Endosomal phosphatidylinositol 3-kinase is essential for canonical GPCR signaling

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Abstract

G protein-coupled receptors (GPCRs), the largest family of signaling receptors, are critically regulated by endosomal trafficking, suggesting that endosomes might provide new strategies for manipulating GPCR signaling. Here we test this hypothesis by focusing on class III phosphatidylinositol (PI) 3-kinase or Vps34, an essential regulator of endosomal trafficking. We verify that Vps34 is required for recycling of the β2-adrenoceptor (β2AR), a prototypical GPCR, and then investigate the effects of Vps34 inhibition on the canonical cAMP response elicited by β2AR activation. Vps34 inhibition impairs the ability of cells to recover this response after prolonged activation, in accord with the established role of recycling in GPCR resensitization. In addition, Vps34 inhibition also attenuates the acute cAMP response, and its effect begins several minutes after initial agonist application. These results establish Vps34 as an essential determinant of both acute and long-term canonical GPCR signaling, and support the potential utility of the endosomal system as a druggable target for signaling.
Introduction

Many signaling receptors, including GPCRs, enter the endosomal system after ligand-induced activation at the plasma membrane (PM), and the endosomal network is known to have myriad effects on cellular GPCR signaling (Pierce et al., 2002; Sorkin and von Zastrow, 2009; von Zastrow and Williams, 2012; Irannejad et al., 2015). Internalized signaling receptors are sorted after endocytosis for recycling back to the PM or subsequent transport to lysosomes for degradation. This sorting decision, by determining whether regulated endocytosis maintains or depletes the surface receptor complement, has been believed to exert long-term control over cellular signaling responsiveness under conditions of prolonged or repeated ligand application (Hanyaloglu and von Zastrow, 2008). In addition, endosomes themselves can function as sites of receptor-mediated signal initiation, which potentially affects both acute and long-term cellular signaling activities (Murphy et al., 2009; Irannejad and von Zastrow, 2014).

PI 3-phosphate (PI3P) is a phosphorylated inositol lipid (phosphoinositide) that is found mainly in endosomal membranes (Gillooly et al., 2000). This lipid, although present in relatively small amount, is a defining feature of endosomal membranes that regulates many aspects of endosome biogenesis, organization and trafficking by recruiting PI3P-binding proteins (Schink et al., 2013). A major pathway of PI3P synthesis is mediated by Vps34, which specifically phosphorylates PI to produce PI3P in endosomal membranes (Shin et al., 2005; Raiborg et al., 2013).

β2ARs are widely considered as prototypic GPCRs (Lefkowitz, 2007). β2ARs internalize rapidly after ligand-induced activation via clathrin-coated pits and have the ability to recycle to the PM with remarkably high efficiency via a retromer-dependent pathway (Goodman et al., 1996; Temkin et al., 2011). Several components of this machinery require 3-phosphorylated phosphoinositides for membrane attachment (Cullen and Korswagen, 2012), and a previous study has implicated Vps34 in promoting β2AR recycling (Awwad et al., 2007). Accordingly, we hypothesized that Vps34 represents an endosomal target that controls β2AR signaling. Chemical
inhibitors used in previous studies of β2AR trafficking (Awwad et al., 2007; Sorensen et al., 1999; Naga Prasad et al., 2001) (wortmannin [WM] and LY294002) have additional cellular targets (Knight, 2010). In the current study, we employed more specific manipulations to examine the trafficking and signaling effects of endosomal PI3P and Vps34, and revealed previously unrecognized roles of this critical endosomal kinase in GPCR signaling. These results demonstrate that both acute and sustained GPCR signaling can be manipulated by targeting the endosomal system.
Materials and Methods

Cell culture, cDNA constructs, and transfection. Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum (UCSF cell culture facility, San Francisco, CA). A plasmid encoding Flag-tagged β2AR was previously described (Cao et al., 1999). A plasmid encoding GFP (enhanced green fluorescent protein) -tagged Hrs 2xFYVE was a kind gift from Harald Stenmark (Gillooly et al., 2000) (Oslo University Hospital, Oslo, Norway). mCherry-FKBP-MTM1 (wild-type, WT) and iRFP-FRB-Rab5 plasmids were obtained from Tamas Balla (NIH, Bethesda, MD) through Addgene (Hammond et al., 2014). A phosphatase-dead mutant of mCherry-FKBP-MTM1 (C375S) was generated by using Quikchange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Nb37-GFP plasmid was previously described (Irannejad et al., 2013). DNA transfection was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, and cells were used for subsequent experiments 24 h after transfection unless otherwise indicated. Cells stably expressing with Flag-tagged β2AR were created as previously described (Lauffer et al., 2010).

