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Phosphoinositide 3-kinase regulates β2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/β-arrestin complex

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Internalization of β-adrenergic receptors (βARs) occurs by the sequential binding of β-arrestin, the clathrin adaptor AP-2, and clathrin. D-3 phosphoinositides, generated by the action of phosphoinositide 3-kinase (PI3K) may regulate the endocytic process; however, the precise molecular mechanism is unknown. Here we demonstrate that βARKinase1 directly interacts with the PIK domain of PI3K to form a cytosolic complex. Overexpression of the PIK domain displaces endogenous PI3K from βARK1 and prevents βARK1-mediated translocation of PI3K to activated β_2ARs. Furthermore, disruption of the βARK1/PI3K interaction inhibits agonist-stimulated AP-2 adaptor protein recruitment to the β_2AR and receptor endocytosis without affecting the internalization of other clathrin dependent processes such as internalization of the transferrin receptor. In contrast, AP-2 recruitment is enhanced in the presence of D-3 phospholipids, and receptor internalization is blocked in presence of the specific phosphatidylinositol-3,4,5-trisphosphate lipid phosphatase PTEN. These findings provide a molecular mechanism for the agonist-dependent recruitment of PI3K to βARs, and support a role for the localized generation of D-3 phosphoinositides in regulating the recruitment of the receptor/cargo to clathrin-coated pits.

Introduction

β-Adrenergic receptors (βARs)* are a member of the large family of G protein–coupled receptors (GPCRs) (Johnson, 1998; Rockman et al., 2002). In the heart, ligand activation of βARs results in the dissociation of the cognate heterotrimeric GTP binding protein (G-protein) into Gα and Gβγ subunits, leading to stimulation of the effector adenylyl cyclase and subsequent physiologic response (Koch et al., 2000). Exposure to agonist also promotes the rapid desensitization of βARs, that not only leads to the attenuation of signaling, but also targets the activated receptor to clathrin-coated pits for internalization (Ferguson et al., 1996; Goodman et al., 1996). An early step in this process involves the rapid phosphorylation of agonist-occupied receptors by a G protein–coupled receptor kinase (GRK, commonly known as the β-adrenergic receptor kinase or βARK) (Lefkowitz, 1998). The phosphorylation of activated βARs by βARK1 requires translocation of the primarily cytosolic βARK to the plasma membrane, a process facilitated by the liberated Gβγ subunits and membrane phospholipids (Pitcher et al., 1998). The second step involves binding of the protein β-arrestin to the phosphorylated receptor resulting in termination of the signal (Lefkowitz, 1998). The binding of β-arrestin serves to target phosphorylated βARs for internalization, through the recruitment of the AP-2 adaptor protein and clathrin to the receptor complex (Laporte et al., 2000). There is increasing evidence that phosphatidylinositol (PtdIns) phospholipids are important molecules in endocytosis of membrane proteins (Czech, 2000). Because a key enzyme for the generation of phospholipids is phosphoinositide 3-kinase (PI3K), it suggests a possible role for PI3K in the internalization of GPCRs. Thus, although the endocytic process is a multistep event involving the coordinate interaction between proteins and as well as the control of lipid modification, the precise molecular mechanisms for this interaction are not well understood.

PI3Ks are a conserved family of lipid kinases that catalyze the addition of phosphate on the third position of the inositol

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*Abbreviations used in this paper: βAR, β-adrenergic receptor; CYP, cyanopindolol; GPCR, G protein–coupled receptor; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol(s).

Key words: phosphatidylinositols; phosphoinositide 3-kinase; AP-2; β-adrenergic receptor; endocytosis

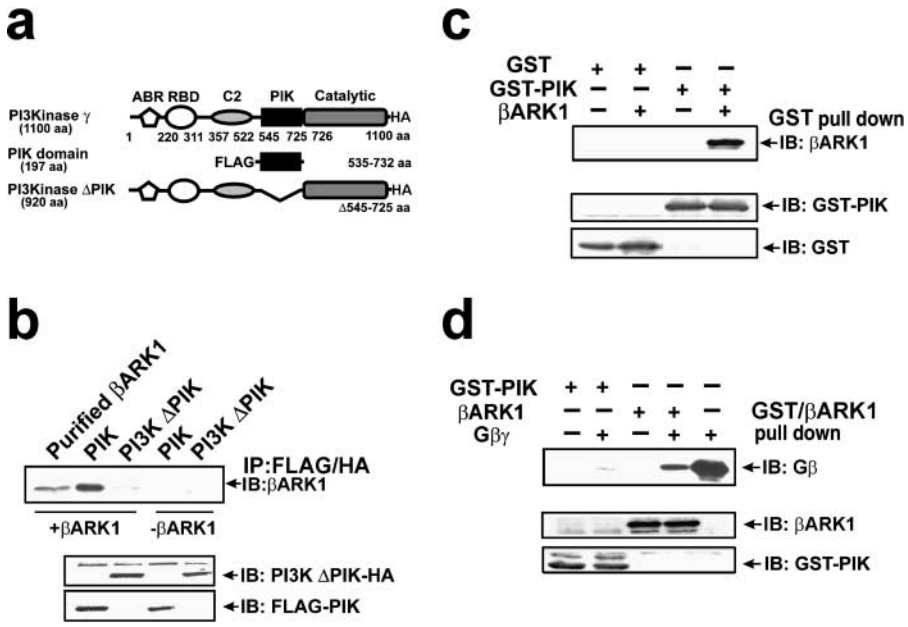

Figure 1. **βARK1 directly interacts with the PIK domain of PI3K.** (a) Schematic representation of full-length PI3Kp110γ and mutants. ABR adaptor binding region, RBD-ras binding domain, C2 similar to PLCδ, which is involved in Ca^{2+}-dependent or -independent phospholipid binding, PIK domain thought to be involved in protein–protein interactions, HA, hemagglutinin tag, FLAG, flag peptide tag. (b) HEK 293 cells cotransfected with plasmids containing βARK1 and FLAG-PIK or PI3KΔPIK cDNAs. FLAG-PIK and PI3KΔPIK were immunoprecipitated from cells, fractionated by SDS-PAGE, and analyzed by immunoblotting with βARK1 monoclonal antibody. βARK1 protein coimmunoprecipitates with FLAG-PIK and not with PI3KΔPIK. (c) GST–PIK fusion protein containing amino acid residues 535–732 of PI3Kγ was produced, which includes the entire PIK domain and flanking upstream 10 amino acids and downstream 7 amino acids. Beads with bound GST alone or GST fusion proteins were incubated with purified βARK1. Beads were washed and bound material was run on SDS-PAGE and immunoblotted with βARK1 monoclonal antibody. Purified βARK1 was found to bind to the GST–PIK fusion protein, whereas no binding was seen with GST alone. (d) PIK domain does not interact with Gβγ subunits of G-protein. Beads with bound GST–PIK fusion protein or βARK1 protein were incubated with purified Gβγ subunits of G-protein. Beads were washed, spun down, and bound material separated by SDS-PAGE electrophoresis followed by immunodetection of Gβ. No interaction between Gβγ and the PIK fusion protein was found, whereas robust interaction between Gβγ and βARK1 purified protein was observed. Purified Gβγ was loaded as a positive control.

ring (Rameh and Cantley, 1999). Stimulation of a variety of receptor tyrosine kinases and GPCRs results in the activation of PI3K and leads to an increase in the level of D-3 PtdIns, which in turn are potent signaling molecules that modulate a number of diverse cellular effects including: cell proliferation, cell survival, cytoskeletal rearrangements, and receptor endocytosis (Martin, 1998; Sato et al., 2001). In this context, GPCR stimulation leads to the activation of the I_B subclass of PI3Ks mediated by the Gβγ subunits of G-proteins (Stoyanov et al., 1995). Studies have suggested a role of phosphoinositides in the process of receptor internalization. For example, deletion of the phosphoinositide binding site from β-arrestin impairs GPCR endocytosis (Gaidarov et al., 1999a), and the binding of PtdIns (3,4,5) P_3 and PtdIns (4,5) P_2 to AP-2 promotes targeting of the receptor-arrestin complex to clathrin-coated pits (Gaidarov and Keen, 1999).

Recently we have shown a possible involvement of PI3K in the regulation of βAR internalization (Naga Prasad et al., 2001). Considering the potential important role of D-3 phosphoinositides in βAR internalization, we sought to determine: (a) whether there was a direct physical interaction between βARK1 and PI3K, and if so, identify the structural domain responsible for this interaction; (b) whether the interaction of PI3K with βARK1 regulates the translocation PI3K to the agonist-occupied βAR; and (c) whether the lipid products of PI3K modulate the internalization of βARs.

