly brighter or dimmer than the others.

Note 3: To find the optimal imaging parameters for deconvolution, use a Nyquist sampling calculator such as www.svi.com/NyquistCalculator. For live cells imaged with a typical 1.45 N.A. objective, a pinhole of 1 AU and an XYZ spacing of $45 \times 45 \times 125$ nm should be satisfactory. Super-resolution techniques capable of live imaging would be desirable but spectral imaging is necessary for the rigorous separation of three transgenic fluorophores during live-cell imaging.

2.6 ENDOSOMAL GPCR PROTEOMICS (FIGURE 5)

2.6.1 Early endosome isolation: Endosomes are isolated by density gradient centrifugation (Guimaraes de Araujo, Fialka, & Huber, 2010; Stasyk et al., 2007, 2010).

1. Disrupt cells by Dounce homogenization on ice for approximately 50 strokes.
2. Add four volumes of homogenization buffer (HB) (250 mM sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, and protease and phosphatase inhibitors) to the cell pellet.
3. Centrifuge the cell lysate at $2000\times g$ for 10 min at 4 °C.
4. Transfer postnuclear supernatant (PNS) carefully to a 15 mL falcon tube and 62% (w/w) sucrose solution (2.351 M sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, in double distilled (dd) H₂O).
5. Add refractive index ~ 1.4463 at 20 °C to the PNS to a final sucrose concentration of 40.6%.
6. Load the diluted PNS on the bottom of an ultracentrifuge tube. 1.5 volume of 35% sucrose (1.177 M sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, in ddH₂O, refractive index ~ 1.3904 at 20 °C), 1 volume of 25% sucrose (0.806 M sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, ddH₂O, refractive index ~ 1.3723 at 20 °C), and 0.5 volume of HB were sequentially overlaid on the top of the PNS and centrifuged at $210,000\times g$ at 4 °C for 1.5 h.

7. Collect the endosomal fraction for endosomal PTHR signaling complex isolation.

2.6.2 Coimmunoprecipitation

1. The endosomal fraction is diluted 1:1 in 3 mM imidazole, pH 7.4, and 1 mM EDTA to reduce the sucrose concentration and centrifuge for 1 h at 100,000× *g*.
2. Resuspend the endosomal fraction in 1× buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 1% n-Dodecyl β-D-maltoside (DDM), and then Dounce for 10–20 strokes (Nobles et al., 2011).
3. Stir for 30 min at 4 °C.
4. Add equal volume of 1× buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM EDTA without detergent, and stir for another 30 min.
5. Centrifuge for 1 h at 100,000× *g* and transfer the supernatant to a new tube.
6. Wash immobilized anti-HA affinity resin with 1× buffer three times.
7. Incubate immobilized anti-HA affinity resin with supernatant for 4 h to overnight with gentle end-over-end mixing or a rocking platform.
8. Spin down the immobilized anti-HA affinity resin with a bench top centrifuge for 30 s at 12,000× *g*. Save the supernatant for analysis of binding efficiency.
9. Wash resin three times with 1 mL 1× buffer with 0.1% DDM.
10. Wash resin three times with 1 mL 1× buffer with 0.01% DDM.
11. Add one bed volume of 0.1 M glycine, pH 2.0–2.8, and incubate for 10–15 min at room temperature.
12. Spin down resin with a bench top centrifuge for 30 s at 12,000× *g*.
13. Transfer the eluate to a new 1.5 mL protein low-bind microcentrifuge tube and neutralize the elution fraction with a 1:10–1:20 volume of 1 M Tris, pH 9.5.
14. Repeat step 11–13 two additional times at 30 °C. Pool the elute fractions in one protein low-bind microcentrifuge tube. If needed, reduce the volume of eluate to about 100 µL using a speed vacuum.

2.6.3 In-solution digestion and peptide desalting

2.6.3.1 In-solution digestion (Kahsai, Rajagopal, Sun, & Xiao, 2014)

1. Add four volumes of methanol (MeOH) and vortex for 30 s.
2. Add one volume of chloroform (CHCl₃) and vortex for 30 s.
3. Add three volumes of ddH₂O and vortex for 30 s.
4. Centrifuge at 10,000 rpm at room temperature in a bench-top microcentrifuge for 2 min.
5. Carefully remove the aqueous top layer.
6. Add four volumes of MeOH and vortex for 30 s.