Inhibitors. PIK-III was synthesized according to the published protocol (Honda et al., 2016). The commercial sources of other inhibitors used in this study were as follows: wortmannin (Sigma, St. Louis, MO); VPS34-IN1 (MedKoo Biosciences, Chapel Hill, NC); SAR405 (ApexBio, Houston, TX); GDC-0941 (Selleck Chemicals, Houston, TX); YM201636 (Cayman, Ann Arbor, MI). Unless otherwise indicated, cells were pre-treated with these inhibitors in serum-free DMEM for either 1 h (100 nM wortmannin, 3 μM PIK-III, 1 μM VPS34-IN1, 3 μM SAR405, or 1 μM GDC-0941) or 3 h (800 nM YM201636) before agonist application. Trafficking and signaling assays were performed in the presence of the indicated inhibitors.

Trafficking assays. Unless otherwise indicated, trafficking assays were performed at 37°C using
HEK293 cells stably expressing Flag-β2AR. Cells plated on coverslips were used to assess receptor localization by fluorescence microscopy. To examine agonist-induced receptor internalization, cells were treated with 10 µM isoproterenol (a β2AR agonist, Sigma) for 25 min. To examine recycling after agonist removal, cells were first treated with isoproterenol for 25 min and then washed with PBS and treated with 10 µM alprenolol (a β2AR antagonist, Sigma) for 45 min. Antagonist was used to prevent any residual agonist effects in the recycling period. Cells were fixed by 4% paraformaldehyde in PBS for 20 min, quenched with Tris-buffered saline for 20 min, and subjected to immunocytochemistry as described below. For acute PI3P depletion by MTM1 recruitment, cells transfected with mCherry-FKBP-MTM1 and iRFP-FRB-Rab5 plasmids were used, and 1 µM rapamycin (Sigma) was applied to cells either 20 min before isoproterenol treatment (for internalization) or at the same timing as alprenolol (for recycling).

Cells were plated on 12-well plates to analyze β2AR trafficking by fluorescence flow cytometry. Both agonist-induced internalization and recycling after agonist removal were tracked quantitatively by determining surface β2AR levels. For internalization, cells were treated with 1 µM isoproterenol for the indicated times. For recycling, cells were first treated with 1 µM isoproterenol for 25 min and then with 10 µM alprenolol for 45 min. Surface β2AR receptors were then labeled with Alexa 488-conjugated M1 anti-Flag antibody (Sigma) at 4°C. Mean fluorescence intensity of each sample (2,000-10,000 cells per sample) was measured using a FACS Calibur instrument (BD Biosciences, San Jose, CA). The percentage of recycled receptors was calculated as follows: 100 X [(Intensity after the recycling period) - (Intensity after the internalization period)] / [(Intensity without agonist addition) - (Intensity after the internalization period)].

To measure β2AR recycling in the continuous presence of agonist, a previously established method (Tsao and von Zastrow, 2000) was used with modifications. Briefly, surface β2ARs were first labeled for 10 min with Alexa 647-conjugated M1 anti-Flag antibody, which requires calcium to bind the Flag epitope. Cells were then treated with 1 µM isoproterenol for 25
min to induce receptor internalization. At this point, cells were washed with calcium, magnesium-free PBS with 0.4% EDTA to dissociate antibodies from surface receptors and specifically label internalized receptors. Cells were then incubated in EDTA-supplemented PBS with 1 µM isoproterenol for 5 min. Control samples without this incubation period were also made. Cells were then chilled on ice, and subjected to flow cytometry as described above to measure the antibody efflux in the 5-min period. Because antibodies bound to receptors that were recycled to the PM in this period eluted immediately, the antibody efflux was correlated to the degree of β2AR recycling.

**Immunocytochemistry and fixed-cell imaging.** Permeabilization and blocking were performed for 20 min with 0.1% Triton-X 100 and 3% skimmed milk in PBS. Cells were then incubated with anti-Flag (M1) antibody (1:1,000; Sigma) and Alexa488-conjugated secondary antibody (1:1000; Molecular Probes, Carlsbad, CA) in the blocking buffer for 1 h each. Fixed samples were mounted with ProLong Gold (Molecular Probes). Cells were imaged with a spinning disc confocal microscope (TE-2000 [Nikon, Tokyo, Japan] with a confocal scanner unit CSU22 [Yokogawa, Tokyo, Japan]) using a 100X NA 1.45 objective. Images were collected using an electron multiplying CCD camera (iXon 897, Andor, Belfast, UK) operated in the linear range controlled by Micro-Manager software (https://www.micro-manager.org).

**Live-cell confocal imaging.** Cells were imaged in DMEM without phenol red (UCSF cell culture facility) with 30 mM HEPES (pH7.4). For MTM1 recruitment experiments, HEK293 cells stably expressing Flag-β2AR were used and imaged with the aforementioned confocal microscope. For Nb37 localization experiments, HEK293 cells were transiently transfected with Flag-β2AR and Nb37-eGFP plasmids. Cells were then pre-treated with DMSO or PIK-III, and then treated with 10 µM isoproterenol. Receptor-expressing cells were randomly chosen for assessing Nb37 localization and imaged after 15 - 30 min of isoproterenol treatment. Cells were imaged with
another spinning disk confocal microscope in the Nikon Imaging Center at UCSF (Ti-E [Nikon]
with a confocal scanner unit CSU22 [Yokogawa]) using a 100X NA 1.49 objective. Images were
collected using an electron multiplying CCD camera (Evolve Delta, Photometrics, Tucson, AZ)
operated in the linear range controlled by Micro-Manager software.