Results

Direct physical interaction of PI3K with βARK1

We postulated that the PIK domain of PI3K (Vanhaesebroeck et al., 2001), which is characterized by five pairs of antiparallel helices, might be an important domain to support protein–protein interactions (Walker et al., 1999). To investigate whether there is a direct physical interaction between βARK1 and PI3K, we created PI3K mutants that contained only the PIK domain (FLAG tagged) or had a deletion of the PIK domain PI3KΔPIK (HA tagged) (Fig. 1 a).

HEK 293 cells were transfected with plasmids containing the FLAG-PIK and PI3KΔPIK-HA cDNAs. PIK and PI3KΔPIK proteins were immunoprecipitated from cell extracts using monoclonal FLAG and HA antibodies, respectively. After the addition of purified βARK1 protein to the immune complexes, the presence of βARK1 was assessed by immunoblotting for βARK1. βARK1 was found to associate with the PIK domain protein and not to full-length PI3K lacking this domain (PI3KΔPIK) (Fig. 1 b). Levels of expression of PIK and PI3KΔPIK proteins were similar (Fig. 1 b, bottom). These data suggest that PI3K and βARK1 form a macromolecular complex within the cell.

To investigate whether there was a direct physical interaction between the PIK domain of PI3K and βARK1, GST–PIK fusion protein was immobilized on sepharose beads and incubated with purified βARK1. Purified βARK1 bound specifically to GST–PIK immobilized beads and not to GST alone (Fig. 1 c). No difference in the level of GST and GST–PIK was found (Fig. 1 c, bottom).

Because PI3Kγ interacts with the βγ subunits of G-proteins, we tested whether the PIK domain might directly interact with $G_{\beta\gamma}$, and thus would compete with βARK1 for these subunits. Purified βARK1 and GST–PIK fusion protein immobilized on Sepharose beads were incubated with purified $G_{\beta\gamma}$. The beads were washed and recovered proteins were analyzed by SDS-PAGE followed by immunoblotting

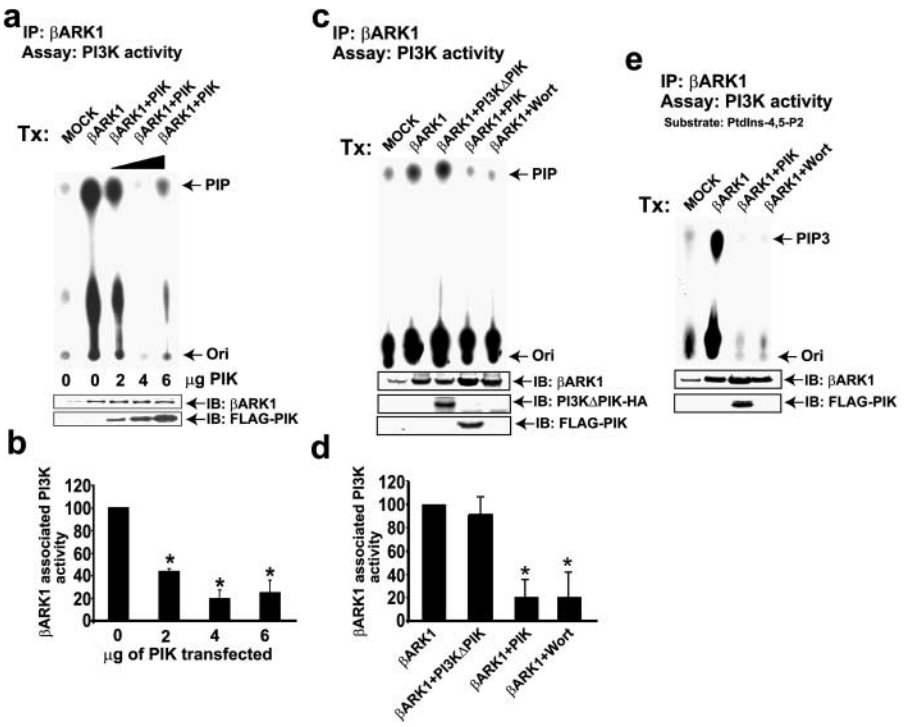

Figure 2. **Overexpression of the 197 amino acid PIK domain of PI3K competes for endogenous PI3K binding to βARK1.** (a) HEK 293 cells were cotransfected with βARK1 and increasing concentrations of PIK domain encoding cDNAs. βARK1 was immunoprecipitated using βARK1 monoclonal antibody and the associated PI3K activity was measured. The lipids were extracted and run on thin layer chromatography plates. Shown is representative autoradiograph of formation of PIP (PtdIns-monophosphate). Ori, Origin. Mock cells were transfected with vector alone. Increasing the quantity of PIK cDNA used for expression leads to displacement of PI3K activity from βARK1 suggesting PIK protein competes out PI3K from βARK1. Bottom panel shows immunoblotting for βARK1 and PIK in cell lysates. (b) Summary results of $n = 5$ experiments. *$P < 0.0005$. The data was normalized to βARK1 associated PI3K activity in cells transfected with βARK1 only. (c) HEK 293 cells were transfected with βARK1 or βARK1 and PIK or βARK1 and PI3KΔPIK encoding cDNAs. βARK1 was immunoprecipitated using βARK1 monoclonal antibody and the associated endogenous PI3K activity was assayed. Extracted lipids were run on TLC plates and a representative autoradiograph showing the formation of PIP is shown here. Mock cells are transfected with vector alone. PIK overexpression lead to displacement of endogenous PI3K activity, whereas PI3KΔPIK overexpression did not alter βARK1 associated PI3K activity. Bottom panels show immunoblotting for βARK1, PI3KΔPIK and PIK in cell lysates. (d) Summary results of $n = 4$ experiments. *$P < 0.001$. The data was normalized to βARK1 associated PI3K activity in cells transfected with βARK1 only. (e) HEK 293 cells were transfected with βARK1 or βARK1 and PIK encoding cDNAs. βARK1 was immunoprecipitated using βARK1 monoclonal antibody and the associated endogenous PI3K activity was assayed using PIP_2 (PtdIns-4,5-bisphosphate) as a substrate. Extracted lipids were run on TLC plates and the autoradiograph showing the formation of PIP_3 (PtdIns-3,4,5-triphosphate) is presented. βARK1 transfected cells were treated with wortmannin (Wort) (50 nM) for 15 min before lysis of cells. Mock cells were transfected with vector alone. Bottom panel shows immunoblotting for βARK1 and PIK from cell lysates. Overexpression of PIK domain or wortmannin treatment led to significant inhibition in the formation of PIP3.

with a G_β polyclonal antibody. Whereas a strong association of $G_{\beta\gamma}$ with βARK1 was found, no appreciable association of $G_{\beta\gamma}$ with the PIK domain was detected (Fig. 1 d). Densitometric quantification showed an 11.5 ± 1.1-fold greater binding ability of βARK1 to Gβγ purified proteins compared with the PIK domain.

To test whether overexpression of the PIK domain could displace βARK1 associated PI3K activity in living cells, experiments were performed in HEK 293 cells cotransfected with plasmids containing the βARK1 cDNA (2 μg) and increasing concentrations of the PIK domain cDNA (ranging from 0 to 6 μg). Cell lysates were immunoprecipitated using the βARK1 monoclonal antibody 48 h after transfection and βARK1 associated PI3K activity was assayed. A robust βARK1 associated PI3K activity was found in the absence of PIK domain protein, but this association could be effectively competed away by increasing the concentration of PIK cDNA (Fig. 2 a). The maximal reduction of βARK1 associated PI3K activity occurred when cells were cotransfected with 4 μg of PIK cDNA (Fig. 2 b). Furthermore, we could coimmunoprecipitate FLAG-tagged PIK protein with βARK1 monoclonal antibodies in the cotransfected HEK 293 cells (data not depicted).

We next tested whether absence of the PIK domain in an otherwise intact PI3K molecule would affect the association of βARK1 with endogenous PI3K in cells. HEK 293 cells were cotransfected with plasmids containing cDNAs for βARK1 (2 μg), βARK1 plus PIK (4 μg), and βARK1 plus PI3KΔPIK (4 μg). Cell lysates were immunoprecipitated with a βARK1 monoclonal antibody and assayed for the associated PI3K activity. Expression of PI3KΔPIK had no effect on the endogenous βARK1/PI3K interaction, whereas overexpression of PIK disrupted this interaction. Treatment with the selective PI3K inhibitor wortmannin (50 nM), abolished this βARK associated PI3K activity (Fig. 2, c and d).