7. Centrifuge at 10,000 rpm at room temperature in a bench-top microcentrifuge for 5 min.
8. Remove the MeOH and speed-vacuum the precipitate for 5 min.
9. Add 9 μ L of protein solubilization solution (8 M urea, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.005% DDM) to the dried precipitate.
10. Vortex for 30 s.
11. Add 1 μ L of 100 mM Dithiothreitol (DTT) to the above solubilization solution. The final concentration of DTT is 10 mM.
12. Incubate at 37 °C for 30 min.
13. Cool down the reduction reaction to room temperature.
14. Add 1.2 μ L of freshly prepared 200 mM iodoacetamide to a final concentration of 20 mM.
15. Incubate the alkylation reaction in dark at room temperature for 20 min.
16. Add 78.8 μ L of trypsin digestion buffer (50 mM Tris-HCl, pH 8.0, and 0.005% DDM). Total volume will be 90 μ L.
17. Dilute modified trypsin (Promega) to 100 ng/mL in trypsin digestion buffer and add 10 μ L to each tube. Incubate the trypsin digestion reaction at 37 °C overnight.
18. When the trypsin digestion reaction is completed, adjust pH of the sample to about 2 using 50% trifluoroacetic acid (TFA). Proceed for peptide desalting step.

2.6.3.2 Peptide desalting

1. Pack two cookies of C18 Empore disk membranes into a GPS-L250 tip (1 cookie for about 4 μ g peptides).
2. Cleaning step: add 80 μ L of MeOH to the stage-tip column, and push out 50% of the solution using a 5 mL syringe.
3. Leave the column at room temperature for 5 min, and then push out the solution slowly (20–40 μ L/min), leave ~5% solution in the stage-tip column.
4. Wetting step: add 40 μ L of 50% acetonitrile (ACN), 0.1% TFA to the stage-tip column, push out the solution slowly (20–40 μ L/min), and leave ~5% solution in the stage-tip column.
5. Equilibration step: add 40 μ L of 5% ACN, 0.1% TFA to the stage-tip column, push out the solution slowly (20–40 mL/min), and leave ~5% solution in the stage-tip column.
6. Binding step: reconstitute the dried peptides in 40 μ L of 0.3% TFA, add more TFA to ensure the pH is lower than 2, and then load the peptides to the equilibrated stage-tip column.
7. Push out the peptide solution slowly (10–20 μ L/min), leave ~5% solution in the stage-tip column. Repeat step (6) and (7) twice.

8. Wash step: add 40 μ L 0.1% TFA to the stage-tip column, push out the peptide solution slowly (10–20 mL/min), leave ~5% solution in the stage-tip column.
9. Elution step: add 40 μ L 50% ACN, 0.1% TFA to the stage-tip column, push out the entire liquid slowly (10–20 μ L/min) to a 1.5 mL low-bind microcentrifuge tube.
10. Peptide reconstitution: speed-vacuum the samples for about 30 min to dryness and reconstitute the peptides in an appropriate buffer to mass spectrometry analysis.

3. DISCUSSION

Endosomal signaling is a new concept in GPCR biology whereby internalized receptors continue to stimulate the production of cAMP via Gs. Methods discussed in this chapter provide direct access to spatiotemporal analyses of GPCR signaling at the single cells level and permit to investigate molecular and cellular mechanisms that govern GPCR signaling when they redistribute in endosomes. In the case of the PTHR, we previously demonstrated that this receptor (R) adopts at least two distinct signaling conformations, R₀ and R_G (Villardaga et al., 2014). R₀-selective ligands (such as PTH) prolong their action via endosomal PTHR/Gs/cAMP signaling and are thought to favor bone-resorption responses associated with sustained calcium release; conversely, R_G selective ligands (such as PTHrP) induce short and transient action that originate receptors localized at the plasma membrane and are believed to favor bone anabolism responses. Live cell microscopy methods described here coupled to those previously reported (Villardaga et al., 2013) are critical to advance the new signaling model of PTHR that is illustrated in Figure 1. Mass spectrometry-based endosomal PTHR proteomics is powerful in revealing the molecular mechanisms of prolonged PTHR endosomal signaling. A number of interesting proteins were identified in the endosome PTHR signaling complexes, including the PTHR, a set of G protein by subunits (G $\beta\gamma$), several GAPs (GTPase-activating proteins) and GEFs (guanine nucleotide exchange factors), PP2A, and β -arrestins. Further investigation of these PTHR interacting proteins may shed light on the molecular mechanisms of prolonged endosomal cAMP production in GPCR signaling.

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FIGURE 1.