**Image analysis.** Images were saved as 16-bit TIFF files and analyzed by using Fiji (Schindelin et
al., 2012). Co-localization between Flag-β2AR and FRB-Rab5 was estimated by calculating
Pearson’s correlation coefficients between the two channels using Coloc 2 plug-in in Fiji. Line
scan analysis was performed with the plot profile function, and the obtained values were
normalized to the maximum value of each channel. The intensities of Nb37 and β2AR were
measured by a custom-written program created by D. Jullié, which works on MATLAB
(MathWorks, Natick, MA). The script is shown in Supplementary Text. Briefly, after selecting a
background region in cytosol and β2AR-containing endosomes, the program created
donut-shaped regions (3 pixel-wide) which include endosome limiting membranes, and
calculated the average fluorescent intensity of each donut. Five endosomes per cell were
randomly chosen for the analysis, and endosomes with negative Nb37 intensities were excluded
from the analysis. The Nb37 intensity of each endosome was then normalized to the β2AR
intensity.

**Luminescence-based real-time cAMP assay.** The experimental procedure was as described
previously except for using wild-type HEK293 cells (Irannejad et al., 2013). Briefly, cells were
transiently transfected with pGloSensor-20F (Promega, Madison, WI) that encodes a
cyclic-permuted luciferase cAMP reporter construct. For acute signaling, cells were treated with
luciferin (GoldBio, St. Louis, MO) in serum-free media for 1 h in a 24-well plate, and
luminescence values in the experimental wells were obtained after adding 1 µM isoproterenol.
Reference wells were made in the same columns in the 24-well plate as the experimental wells
and treated with 5 µM forskolin (Sigma). The luminescence values obtained in experimental wells were normalized to the maximum and minimum values in the reference wells (i.e., the maximum and minimum values of the reference wells were set to 100% and 0 %, respectively). For repeated signaling, cells were lifted and seeded on a 12-well plate one day after transfection, and the experiments were performed on the next day. Cells were treated with DMSO or 5 µM PIK-III and luciferin in serum-containing media for 1 h, and then treated with 1 µM isoproterenol for 1 h (first treatment). Cells were washed three times with serum-containing media without isoproterenol, and 10 min after wash-out, re-challenged with 1 µM isoproterenol (second treatment). Luminescence was recorded after both the first and second treatment, and the increase of luminescence values after second treatment was normalized to that after the first treatment in the same well.

**Biochemical cAMP assay.** After the indicated time of isoproterenol or forskolin treatment, cells were washed with ice-cold PBS and lysed by 0.1 M HCl for 10 min at room temperature. The cAMP concentration in the lysate was determined by using Direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer’s instructions. The cAMP concentration was normalized to the protein concentration determined by Coomassie Plus Protein Assay (Pierce, Waltham, MA), and displayed as % of the cAMP level in the reference sample for each independent experiment. The reference sample of each experiment was described in figure legends.

**Statistics and reproducibility.** Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA). Statistical significance was determined with either two-tailed Student’s t test or one-way ANOVA and considered to be significant if P values are less than 0.05 (for detail, see figure legends). Independent experiments were performed on different days. Representative microscopic data were from at least two independent experiments.
Results

To specifically investigate effects of endosomal PI3P, we employed a system to inducibly hydrolyze endosomal PI3P through rapamycin-induced recruitment of the inositol 3-phosphatase myotubularin 1 (MTM1) to endosomes in cells expressing MTM1 fused to the FK506 binding protein (FKBP) and the FKBP12-rapamycin-binding (FRB) domain fused to Rab5 (Hammond et al., 2014) (Fig. 1A). FKBP-MTM1 mainly localized in the cytoplasm but was acutely recruited to endosomes after rapamycin addition (Fig. 1B, right panels). We confirmed effective depletion of endosomal PI3P in HEK293 cells by using a PI3P probe protein (2xFYVE domain of Hrs) (Gillooly et al., 2000). Before rapamycin addition, 2xFYVE showed punctate localization corresponding to PI3P-containing endosomal membranes. After 5 minutes of rapamycin treatment, the 2xFYVE probe lost its punctate localization and showed even distribution in cytoplasm, concomitant with FKBP-MTM1 accumulation on endosomes (Fig. 1B, left panels). This effect was dependent on the phosphatase activity of MTM1 because a phosphatase-dead mutant of MTM1 (C375S) (Fili et al., 2006) did not cause dissociation of the 2xFYVE probe following rapamycin-induced recruitment (Fig. 1B, compare ‘wild-type (WT)’ to ‘C375S’).