Because the PIK domain is shared by all members of the PI3K family, we wanted to confirm that the βARK1-associated endogenous PI3K activity was contributed by Class I PI3K. To test this, HEK 293 cells were transfected with plasmids containing cDNAs for βARK1 (2 μg) or βARK1 plus PIK (4 μg). Cell lysates were immunoprecipitated with a βARK1 monoclonal antibody and assayed for the associated PI3K activity. However, in this experiment PtdIns-4,5-P_2 was used as the substrate instead of PtdIns, as in vitro, PtdIns-4,5-P_2 can be phosphorylated only by Class I PI3K (Fruman et al., 1998) and not by either the Class II or Class III PI3K enzymes. As shown in Fig. 2 e, robust generation of PtdIns-3,4,5-P_3, the product of Class I PI3K catalytic activity, was seen associated with βARK1 and the coexpression of PIK completely displaced the

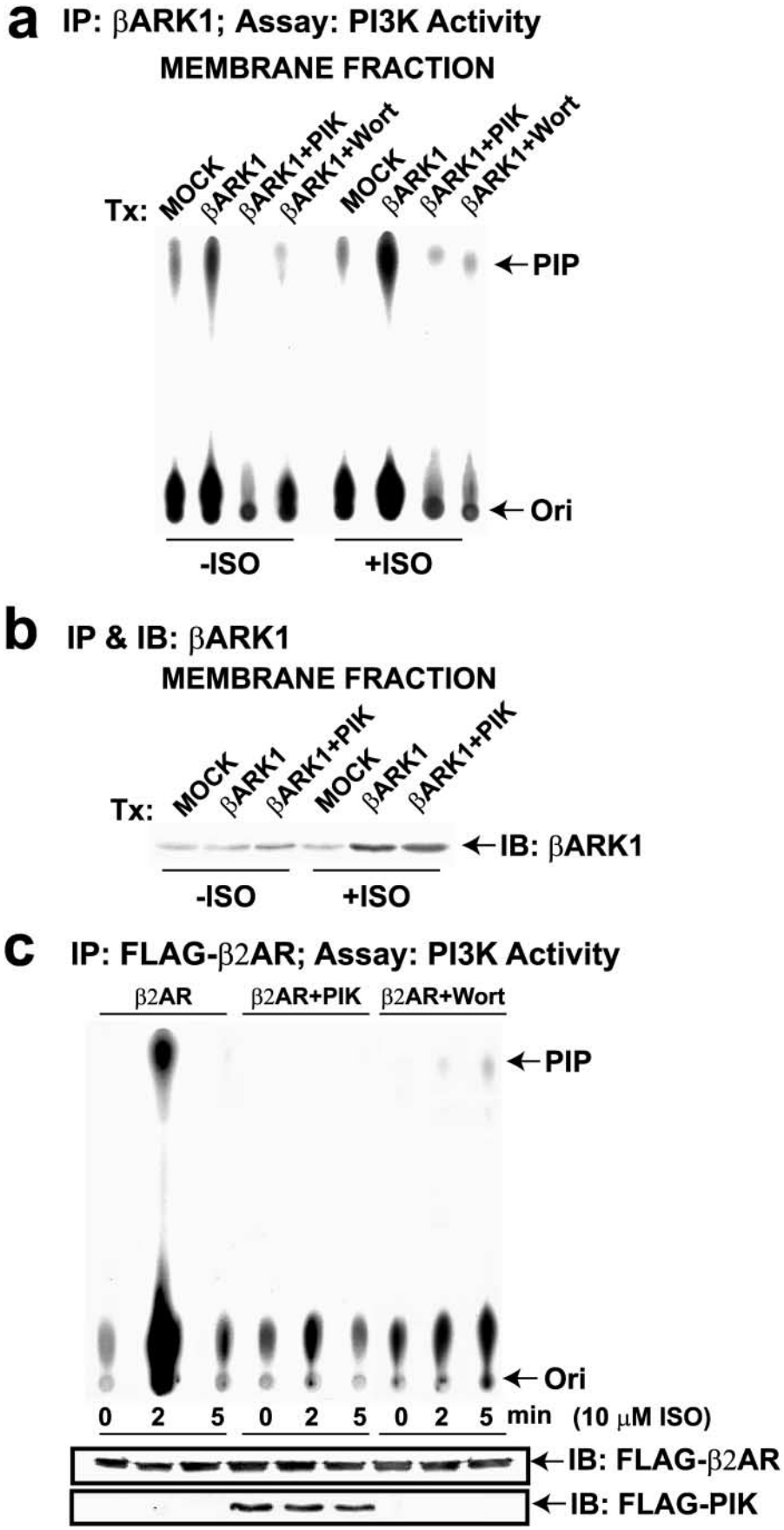

Figure 3. **Overexpression of PIK domain blocks βARK1 mediated translocation of PI3K to the membrane and to the β_2AR.** (a) HEK 293 cells were transfected with βARK1 or βARK1 and PIK encoding cDNAs. βARK1 was immunoprecipitated using βARK1 monoclonal antibody from the membrane fraction of unstimulated and stimulated (10 μM isoproterenol for 2 min) cells. βARK1 associated PI3K activity was assayed from these membranes and extracted lipids were separated by thin layer chromatography. The significant increase in βARK1 associated PI3K activity found in the membrane fraction after agonist was completely abolished in presence of PIK domain peptide and with wortmannin (50 nM) treatment of βARK1 transfected cells (Wort). (b) Membrane fraction of cells transfected with βARK1 or βARK1 and PIK encoding cDNAs were immunoprecipitated with a βARK1 monoclonal antibody and immunoblotted for βARK1. Overexpression of PIK did not inhibit βARK1 translocation to the membrane after isoproterenol stimulation. (c) HEK 293 cells were transfected with β_2AR or β_2AR and PIK encoding cDNAs. The transfected cells were split into separate dishes and individually treated with 10 μM isoproterenol for indicated times. Before isoproterenol treatment, a set of β_2AR-transfected cells were treated with wortmannin (Wort) (50 nM). At indicated time points FLAG-β_2AR was immunoprecipitated and the associated PI3K activity was measured. Shown is a representative autoradiograph of the thin layer chromatography plate showing the formation of PIP. Lower panel, level of expression of β_2AR and PIK domain protein in cell lysates as assessed by immunoblotting.

βARK1 associated PI3K activity (Fig. 2 e). Additionally, treatment of cells with wortmannin (50 nM) prior to cell lysis also inhibited the PI3K activity that was coimmunoprecipitated along with βARK1 (Fig. 2 e). Taken together, these data demonstrate that overexpression of the PIK domain can disrupt the interaction between βARK1 and PI3K, and that the lipid kinase activity belongs to the Class I PI3K family.

Overexpression of PIK blocks βARK1-mediated translocation of endogenous PI3K

Our results suggest that overexpression of the PIK domain should block the βARK1-mediated translocation of PI3K to the membrane. In order to test this hypothesis, HEK 293 cells were cotransfected with the βARK1 (2 μg), βARK1, and PIK domain (4 μg) containing plasmids, and endogenous βARs were stimulated with 10 μM isoproterenol for 2 min. Cytosolic and membrane fractions were prepared and analyzed for βARK1 associated PI3K activity. After isoproterenol stimulation, robust βARK1-associated PI3K activity that was wortmannin (50 nM) sensitive could be seen in the membrane fraction (Fig. 3 a). In contrast, overexpression of the PIK domain effectively abolished the agonist-induced translocation of PI3K to the membrane by disrupting the βARK1/PI3K interaction. No change in βARK1 associated PI3K activity was found in cytosolic fractions after isoproterenol stimulation (data not depicted).

To investigate whether βARK1 recruitment to the membrane would be inhibited in presence of PIK domain protein, membrane fractions were prepared from the HEK293 cells cotransfected with the βARK1 (2 μg), βARK1, and PIK domain (4 μg) containing plasmids. As shown in Fig. 3 b, overexpression of PIK had no effect on the membrane recruitment of βARK1 after isoproterenol (10 μM) stimulation. These data show that the overexpression of PIK domain protein interrupts the βARK1/PI3K interaction and does not affect agonist-dependent βARK1 translocation to the membrane.

To test whether disruption of the βARK1/PI3K interaction would prevent the recruitment of PI3K to activated βARs, HEK 293 cells were transfected with plasmids containing cDNAs encoding FLAG epitope–tagged β_2AR (FLAG-β_2AR, 2 μg) or FLAG-β_2AR (2 μg) and PIK domain (4 μg), and assayed for β_2AR associated PI3K activity after stimulation with 10 μM isoproterenol. HEK 293 cells are known to contain adequate levels of βARK1 to support agonist-induced translocation and receptor phosphorylation (Menard et al., 1997). FLAG-tagged β_2ARs were immunoprecipitated from cell extracts and associated endogenous PI3K activity measured. Significant FLAG-β_2AR–associated PI3K activity was observed as early as 2 min after agonist stimulation, with a decline by 5 min in the cells transfected with β_2AR alone (Fig. 3 c). In contrast, no FLAG-β_2AR–associated PI3K activity was found when either the PIK domain was overexpressed or the transfected cells were treated with wortmannin before isoproterenol stimulation (Fig. 3 c). These data indicate that overexpresssion of PIK domain displaces PI3K from βARK1 complex, thereby preventing its recruitment to the agonist-occupied receptor complex.