Signaling modes of parathyroid hormone (PTH) receptor (PTHR). (A) Examples of time courses of cAMP production in cells briefly challenged by PTHrP (short) or PTH (long), the two native agonists for PTHR. A 3D view of PTH-TMR and a PTHR N-terminally tagged with GFP (PTHR-GFP) in live HEK-293 cells by spinning-disc confocal microscopy 30 min after ligand washout. PTH-TMR (red) and PTHR-GFP (green) colocalized within endocytic compartments at a time point where cAMP levels remained elevated. (B) Proposed model of PTHR signaling. PTH-bound PTHR (green) generating cAMP (orange) by activation of

adenylate cyclases at the plasma membrane internalizes to early endosomes in a process that involves binding of β -arrestins. Signaling endosomal complexes containing PTH, PTHR, and β -arrestins mediated activation of ERK1/2 signaling that causes inhibition of cAMP-specific phosphodiesterases, thus permitting sustained cAMP signaling. Generation of cAMP is stopped by the negative feedback actions of PKA and v-ATPase, which engages sorting of the receptor to retrograde trafficking domains via the retromer complex. See Introduction for more details. (See color plate)

FIGURE 2.

Real-time measurement of cAMP production in live cells. (A) Examples of averaged time courses of cAMP production recorded by wide-field FRET microscopy and using HEK293 cells expressing the PTHR and the FRET-based cAMP biosensor, EPAC-CFP/YFP. Individual cells were continuously perfused with buffer or with PTH (100 nM) and treated without (left panel) or with (right panel) Bafilomycin for the time indicated on the *x*-axis. (B) Similar cAMP recording as in (A) and performed in cells stably transiently transfected to express the retromer Vps26, Vps29, and Vps35 subunits (+retromer). (C) Averaged time

courses of PTH-mediated interaction between PTHR and β -arrestin2 recorded by changes of the normalized FRET ratio F_{YFP}/F_{CFP} in HEK293 cells transiently expressing PTHR-CFP with β -arrestin2-YFP treated without (left) or with (right) Bafilomycin. (D) Comparison of averaged time courses of PTH-mediated interaction between PTHR and either β -arrestin2 or retromer subunit Vps29 recorded by changes of the normalized Förster resonance energy transfer (FRET) ratio F_{YFP}/F_{CFP} in HEK-293 cells transiently expressing PTHR-CFP with either β -arrestin2-YFP or Vps29-YFP along with Vps26 and Vps35. Data represent mean values $\pm$ s.e.m. of five independent experiments and $n > 80$ cells for each condition.

FIGURE 3.

pH measurement of endosomes containing the parathyroid hormone (PTH) receptor (PTHR), PTH-bound PTHR. (A) Real-time confocal microscopy of HEK293 stably coexpressing PTHR and Rab5-GFP or Rab7-GFP and briefly (20 s) challenged by PTH-TMR. Micrographs represent Rab5-GFP (left column), PTH-TMR (middle column) and overlay image with Rab5-GFP in green (white in print versions), PTH-TMR in red (gray in print versions). (B) Corresponding Pearson's correlation coefficient quantification. (C) Linear dependence in fluorescence emission of PTH-FITC over the pH range 4.0–8.0. (D) HEK cells expressing PTHR-CFP were briefly challenged with PTH-FITC to record pH variation along the endocytic pathway in the absence (black) or presence of either Bafilomycin A1 (light gray) or H89 (dark gray). pH estimates were made using the pH standard plot shown in (C).

FIGURE 4.

Imaging endosomal parathyroid hormone (PTH) receptor (PTHR) in complex with β -arrestins or retromer. (A) HEK293 cells expressing GFP-PTH1R, Vps29^{YFP}, and β -arr1^{tom} were challenged with a brief pulse of PTH and endosomes were visualized via confocal microscopy using spectral and spatial deconvolution. Endosomes showed changes in colocalization of GFP-PTH1R, Vps29^{YFP}, and β -arr1^{tom} as a function of time after ligand challenge white bar, 1 μ m. (B) Individual endosomes were classified according to time after ligand challenge and colocalization (Pearson's, JaCoP plugin, ImageJ) was measured for

each endosome. We used Pearson's analysis to quantify the change in PTHR localization from arrestin-to retromer-labeled endosomal domains.

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FIGURE 5.

Endosomal parathyroid hormone (PTH) receptor (PTHR) proteomics. (A) Flow chart for analyzing endosomal PTHR signaling complexes by LC/MS/MS. (B) A representative PTHR peptide (PTHR: ⁴⁸⁹SGSSSYSGPMVSHTSVTNVGPR⁵¹¹) identified in LC/MS/MS analysis. (C) A representative β -arrestin1 peptide (β -arrestin1: ²⁶DFVDHIDLVDLPVDGVVLVDPEYLK⁴⁹) identified in LC/MS/MS analysis. The position, sequence, charge state, Xcorr (SEQUEST cross-correlation value), observed and theoretic *m/z*, and precursor mass error (ppm) for the identified peptides were listed. The peak heights are the relative abundances of the corresponding fragmentation ions, with the annotation of the identified matched amino terminus-containing ions (b ions) in blue and the carboxyl terminus-containing ions (y ions) in red. (See color plate)