Endosomal recruitment of MTM1 (WT) did not detectably perturb agonist (isoproterenol)-induced endocytosis of β2ARs, as indicated by robust redistribution of Flag-β2ARs from PM to endosomes, indistinguishable from that observed after recruitment of the control, phosphatase-dead MTM1 (C375S) (Fig. 1C, ‘Agonist’ condition, top rows). However, MTM1 (WT) recruitment impaired β2AR recycling, indicated by retention of Flag-β2AR in endosomes after isoproterenol removal, whereas efficient recycling was observed after recruitment of the phosphatase-dead MTM1 (C375S) (Fig. 1C, ‘Agonist to antagonist’ condition, bottom panels). These results, verified across multiple samples (Fig. 1D), suggest that endosomal PI3P is specifically required for β2AR recycling but not endocytosis.

To examine the role of Vps34 activity, we used PIK-III, a potent chemical inhibitor of
Vps34 that has high selectivity for Vps34 over other kinases (Dowdle et al., 2014; Honda et al., 2016). PIK-III application produced a pronounced redistribution of the 2xFYVE probe from endosomes to the cytoplasm (Fig. 2A). We then quantified effects of specific Vps34 inhibition on β2AR trafficking using fluorescence flow cytometry. PIK-III did not detectably change basal surface β2AR levels over the time interval required for our experiments, similar to the broad-spectrum PI 3-kinase inhibitor WM (Fig. 2B). These inhibitors also had little effect on isoproterenol-induced reduction of β2ARs from the PM measured 5 minutes after agonist application (Fig. 2 C and D), a time point at which net β2AR internalization is dominated by endocytic rate. However, both PIK-III and WM significantly reduced surface concentration of β2ARs at later time points (Fig. 2C), suggesting a selective inhibition of receptor recycling. We verified this by direct measurement of β2AR recycling after agonist removal (Fig. 2E). The similar degree of inhibition produced by PIK-III and WM suggests that Vps34 is the major PI 3-kinase isoform controlling β2AR trafficking in this cell system. We also confirmed the PIK-III effects by immunofluorescence microscopy. PIK-III caused clearly detectable retention of β2AR in intracellular structures after agonist removal (Fig. 2F), consistent with the recycling defect observed by flow cytometry.

Internalized β2ARs are efficiently recycled not only after agonist removal but also in the continuous presence of agonist (Morrison et al., 1996; Tsao and von Zastrow, 2000). To analyze β2AR recycling in the presence of agonist, we employed a previously established ‘loss of internal receptor’ assay (Tsao and von Zastrow, 2000). In this assay, Flag-tagged β2ARs are labeled with fluorochrome-conjugated anti-Flag antibody, and subsequently internalized by isoproterenol application. The recycling of Flag-tagged β2AR is then estimated by the efflux of anti-Flag antibody to calcium-depleted medium. PIK-III essentially abolished this antibody efflux, providing evidence that PIK-III blocks β2AR recycling in the presence of isoproterenol (Fig. 2G).

To further confirm the role of Vps34 in β2AR recycling, we used two additional recently reported selective Vps34 inhibitors, VPS34-IN1 (Bago et al., 2014) and SAR405 (Ronan et al.,
Both inhibitors suppressed β2AR recycling after agonist removal as well as PIK-III (Fig. 3A). In marked contrast, a selective inhibitor of class I PI3Ks, GDC-0941 (Folkes et al., 2008) did not affect β2AR recycling, indicating that class I PI3Ks are not required for β2AR recycling (Fig. 3A).

Notably, PI3P is a precursor for producing another phosphoinositide phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), which also regulates trafficking and ion homeostasis at endosomes (McCartney et al., 2014). Reduction of PI3P levels thus potentially affects PI(3,5)P₂ production, and moreover, MTM1 has a phosphatase activity toward PI(3,5)P₂ as well as PI3P (Fili et al., 2006). Thus we considered the possibility that Vps34 inhibition affects β2AR recycling through depleting PI(3,5)P₂ rather than PI3P itself. To test this, we used YM201636 to selectively block PIKfyve that generates PI(3,5)P₂ from PI3P (Jefferies et al., 2008), and measured β2AR recycling by flow cytometry. YM201636, in contrast to PIK-III, did not significantly affect β2AR recycling (Fig. 3B). These observations verify that PI3P is the key phosphoinositide product mediating the Vps34 effect on β2AR recycling.

We next investigated whether Vps34 affects β2AR signaling. The key biochemical step initiating canonical β2AR signaling is receptor-mediated activation of the heterotrimeric G protein, Gₛ (Rasmussen et al., 2011). Ligand-activated β2ARs activate Gₛ first in the PM and then in the endosome limiting membrane after endocytosis, with both events occurring sequentially over a period of several minutes (Irannejad et al., 2013). Accordingly, we reasoned that Vps34 might affect canonical β2AR signaling by enhancing or inhibiting the endosome phase of Gₛ activation. To test this we applied a previously described conformational biosensor, Nb37-GFP, which detects Gₛ activation in intact cells by local recruitment (Irannejad et al., 2013). Recruitment of Nb37-GFP, indicative of Gₛ activation, was clearly evident at β2AR-containing endosomes (Supplemental Fig. 1A) and localized to the limiting membrane (Insets in Supplemental Fig. 1A and line scans). PIK-III did not detectably change this behavior (Supplemental Fig. 1A). We quantified the intensity of Nb37-GFP signal in endosomal limiting membranes and revealed that
Nb37-GFP was similarly recruited irrespective of PIK-III application (Supplemental Fig. 1B). This suggests that Vps34 is not essential for Gs activation by β2AR at endosomes.