Attenuation of β_2AR sequestration by PIK

Previous studies have suggested a role for PI3K in β_2AR internalization (Naga Prasad et al., 2001). We postulated that disruption of the endogenous βARK1/PI3K interaction by the PIK protein would attenuate β_2AR endocytosis. To test this, agonist-dependent sequestration was studied by [^{125}I]-cyanopindolol binding in HEK 293 cells cotransfected with plasmids containing either the FLAG-β_2AR cDNA, or FLAG-β_2AR and FLAG-PIK cDNAs. A significant (>60%) attenuation in the rate of β_2AR sequestration occurred when the PIK domain protein was overexpressed (Fig. 4 a). Interestingly, overexpression of PIK domain protein was effective in attenuating the early processes of β_2AR sequestration as the initial phase (0–5 min) was significantly impaired.

We further evaluated β_2AR endocytosis using confocal microscopy in transfected cells using β_2AR tagged with the YFP (β_2AR-YFP) in the presence and absence of PIK. β_2AR internalization was followed in the same cell after agonist stimulation. Before agonist, the distribution of β_2AR-YFP was found distinctly at the plasma membrane (Fig. 4 b, panel 1). After agonist treatment, there was redistribution of the β_2AR-YFP into membrane puncta consistent with entry into clathrin coated pits (Fig. 4 b, panel 2) (Laporte et al., 1999). With time, this was followed by the formation of cytoplasmic aggregates (Fig. 4 b, panel 3), and then with the complete loss of membrane fluorescence (Fig. 4 b, panels 3 and 7, arrowheads). In marked contrast, coexpression with the PIK domain completely prevented redistribution of β_2AR-YFP fluorescence into membrane puncta and blocked the formation of intracellular aggregates after isoproterenol stimulation (Fig. 4 b, panels 6 and 8).

To definitively show that only those cells that contained the PIK domain protein failed to undergo agonist-stimulated βAR internalization, dual labeling experiments were performed after cotransfection with plasmids encoding HA tagged β_2AR (HA-β_2AR) and PIK–GFP. After isoproterenol stimulation, cells were fixed and HA-β_2AR was visualized by Texas red staining and PIK was visualized by GFP fluorescence. Cells that showed restricted distribution of Texas red staining (HA-β_2AR expression) to the membrane (Fig. 4 c, panel 1), also had GFP fluorescence indicating PIK protein expression (Fig. 4 c, panel 2). In contrast, cells that lacked GFP fluorescence (i.e., PIK protein expression), showed marked agonist-induced β_2AR internalization (Fig. 4 c, panel 1 and 2, arrowheads) as clearly seen in the overlay (Fig. 4 c, panel 3, arrowheads).

PI3K is not necessary for β-arrestin recruitment

To test whether PI3K activity was necessary for the recruitment of β-arrestin to the receptor complex, and whether overexpression of the PIK protein alters agonist-induced receptor phosphorylation, we used a HEK 293 cell line with stable expression of both β_2AR-HA and β-arrestin2–GFP proteins. Double stably expressing cells were transfected with the plasmid containing FLAG-PIK cDNA and then split in separate dishes. Confocal microscopy was used to visualize fluorescence in cells with 10 μM isoproterenol and subsequently fixed. All cells show β-arrestin2–GFP fluorescence, whereas a smaller percentage shows Texas red staining of the FLAG epitope (Fig. 5 a, panels 1 and 2). In the absence of isoproter-

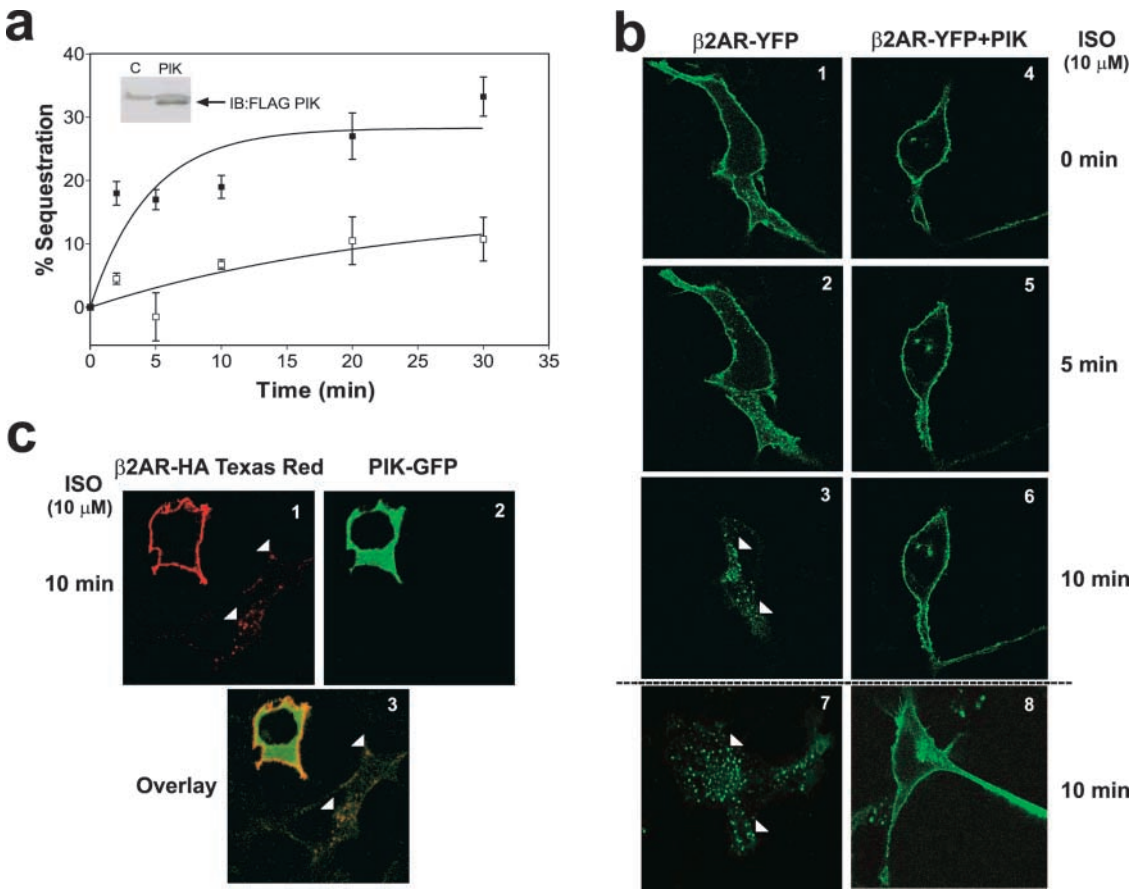

Figure 4. **Attenuation of β_2AR sequestration upon coexpression of PIK.** (a) Agonist-promoted (1 μM isoproterenol) β_2AR sequestration by [^{125}I]-CYP binding was performed in HEK 293 cells transfected with β_2AR and either the empty vector (■) or PIK (□) encoding cDNAs over a time course of 0–30 min. Receptor expression (fmol/mg of whole-cell protein) was: β_2AR + vector, 330 ± 80; β_2AR + PIK, 303 ± 51. $n = 5$, $P < 0.0001$ versus β_2AR. Inset shows expression of PIK protein. (b) Endocytosis of β_2AR-YFP in live cells was monitored for 10 min after isoproterenol (10 μM) stimulation in the absence or presence of PIK domain protein coexpression using confocal microscopy. Panels on the left represent cells transfected with the β_2AR-YFP alone (panels 1–3 show the same cell monitored at 0, 5, and 10 min after stimulation. Panel 7 is an example of another cell after 10 min of isoproterenol). Panels on the right represent cell transfected with β_2AR-YFP and PIK (panels 4–6 show the same cell monitored as above). Panel 8 is an example of another cell after 10 min of isoproterenol). In the absence of PIK, isoproterenol causes the internalization β_2ARs as shown by the formation of distinct cytoplasmic aggregates (arrowheads) and complete loss of membrane fluorescence. In contrast, in the presence of PIK domain protein, there is no redistribution of β_2AR-YFP after agonist stimulation, indicating that the process of receptor endocytosis is completely inhibited. (c) Dual staining was performed in cells transfected with plasmids containing HA-β_2AR and GFP-PIK cDNAs. After 10 min of 10 μM isoproterenol, cells were fixed with 4% paraformaldehyde, stained for HA-β_2AR receptor with Texas red, and PIK was visualized by GFP fluorescence. Panel 1 shows two cells, one with only membrane distribution of HA-β_2AR, and another cell with complete redistribution of β_2ARs into aggregates (arrowheads). Panel 2, shows that the cell with GFP fluorescence (i.e., PIK domain expression) failed to undergo β_2AR internalization, whereas the cell not expressing GFP-PIK showed HA-β_2AR aggregates. Panel 3 is an overlay of panels 1 and 2.

enol (Fig. 5 a, panels 1 and 2) cells have a cytosolic distribution of PIK as well as β-arrestin2-GFP. With isoproterenol, there was marked redistribution of GFP fluorescence to the membrane, indicating recruitment of β-arrestin to the membrane (Fig. 5 a, panel 3). Importantly, cells that contained PIK proteins did not affect the membrane recruitment of β-arrestin (Fig. 5 a, panels 3 and 4, arrowheads), suggesting that D-3 phosphoinositide molecules are not necessary for arrestin recruitment to activated receptors.

Overexpression of PIK domain does not inhibit clathrin-mediated transferrin uptake or downstream signaling of PI3K

To determine whether the overexpression of the PIK domain in cells nonspecifically affect other clathrin-mediated processes, we assessed the ability of transferrin to undergo endocytosis. Transferrin receptors are known to constitutively localize and internalize via clathrin-coated vesicles (van Dam and Stoorvogel, 2002). HEK 293 cells were transfected with plasmids encoding PIK–GFP. Transfected cells were incubated with Transferrin–Texas red conjugate at 37°C for 30 min and then fixed. As shown in Fig. 5 b, transferrin uptake was unaffected by the presence of PIK–GFP (Fig. 5 b, panels 1 and 2), indicating that overexpression of PIK domain does not inhibit other clathrin mediated processes.

To investigate whether overexpression of PIK domain protein in cells would alter downstream PI3K signaling, PKB activation was measured in HEK 293 cells stably expressing the PIK domain protein. Cells stably expressing PIK and wild-type HEK 293 cells were transfected with the plasmid con-

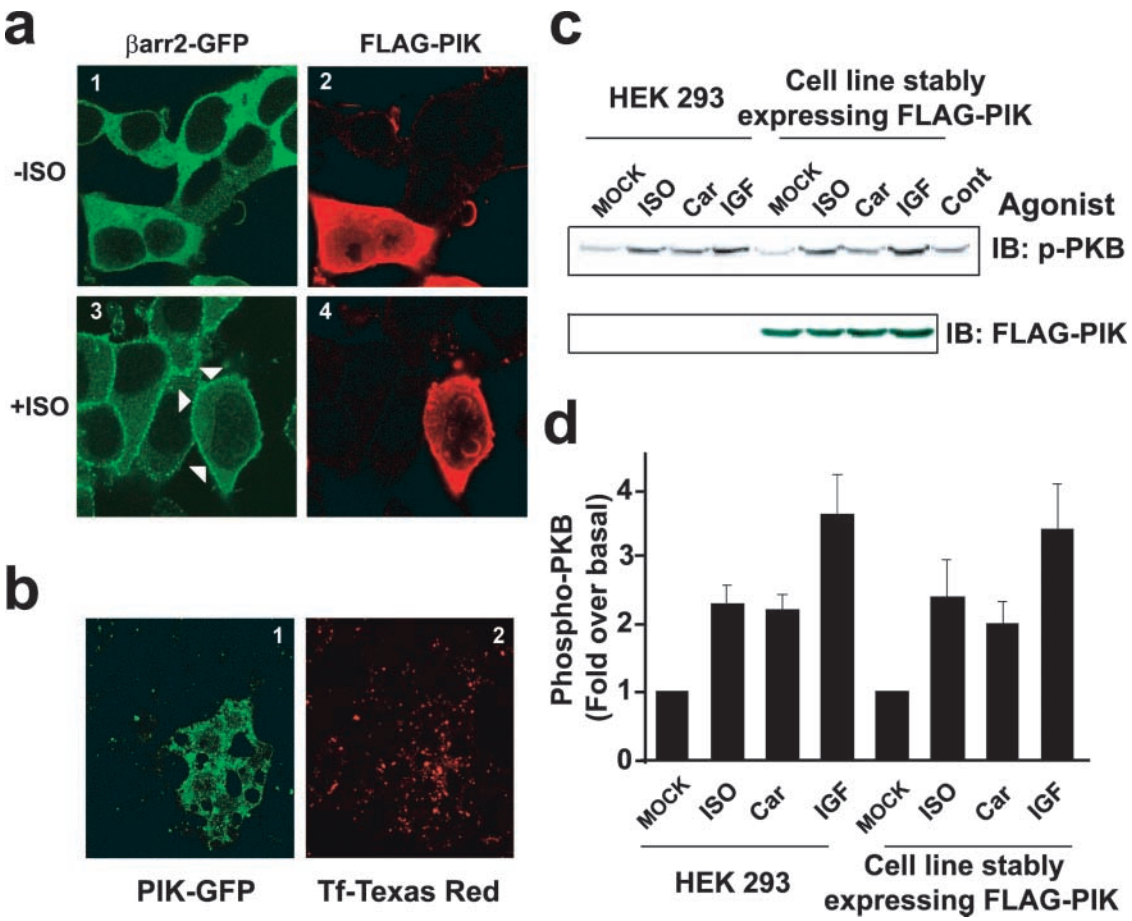

Figure 5. **Overexpression of PIK neither inhibits β-arrestin2 recruitment to the receptor complex, transferrin uptake, or downstream PI3K signaling.** (a) Confocal microscopy was used to visualize fluorescence in HEK 293 double stably expressing (HA-β_2AR-HA and β-arrestin 2-GFP) cells transfected with the FLAG-PIK plasmid. After stimulation with 10 μM isoproterenol for 10 min, cells were fixed and stained with Texas red. All cells show β-arrestin2–GFP (βarr2-GFP) fluorescence whereas a smaller percentage show Texas red staining of the FLAG epitope. Panels 1 and 2 show that in the absence of isoproterenol, both PIK and β-arrestin2–GFP were distributed in the cytoplasm. Panels 3 and 4, after the addition of isoproterenol, the presence of PIK domain protein had no effect on the marked redistribution of β-arrestin–GFP fluorescence to the membrane (arrowheads). (b) HEK 293 cells were transfected with GFP–PIK (4 μg) and plated on to glass-bottom petri dishes. Serum-starved cells were incubated with Transferrin (Tf) conjugated to Texas red for 30 min at 37°C. The cells were thoroughly washed with PBS and fixed in 4% paraformaldehyde. Fixed cells were visualized using laser scanning confocal microscope. Panel 1 shows PIK domain expression in cells as visualized by GFP fluorescence, whereas panel 2 shows the same set of cells that took up Transferrin (Tf) as visualized by Texas red. Cells that overexpressed the PIK domain also had robust Transferrin (Tf) uptake. (c) HEK 293 cells or cells stably expressing PIK were transfected with β_2AR encoding cDNA and treated with various agonists. Clarified lysates (50 μg) were immunoblotted for phospho-PKB. MOCK, treated with ascorbic acid; IGF, treated with insulin growth factor (10 nM); Car, treated with carbachol (1 mM); ISO, treated with isoproterenol dissolved in ascorbic acid (10 μM); Cont, positive control for phospho-PKB. (d) Summary results of densitometric analysis of phospho-PKB after stimulation by various agonists (n = 3–5). The data is represented as fold over basal (MOCK).

taining cDNA for the FLAG-β_2AR, and stimulated with various GPCR or growth factor agonists. Agonist stimulation in the absence of PIK resulted in a significant increase of pPKB over mock treatment (Fig. 5, c and d). Importantly, there was robust PKB activation in the cell line overexpressing the PIK domain protein (Fig. 5, c and d). These data show that overexpression of the PIK domain in cells does not interfere with cellular signaling downstream of PI3K.