Following β2AR activation, an initial phase of cytoplasmic cAMP accumulation occurs from the PM and then receptors internalize to initiate a second signaling phase from endosomes (Irannejad et al., 2013; Tsvetanova and von Zastrow, 2014). The cellular cAMP response is desensitized by prolonged agonist exposure (Pierce et al., 2002), and recycling of internalized β2ARs to the PM after agonist removal promotes resensitization of the cellular cAMP response to subsequent agonist challenge (Yu et al., 1993; Pippig et al., 1995; Odley et al., 2004). Accordingly, to investigate potential signaling consequences of Vps34 activity, we investigated the effects of specific Vps34 blockade, and used three different experimental protocols to examine both acute and long-term β2AR-mediated cAMP responses.

We first examined the isoproterenol-induced cAMP response elicited within seconds after acute application, which we reasoned would sample primarily signaling from the PM. To do so we used a biosensor (Glosensor) that offers very rapid detection without cell lysis for sample preparation as required for standard biochemical determination of cAMP (Fan et al., 2008). Blocking Vps34 activity with PIK-III did not detectably affect this immediate signal (Fig. 4A). We next examined the isoproterenol response elicited after a 60-minute interval of isoproterenol pre-exposure followed by washout, which we reasoned would sample the resensitized β2AR pool present in the PM. Vps34 blockade significantly reduced this response (Fig. 4B). Together these results are consistent with the essential role of Vps34 in β2AR recycling and the previously established role of recycling in promoting β2AR resensitization.

We then investigated the effect of Vps34 blockade on the acute isoproterenol-induced cAMP response when measured 5 - 10 minutes after initial application, which we reasoned could include signaling initiated from both the PM and endosomes. It was possible to use standard biochemical determination of cAMP over this time scale, facilitating more direct and quantitative comparison of Vps34 effects on the cAMP response than using the Glosensor. PIK-III had no
detectable effect on the cAMP response measured 1 minute after isoproterenol application, consistent with the Glosensor results. However, when measured after 5 - 10 minutes, PIK-III caused a pronounced inhibition (Fig. 4C). Another selective Vps34 inhibitor, VPS34-IN1, caused essentially the same effects as PIK-III (Fig. 4C). This effect is specific to isoproterenol-induced cAMP response because Vps34 inhibition did not attenuate the cAMP response caused by receptor-independent adenyl cyclase activation with forskolin (Awad et al., 1983) (Fig. 4D). Thus Vps34 activity not only supports long-term cellular β2AR signaling, as indicated by its effect on resensitization, but it also plays an essential role in supporting the acute signaling response.
Discussion

Since it was first identified in yeast as a gene required for vacuolar protein sorting (Herman and Emr, 1990), Vps34 has long been recognized to regulate endosomal trafficking. In line with this, a previous study showed that broad-spectrum PI 3-kinase inhibitors (LY294002 and WM), which block Vps34 and other PI 3-kinases, suppress β2AR recycling (Awwad et al., 2007). However, since such inhibitors are not specific for Vps34, it was not possible previously to interrogate the role of Vps34 or endosomal PI3P specifically. In this study, we verify the role of Vps34 and PI3P by using recently developed specific manipulations. We demonstrate that both acute PI3P depletion in endosomal membranes (Fig. 1) and specific Vps34 inhibition (Fig. 2 and 3) suppress β2AR recycling. In addition we show that a highly selective inhibitor of class I PI 3-kinases, which are blocked by broad-spectrum inhibitors, does not affect β2AR recycling (Fig. 3A). Together, our results unambiguously show that Vps34 is essential for β2AR recycling. Although Vps34 might have additional kinase-independent effects, our kinase inhibitor and acute PI3P depletion data presented here indicate that the kinase activity of Vps34 to produce PI3P is required for β2AR recycling.

We then demonstrate a fundamental role of Vps34 in the cAMP signaling response triggered by β2AR activation. Figure 5 summarizes our proposed model regarding this. The acute cAMP response by single agonist application reaches maximum before 5 minutes at the latest, but then is rapidly desensitized and returns to steady-state levels. After agonist wash-out, cellular responsiveness is recovered at least in part by β2AR recycling to the PM. Vps34, by promoting β2AR recycling, supports this resensitization (Fig. 5A). Our data also reveal an additional role of Vps34 in promoting the acute cellular cAMP response, and they resolve a second component of the acute response that is specifically Vps34-dependent (Fig. 5B).