Role of D-3 PtdIns in the recruitment of adaptin

Since overexpression of PIK leads to attenuation of β_2AR endocytosis, we wanted to determine whether the βARK1-mediated localization of PI3K within the activated receptor complex is responsible for the generation of D-3 phosphorylated phosphoinositides that promote recruitment of AP-2 to the agonist-occupied receptor. To test this hypothesis, HEK 293 cells were transfected with FLAG-β_2AR or FLAG-β_2AR, and PIK plasmids and then stimulated with isoproterenol. The FLAG-epitope was immunoprecipitated from cell extracts and blotted for the associated AP-2 adaptin and clathrin proteins. There was a significant increase in the association of AP-2 adaptin to the agonist-stimulated receptor within 2–5 min, which returned to basal levels by 10 min (Fig. 6 a). In contrast, overexpression of the PIK peptide completely abolished the recruitment of adaptin to the agonist-stimulated β_2AR complex (Fig. 6 a). Although the levels of clathrin that coimmunoprecipitated with the receptor showed only modest changes after agonist, this effect appeared to be attenuated in the presence of the PIK peptide (Fig. 6 a). Similar levels of adaptin, clathrin, β_2AR,

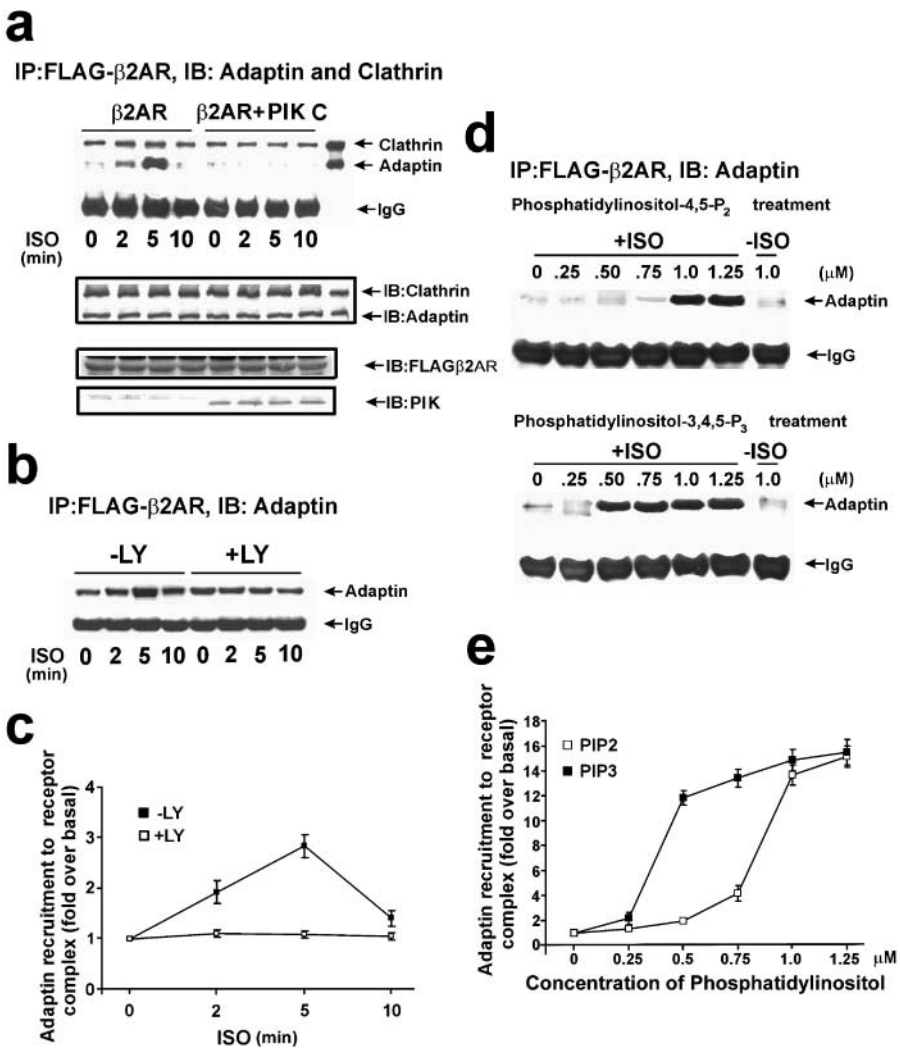

Figure 6. **Generation of D-3 PtdIns are necessary for the efficient recruitment of adaptin to the β_2AR complex.** (a) HEK 293 cells were transfected with FLAG-β_2AR, or FLAG-β_2AR and PIK encoding cDNAs. Transfected cells were treated with isoproterenol (10 μM) for 0, 2, 5, and 10 min β_2AR was immunoprecipitated using the FLAG epitope from the cells lysates and immunoblotted with antibodies for AP-2 adaptin protein and clathrin. Presence of PIK domain protein inhibits AP-2 adaptin association with the β_2AR complex. Bottom panels show expression of β_2AR and PIK protein in transfected cells and equal quantities of adaptin and clathrin loading. IgG, represents heavy chain of the antibody. C, positive control for clathrin and AP2 adaptin proteins. (b) FLAG-β_2AR expressing stable cells were treated with LY294002 (a selective PI3K inhibitor) for 15 min before isoproterenol stimulation. The cells were stimulated for 0, 2, 5, and 10 min and β_2AR was immunoprecipitated using a FLAG epitope. Lysates were immunoblotted for AP-2 adaptin protein. Presence of LY294002 inhibited adaptin recruitment to the receptor complex. –LY, untreated cells, +LY cells treated with LY294002. (c) Summary results of densitometric analysis of adaptin recruitment to β_2AR in presence and absence of LY294002 ($n = 7$). The data is represented as fold over basal. (d) FLAG-β_2AR expressing stable cells were treated with the permeablizing reagent saponin and the phospholipids as shown, followed by isoproterenol for 5 min. FLAG epitope was immunoprecipitated from the cell lysates and blotted with adaptin antibodies. The upper panel shows cells treated with phosphatidyinositol-4,5-P_2, while lower panel were treated with PtdIns-3,4,5-P_3. IgG, heavy chain of antibody. (e) Summary results of densitometric analysis of adaptin recruitment to β_2AR complex in presence of phosphatidyinositol-4,5-P_2 (PIP2) or PtdIns-3,4,5-P_3 (PIP3) ($n = 7$). Data is represented as fold over basal.

and PIK were observed in the appropriately transfected cells (Fig. 6 a, bottom).

Because the recruitment of the AP-2 adaptor protein was inhibited in presence of PIK overexpression, we investigated whether inhibition of PI3K activity would affect the recruitment of adaptin to the receptor complex. To test this, we used a HEK 293 cell line with stable expression of FLAG-β_2AR. Cells were treated with LY294002, an inhibitor of PI3K activity or DMSO as control for 15 min and then stimulated with isoproterenol. A significant increase in the association of adaptin protein with the receptor complex is observed at 5 min in the absence of LY294002, which was completely blocked with LY294002 pretreatment (Figs. 6, b and c). These data are consistent with our data showing that disruption of the βARK/PI3K interaction with overexpression of PIK leads to a loss in the receptor associated PI3K activity, a reduction in adaptin recruitment, and attenuation in receptor sequestration

To directly demonstrate that the generation of D-3 PtdIns phospholipids are important for the recruitment of AP-2 adaptor proteins to the agonist-occupied receptor complex, the same HEK 293 cells stably expressing FLAG-β_2AR were permeabilized with saponin (Jones et al., 1999) and then incubated with increasing concentrations of the phosphorylated lipids, PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3. After stimulation with isoproterenol, FLAG-β_2AR was immunoprecipitated and the immune complexes immunoblotted for the presence of adaptin. The efficiency of recruitment of adaptin to the receptor was significantly enhanced in the presence of PtdIns-3,4,5-P_3 compared with a similar concentration of PtdIns-4,5-P_2 (Figs. 6, d and e). At high concentrations of PtdIns-4,5-P_2, the preferential effect of PtdIns-3,4,5-P_3 was lost consistent with previous studies showing that the AP-2 adaptor protein has a higher affinity for PtdIns-3,4,5-P_3 compared to other phosphoinositides (Gaidarov et al., 1996).

Attenuation of β_2AR endocytosis upon inhibition of PtdIns-3,4,5-P_3 production in cells

To directly test whether the local production of PtdIns-3,4,5-P_3 is required for receptor internalization, we utilized the lipid phosphatase PTEN (phosphatase and tensin homo-

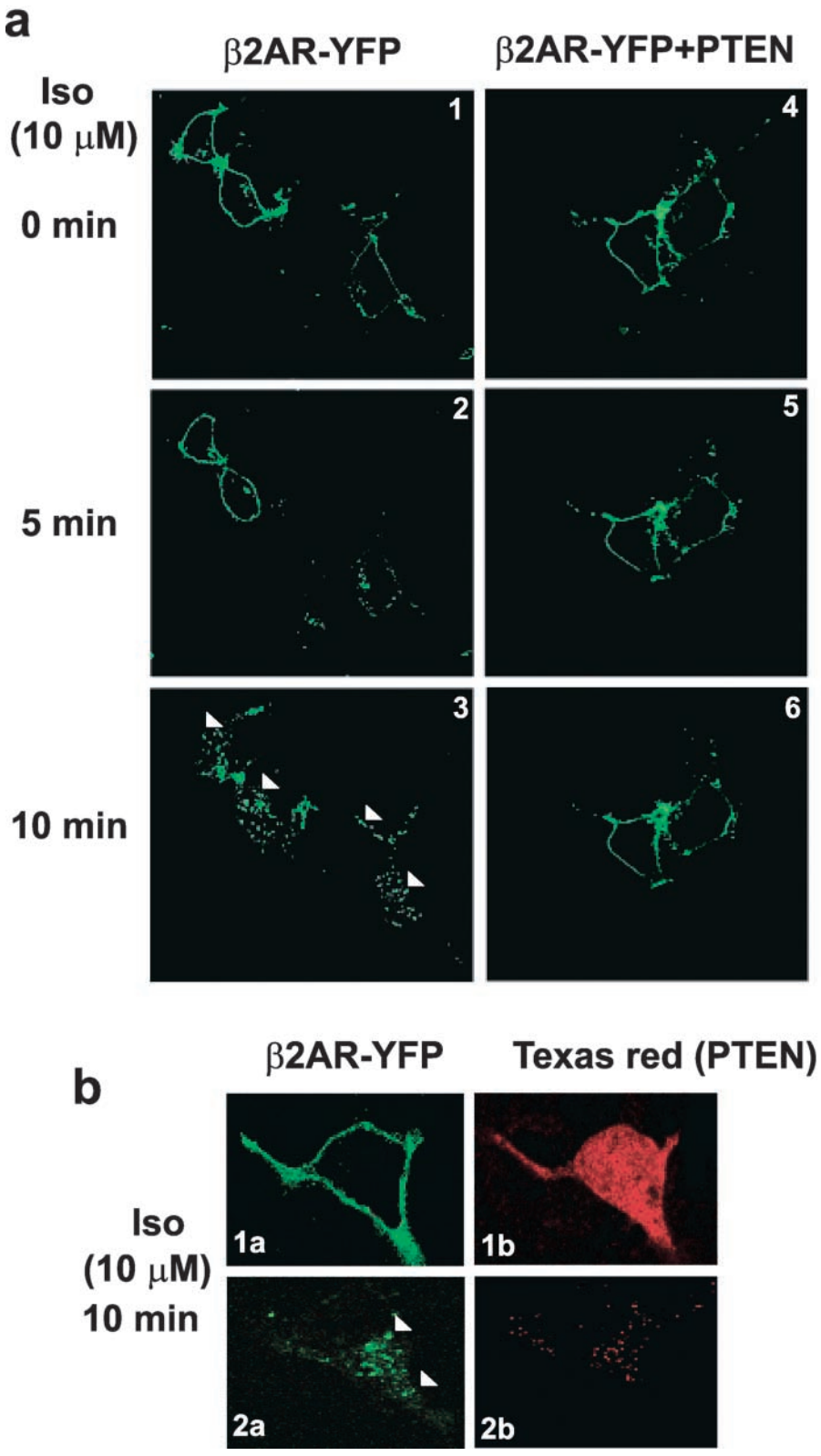

Figure 7. **Depletion of D-3 phosphatidylinositols leads to attenuation in β_2AR receptor internalization.** (a) Endocytosis of β_2AR-YFP in live cells was monitored for 10 min after isoproterenol (10 μM) stimulation in the absence or presence of PTEN protein coexpression using confocal microscopy. Panels on the left represent cells transfected with the β_2AR-YFP alone (panels 1–3 show the same cell monitored at 0, 5, and 10 min after stimulation). Panels on the right represent cell transfected with β_2AR-YFP and PTEN (panels 4–6 show the same cell monitored as above). In the absence of PTEN, isoproterenol causes the internalization β_2ARs as shown by the formation of distinct cytoplasmic aggregates (arrowheads) and complete loss of membrane fluorescence. In contrast, in the presence of PTEN protein, there is no redistribution of β_2AR-YFP after agonist stimulation, indicating that the process of receptor endocytosis is completely inhibited. (b) Dual staining was performed in cells transfected with plasmids β_2AR-YFP and PTEN cDNAs. After 10 min of 10 μM isoproterenol, cells were fixed with 4% paraformaldehyde, stained for PTEN using Texas red and β_2AR was visualized by GFP fluorescence. Panel 1 (a and b) shows the same cell with intact membrane β_2AR visualized by GFP fluorescence (panel 1 a) and PTEN Texas red fluorescence (panel 1 b). Panel 2 (a and b) shows the cell with complete redistribution of β_2ARs into aggregates (arrowheads) visualized by GFP fluorescence and very little PTEN expression as visualized by Texas red fluorescence (panel 2 b). Cells from panels 1 and 2 are from the same petri dish but from different fields of view.

logue deleted on chromosome 10) (Vanhaesebroeck et al., 2001). PTEN is known to use PtdIns-3,4,5-P_3 as its primary substrate converting it to PtdIns-4,5-P_2, thus depleting PtdIns-3,4,5-P_3 from the membrane (Vanhaesebroeck et al., 2001). HEK 293 cells were transfected with plasmids containing cDNAs with either β_2AR-YFP or β_2AR-YFP plus PTEN, and β_2AR endocytosis was evaluated using laser scanning confocal microscopy. β_2AR internalization was followed in the same cell after agonist stimulation. Before agonist, the distribution of β_2AR-YFP was found distinctly at the plasma membrane (Fig. 7 a, panel 1). After agonist stimulation, there was a progressive redistribution of the β_2AR-YFP into membrane puncta, followed by the formation of cytoplasmic aggregates and complete loss of membrane fluo-

rescence by 10 min (Fig. 4 b, panels 2 and 3). In contrast, coexpression of β_2ARs with the PTEN completely prevented the agonist induced redistribution of β_2AR-YFP fluorescence into membrane puncta and intracellular aggregates (Fig. 7, panels 4–6).

To show that only those cells that contained the PTEN failed to undergo agonist-stimulated βAR internalization, dual labeling experiments were performed in cells co-transfected with plasmids containing β_2AR-YFP and PTEN. After isoproterenol stimulation cells were fixed and β_2AR was visualized by GFP fluorescence and PTEN expression was visualized by Texas red staining. Cells that had the restricted membrane distribution of GFP fluorescence (β_2AR) also had the Texas red staining (Fig. 7 b, panel 1, a and b). In contrast, cells that showed β_2AR internalization lacked Texas red staining (PTEN expression) (Fig. 7 b, panel 2, a and b, arrowheads).

Discussion

In the present investigation, we provide evidence for a direct protein–protein interaction between PI3K and βARK1, and show that the region of the PI3K molecule that provides the necessary structure for this interaction is the PIK domain. Moreover, we show that the interaction between PI3K and βARK1 is not dependent on $G_{\beta\gamma}$ and that overexpression of PIK domain competitively displaces PI3K from the βARK1/PI3K complex leading to a loss in βARK1 associated PI3K activity. Although overexpression of PIK domain protein disrupts the βARK1/PI3K interaction, it does not inhibit the Gβγ mediated translocation of βARK1 to agonist-occupied receptors or alter other Gβγ-dependent cellular processes. Finally, these experiments demonstrate that overexpression of the PIK domain markedly attenuates β_2AR endocytosis in the early phase after agonist stimulation, and suggest that impairing the local production of PtdIns-3,4,5-P_3 lipid molecules within the agonist-occupied receptor complex affects the recruitment of critical molecules necessary for efficient receptor endocytosis. Furthermore, we show that overexpression of PTEN, which uses PtdIns-3,4,5-P_3 as a substrate, leads to complete inhibition of β_2AR internalization, providing strong evidence that the local production D-3 phosphoinositides by PI3K within the agonist-occupied receptor complex is required for endocytosis.

Agonist-occupied βARs are phosphorylated by βARK1 after translocation of βARK1 to the membrane (Lefkowitz, 1998). Subsequently, the phosphorylated receptor can bind with high affinity to β-arrestin (Ferguson et al., 1996), which then recruits AP-2 adaptor molecules and clathrin; two required components in the formation of the endocytic vesicle (Goodman et al., 1996; Laporte et al., 1999, 2000). Previous studies have shown that the association of adaptor proteins with clathrin are critical to the formation of the clathrin lattice complex (Schmid, 1997; Brodsky et al., 2001). Because the recruitment of AP-2 adaptor proteins are at least in part regulated by D-3–phosphorylated phosphoinositides (Gaidarov and Keen, 1999), the generation of these lipids not only play an important role in targeting of the agonist-stimulated receptor to the clathrin coated pit, but are likely important in the initiation/nucleation of the clathrin lattices at sites of endocytosis. This is consistent with data from in vitro studies showing that AP-2 in the assembled coat structure (which is very similar to clathrin-coated pit), binds PtdIns-3,4,5-P_3 with greater affinity compared with other phosphoinositides including PtdIns-4,5-P_2 (Gaidarov et al., 1996).