What is the underlying mechanism of this Vps34-dependent component? We previously showed that β2AR recycling events are observed after 3 - 5 minutes of agonist application, and reach steady-state within 10 minutes (Yudowski et al., 2009). This is consistent with the time
course of the Vps34-dependent component of acute signaling that is established in the present work. Also, our data indicate that Vps34 is required for β2AR recycling in the continuous presence of agonist (Fig. 2G). We thus speculate that VPS34 / PI3P-mediated fast recycling enhances cAMP production (Fig. 5C). β2AR recycling supplies receptors to the PM, and the recycled receptors are subjected to the further round of endocytosis. This rapid cycling between two compartments would sustain cAMP responses by supplying functional receptors to the PM and then to endosomes. Alternatively, although Vps34 inhibition does not detectably affect endosomal Gs activation (Supplementary Fig. 1), it could affect endosomal cAMP production downstream of Gs activation. Indeed, after 5-minutes of isoproterenol application, Vps34 inhibition had little effect on the surface β2AR density (Fig. 2C) but reduced the cAMP levels by ~40% (Fig. 4C). Thus it is possible that Vps34 inhibition reduces the acute cAMP response separately from receptor recycling at this time point, presumably by affecting the endosome signal (Fig. 5C). Further studies will be needed to clarify precisely how Vps34 impacts the acute cAMP response.

Vps34 inhibition increases the endosomal β2AR pool (Fig. 2C) and reduces the cAMP response after 10-minutes of isoproterenol application (Fig. 4C). One interpretation of these data is that the endosomal β2AR pool cannot activate the cAMP response as strongly as the surface β2AR pool. Indeed, our previous report suggested that endosomal β2AR activation makes a relatively small, but functionally distinct, contribution to the cellular cAMP response (Irannejad et al., 2013; Tsvetanova and von Zastrow, 2014). It is also possible that the endosome signaling component is normally transient, as is the surface component, so that inhibiting recycling through Vps34 blockade causes the net response to dissipate. It will be interesting to investigate these possibilities in future studies.

The present results show that the endosomal system can indeed be targeted to manipulate canonical GPCR signaling, and identify an essential role of the endosomal PI3-kinase Vps34 in promoting both acute and long-term cellular responses mediated by a
canonical GPCR cascade. While class I PI 3-kinases are well known to promote signaling at the PM (Marat and Haucke, 2016), Vps34 has been studied primarily in the context of membrane trafficking and its signaling functions are only beginning to emerge (Schink et al., 2013). Previous work on receptor-linked kinases such as the TGF-β receptor implicates Vps34 in recruiting PI3P-dependent signaling scaffolds to endosomes (Tsukazaki et al., 1998), and Vps34 was also shown to function as a non-canonical endosomal effector of yeast GPCR signaling (Slessareva et al., 2006). We propose that the present results, by establishing Vps34 as a druggable endosomal target essential for both acute and sustained activation of canonical cellular GPCR signaling, open the door to the endosomal system as a target for future therapeutic intervention.
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Authorship Contributions

Participate in research design: Uchida, von Zastrow
Conducted experiments: Uchida
Contributed new reagents or analytic tools: Rataganira, Jullié, Shokat
Performed data analysis: Uchida, von Zastrow
Wrote or contributed to the writing of the manuscript: Uchida, Rutaganira, Jullié, Shokat, von Zastrow
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Footnotes

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**Figure Legends**

**Fig. 1.** Endosomal PI3P is required for β2AR recycling but not endocytosis. (A) Schematic of the rapamycin (rapa)-induced recruitment of MTM1 to Rab5-containing endosomes. (B) Representative images of live cells expressing GFP-2xFYVE (a PI3P probe protein), mCherry-FKBP-MTM1 (either wild type (WT) or a phosphatase-dead mutant (C375S)) and iRFP-FRB-Rab5. Cells were imaged before and after 5-min of rapa treatment by confocal microscopy. Scale bar, 10 µm. (C and D) Trafficking of β2AR examined by immunofluorescence microscopy. (C) Cells stably expressing Flag-β2AR were transfected with mCherry-FKBP-MTM1 (WT or C375S) and iRFP-FRB-Rab5. In the ‘agonist’ condition, cells were treated with rapa for 20 min, and then with a β2AR agonist isoproterenol (iso) for 25 min. In the ‘agonist to antagonist’ condition, cells were first treated with iso for 25 min, and then with a β2AR antagonist alprenolol and rapa for 45 min. Following fixation, cells were stained for Flag epitope. Representative confocal images are shown. Insets show the boxed areas at higher magnification. Arrows show β2AR localization to Rab5-containing endosomes. Scale bar, 10 µm. (D) Quantification of the results. Pearson’s correlation coefficients between β2AR and FRB-Rab5 were calculated (n = 11 [WT, agonist], 11 [C375S, agonist], 21 [WT, agonist to antagonist], or 19 [C375S, agonist to antagonist] cells from two independent experiments, ***P < 0.001 by two-tailed t-test). Error bars represent SD.

**Fig. 2.** Vps34 is required for efficient β2AR recycling but not endocytosis. (A) Representative images of HEK293 cells expressing GFP-2xFYVE. Cells were treated with PIK-III or DMSO for 1 h, fixed, and imaged by confocal microscopy. Scale bar, 10 µm. (B) Effects of PI 3-kinase inhibitors on basal surface β2AR levels. Cells stably expressing Flag-tagged β2AR were treated with the indicated inhibitors for 1 h. Surface receptor levels were then determined by flow cytometry, and expressed as a percentage of the levels in DMSO-treated cells (n = 4 independent experiments). (C) Effects of PI 3-kinase inhibitors on agonist-induced reduction of surface β2AR
levels. After 1-h pre-treatment of inhibitors, cells were treated with iso for the indicated periods, and surface receptor levels were determined. Basal receptor levels shown in (B) were included in a graph as ‘0 min’ for comparison, and data were expressed as a percentage of the basal receptor levels in DMSO-treated cells. Data are from \( n = 3 \) (5 to 40 min) or 4 (0 min) independent experiments. PIK-III or WM treatment significantly reduced surface \( \beta_2 \)AR levels compared to DMSO control at 10, 20, and 40 min after iso treatment (*\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) by two-way ANOVA and Tukey’s post-hoc tests). (D) The efficiency of \( \beta_2 \)AR endocytosis was estimated from the percentage reduction in surface receptor levels after the shortest (5-min) iso application in (C). Data were from \( n = 3 \) independent experiments. (E) Direct measurement of \( \beta_2 \)AR recycling after agonist removal and antagonist treatment. After 1-h treatment of the indicated inhibitor, cells were treated with iso for 25 min, and then with alprenolol for 45 min. The percentage of recycled receptors was calculated as described in methods, and shown in bar graphs. (\( n = 3 \) independent experiments, ***\( P < 0.001 \) compared to DMSO control by one-way ANOVA and Tukey’s post-hoc tests). (F) Recycling defect in PIK-III-treated cells was verified by fluorescence microscopy. After 1-h treatment of PIK-III or DMSO, cells were treated with iso for 25 min (in the ‘agonist’ condition) and then with alprenolol for 45 min (in the ‘agonist to antagonist’ condition). Following fixation, cells were stained for Flag epitope. Representative confocal images are shown. Scale bars, 10 \( \mu \)m. (G) \( \beta_2 \)AR recycling in the continuous presence of agonist. Surface receptors were labeled with Alexa647-conjugated anti-Flag antibody, and then internalized by 1 \( \mu \)M iso for 25 min. After stripping antibodies bound to residual surface receptors by calcium-depleted medium, cells were further incubated for 5 min in calcium-depleted medium. \( \beta_2 \)AR recycling was then estimated by measuring the antibody efflux in this 5-min period. (\( n = 3 \) samples from one experiment, **\( P < 0.01 \) by two-tailed \( t \)-test. Similar results were obtained in 2 other independent experiments). Error bars mean SD.

**Fig. 3.** Vps34, but not class I PI 3-kinases or PIKfyve, is required for \( \beta_2 \)AR recycling. (A and B)
After 1-h (A) or 3-h (B) treatment of the indicated inhibitor, cells were subjected to the recycling assay as in Fig. 2E. \( n = 7 \) [DMSO] or 3 [each inhibitor] independent experiments in (A), and \( n = 3 \) independent experiments in (B). ***\( P < 0.001 \) compared to DMSO control by one-way ANOVA and Tukey’s post-hoc tests). Error bars mean SD.

**Fig. 4.** Vps34 supports not only long-term cellular β2AR signaling but also the acute signaling response. (A) Luminescence-based cAMP assay examining the initial accumulation of cytoplasmic cAMP induced by iso application. Wild-type HEK293 cells were transiently transfected with pGloSensor-20F. Cells were pre-treated with PIK-III or DMSO, and then iso or forskolin (fsk) was added (time = 0 sec). cAMP responses in iso-treated cells are shown. Data are normalized to the luminescence of fsk-treated wells \( (n = 6 \) independent experiments). (B) Luminescence-based cAMP assay examining resensitization of cellular cAMP response after prolonged iso treatment and wash-out. Cells were pre-treated with PIK-III or DMSO, and then treated with iso for 1 h. After a 10-min wash-out period, cells were re-challenged with iso. Luminescence increased by the second stimulation was normalized to that of the first stimulation. Normalized luminescence is significantly reduced in PIK-III-treated cells after 70 - 150 seconds of iso application compared to DMSO-treated cells \( (n = 3 \) independent experiments, \( * P < 0.05 \) by two-tailed \( t\)-test). (C and D) Biochemical determination of cAMP levels. HEK293 cells were pre-treated with the indicated inhibitor, and then treated with iso (C) or fsk (D) for the indicated times. Cells were then lysed, and cAMP concentration in the lysates was determined by ELISA and normalized to protein concentration. In (C), data are expressed as a percentage of the cAMP levels in cells pre-treated with DMSO and then iso for 1 min \( (n = 8 \) [DMSO] or 4 [PIK-III and VPS34-IN1] independent experiments, \( * P < 0.05, ** P < 0.01 \) compared to DMSO control by one-way ANOVA and Tukey’s post-hoc tests). In (D), data are expressed as a percentage of the cAMP levels in cells pre-treated with DMSO and then fsk for the indicated times \( (n = 4 \) independent experiments). Error bars mean SEM.
Fig. 5. Summary and model of Vps34 effects in β2AR-mediated cAMP response. (A) There are two aspects of cAMP response triggered by β2AR agonist (iso) application: One is acute response by single application, and the other is repeated response by a second (or more) application (boxed by a dotted line) after initial application and wash-out. The time course of agonist application and wash-out is indicated by black/white boxes above the graphs. In both cases, cAMP responses reach maxima less than 5 min and are rapidly desensitized. β2AR recycling promotes resensitization of the cellular responsiveness to subsequent agonist application in the wash-out period, thus supports the repeated response. We demonstrated that efficient β2AR recycling requires Vps34, and in line with this, Vps34 supports the repeated cAMP response. (B and C) According to our data showing Vps34 inhibition reduces cAMP levels after 5-10 min of acute agonist application, the acute cAMP response seems to be divided to two phases: Vps34-independent initial phase and Vps34-dependent later phase. Our models regarding the role of Vps34 in the acute cAMP response are shown in (C). In the initial phase, β2ARs activate cAMP synthesis in the PM and endosomes, but are not recycled yet. In the later phase, the recycling of β2AR begins in a Vps34 / PI3P-dependent manner, and the recycled receptors are subjected to the next round of receptor cycling between the PM and endosomes (indicated by red arrows). The continuous cycling of β2ARs, which is driven by Vps34, would enhance cAMP production from both locations in the later phase. It is also possible that Vps34 directly enhances endosomal cAMP production downstream of Gs activation (indicated by another red arrow with a question mark).
Figure 1

A

Before rapa

After rapa

B

WT

C375S

C

Agonist

Agonist to antagonist

D

Pearson's Coefficients

(β2AR / FRB-Rab5)
**Figure 2**

(A) Representative immunofluorescence images of 2xFYVE expression in DMSO and PIK-III treated cells. Scale bar: 10 μm.

(B) Basal surface β2AR levels (% of DMSO 0 min) for DMSO, PIK-III, and WM treatments.

(C) Graph showing the surface β2AR levels (% of DMSO 0 min) over time (min) for DMSO, PIK-III, and WM treatments. Key markers indicate statistical significance.

(D) Graph showing the % reduced surface β2AR (5-min agonist) for DMSO, PIK-III, and WM treatments.

(E) Graph showing the % recycled (45-min antagonist) for DMSO, PIK-III, and WM treatments. Key markers indicate statistical significance.

(F) Representative images of β2AR expression under agonist and antagonist conditions for DMSO and PIK-III treatments. Scale bar: 10 μm.

(G) Graph showing the % antibody flux (5-min) for DMSO and PIK-III treatments. Key markers indicate statistical significance.
Figure 3

A

% recycled (45-min antagonist)

DMSO
VPS34-IN1
SAR405
GDC-0941

B

% recycled (45-min antagonist)

DMSO
YM201636

***

***
Figure 4

**A**

- DMSO/PIK-III iso
- (min) -60 0
- Measure luminescence

Normalized luminescence (% of 5 μM Fsk)

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<th>Time (sec)</th>
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<th>PIK-III</th>
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</tr>
<tr>
<td>150</td>
<td>50</td>
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</table>

**B**

- DMSO/PIK-III iso wash iso
- (min) -60 0 60 70
- Measure luminescence

Normalized luminescence (% of 1st stimulation)

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>DMSO</th>
<th>PIK-III</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>120</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

**C**

- DMSO/PIK-III/ VPS34-IN1 iso
- (min) -60 0 1 5 10
- Lysis, ELISA

cAMP concentration (% of DMSO 1 min)

- **1 min**
  - DMSO: 100
  - PIK-III: 90
  - VPS34-IN1: 90

- **5 min**
  - DMSO: 50
  - PIK-III: 40
  - VPS34-IN1: 40

- **10 min**
  - DMSO: 20
  - PIK-III: 10
  - VPS34-IN1: 10

**D**

- DMSO/PIK-III fsk
- (min) -60 0 5 10
- Lysis, ELISA

cAMP concentration (% of DMSO 1 min)

- **5 min**
  - DMSO: 150
  - PIK-III: 120

- **10 min**
  - DMSO: 200
  - PIK-III: 160

* * *
Figure 5

A

**Acute response**

\[ \text{iso} \]

![](chart1)

\[ \text{[cAMP]} \]

10 20 (min)

**Repeated response**

\[ \text{iso} \ (-) \text{iso} \]

![](chart2)

\[ \text{[cAMP]} \]

1 (hr)

Dependent on VPS34-mediated β2AR recycling

B

**VPS34-independent**

\[ \text{VPS34-dependent} \]

![](chart3)

\[ \text{[cAMP]} \]

10 20 (min)

C

**VPS34-independent phase**

\[ \sim 2.5 \text{ min} \]

![Diagram](chart4)

**VPS34-dependent phase**

\[ \sim 10 \text{ min} \]

![Diagram](chart5)