Whether the recruitment of AP-2 to agonist-stimulated receptor initiates the nucleation of a new clathrin-coated pit or targets the receptor to a preexisting pit, remains controversial. For example, AP180, a neuronal specific clathrin adaptor protein needs to bind clathrin and phosphoinositides simultaneously to initiate nucleation (Ford et al., 2001), and the assembled receptor/β-arrestin/AP-2 complex may subserve this role and initiate nucleation of the clathrin-coated pit (Laporte et al., 2000). In contrast, studies in living cells have suggested that the targeting of activated GPCRs is to preexisting clathrin-coated pits (Scott et al., 2002). Furthermore, studies using a clathrin–green fluorescent fusion protein suggest that clathrin-coated pits form repeatedly at defined sites and the mobility of these pits is limited by the actin cytoskeleton (Gaidarov et al., 1999b). Studies using receptor tail like synthetic peptides (crosslinked to UV photoreactive molecules) showed enhanced binding to the μ2 subunit of the AP-2 complex in presence of D-3 phosphoinositides, suggesting that phospholipids may provide a mechanism for increasing the specificity in sorting and clathrin coat assembly (Rapoport et al., 1997).

In this study we show that expression of PIK does not block β-arrestin recruitment to the receptor, nor does it impair the ability of βARK to phosphorylate activated receptors, as β-arrestin is only recruited to GRK phosphorylated receptors (Ferguson et al., 1996). Previous studies have shown that deletion of the phosphoinositide binding domain on β-arrestin impairs the formation of clathrin coated pits, but that this process is not altered by wortmannin treatment (Gaidarov et al., 1999a). This suggests that β-arrestin can bind PtdIns-4,5-P_2, which is present on the plasma membrane in much greater concentration than PtdIns-3,4,5-P_3. Consistent with these findings is our observation showing that even in the absence of receptor-associated PI3K activity, membrane PtdIns-4,5-P_2 is sufficient to recruit β-arrestin in an agonist-dependent manner.

Importantly, overexpression of the PIK domain does not block transferrin uptake, whose receptor constitutively localized within clathrin coated vesicles (van Dam and Stoorvogel, 2002). This suggests that recruitment of PI3K by βARK1 to the agonist occupied receptor is a βARK1 specific process. Furthermore, overexpression of the PIK domain did not affect activation of PKB suggesting a specific role in displacement of PI3K from βARK1. Thus, agonist dependent phosphorylation of PKB in PIK expressing cells may enable signaling either by direct receptor stimulation or through transactivation of receptor tyrosine kinases, as shown for other GPCRs (Kowalski-Chauvel et al., 1996; Saward and Zahradka, 1997).

To test the hypothesis that the local generation of PtdIns-3,4,5-P3 is a necessary step for β_2AR endocytosis, we performed experiments in cells that had overexpression of PTEN, a PtdIns-3,4,5-P_3 lipid phosphatase. Because PtdIns-3,4,5-P_3 generated by PI3K within the receptor complex

would be immediately hydrolyzed by PTEN we could determine the importance for PtdIns-3,4,5-P_3 in supporting the internalization process. The presence of PTEN resulted in significant inhibition of β_2AR endocytosis showing the requirement for PI3K activity within the receptor complex for effective agonist-induced endocytosis. We postulate that inhibition of PtdIns-3,4,5-P_3 production within the receptor complex prevents the effective interaction of various components needed for receptor endocytosis particularly, adaptin leading to an impairment in receptor endocytosis.

The crystal structure of PI3Kγ shows the PIK domain to be centrally positioned with a solvent exposed surface suitable for protein-protein interactions (Walker et al., 1999). Therefore, it is possible that other molecules containing a PIK domain, such as enzymes belonging to the family of PI3K (all the classes of PI3K), can potentially interact with βARK1 depending up the tissue and the abundance of the various isoforms. This is consistent with our previous data where we have shown in HEK 293 cells that βARK1 could also interact with the PI3Kα isoform when overexpressed (Naga Prasad et al., 2001). Interestingly, it has recently been shown that another PI3K, the class II PI3K C2α, interacts with clathrin and regulates clathrin-mediated membrane trafficking particularly in the process of vesicle uncoating (Gaidarov et al., 2001). This suggests possible redundancy for the production of phosphoinositides within the receptor complex, a finding not surprising considering that receptor sequestration is a multistep process, highly regulated by many molecules at different stages (Brodsky et al., 2001).

Taken together, these data show that overexpression of the PIK domain displaces endogenous PI3K from βARK1 leading to impairment of PI3K translocation to the receptor after agonist stimulation. The loss in receptor associated PI3K activity impairs the ability of the agonist-occupied receptor/PI3K complex to generate D-3 phospholipid molecules. We propose that the products of PI3K play a critical role in determining the dynamics of receptor endocytosis. Agonist-induced recruitment of class I PI3Ks by βARK1 to the receptor complex functions to increase the production of D-3 phospholipid molecules, that in turn regulates the recruitment of AP-2 and cargo (i.e., receptor/β-arrestin complex) to clathrin-coated pits on the membrane. The generation of PtdIns-3,4,5-P_3 by PI3K within the activated receptor complex promotes more efficient recruitment of AP-2 and receptor sequestration. The rise in the local concentration of PtdIns-3,4,5-P_3 within the receptor complex, which enhances the recruitment of AP-2 to the complex, likely plays a significant role in the initiation/nucleation of new clathrin-coated pits. The efficiency of clathrin coated pit formation will depend on the association of the various critical components that, in part, are regulated by their affinity to bind newly generated D-3 phospholipids.

Materials and methods

Cell culture

HEK 293 cells were maintained and transfected as previously described (Naga Prasad et al., 2001). Cells were harvested 24 h after transfection, replated in triplicate, allowed to grow overnight, and serum starved either overnight or 2–4 h before agonist stimulation. FLAG-PIK domain expressing permanent HEK 293 cell line was prepared by selecting cells against Geneticin (Life Technolgies) as described earlier (Laporte et al., 2000). All cells with stable FLAG-PIK expression showed both diffuse cytoplasmic distribution of PIK and inhibition of β_2AR-YFP internalization as visualized by confocal microscopy (unpublished data). HA-β_2AR and β-arrestin double stable cell lines were prepared by selecting the cells against two different antibiotics (Menard et al., 1997). Cell lines with stable expression of FLAG-β_2AR, a gift from Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC) and HA-β_2AR were used for the confocal experiments.

Plasmid constructs

PIK and PI3KΔPIK mutants of PI3K were prepared by PCR amplification using the full-length p110γ cDNA as template (Fig. 1 a). The PIK domain was amplified using *Pfu* platinum turbo *Taq* high fidelity enzyme (STRATAGENE) with the 5′ primer (5′-TCTCGAGGATCCGCCGCCATGGACTACA AGGACGACGATGATAAGCACCCGATAGCCCTGCCT-3′) containing XhoI and BamHI sites for subcloning, followed by Kozak consensus sequence and a FLAG epitope tag and the 3′ primer (5′-GTCGACCTAGTCGTGCAGCATGGC-3′) containing consensus stop codon with a SalI site for subcloning. The PCR product was subcloned in zero-blunt TOPO vector (Invitrogen) and sequence verified for authenticity. After digestion with the restriction enzymes BamHI and SalI, the PIK domain fragment was subcloned into the following expression plasmids: the pRK5 mammalian expression vector, the pGEX-4T1 bacterial expression vector to generate GST fusion proteins, and the EGFP vector pEGFP-C1.

PI3KΔPIK was constructed using two PCR reactions that selectively amplified upstream and downstream from the PIK domain (Fig. 1 a). PI3K upstream from PIK was amplified using the forward primer (5′-TGCGGATCCGCCACCATGGAGCTGGAGAACTATAAACAG-3′) containing a BamHI site and Kozak consensus sequence, and the reverse primer (5′-TTCTGCTCGACCGCGGTCCCCTTCCGG-3′) containing a SacI site. The region of PI3K downstream of the PIK domain was amplified using the forward primer (5′-CTGAGGGGCCGCGGCACAGCCATG-3′) containing a SacI site and the reverse primer (5′-ACCCGGGATCCTTAAGCGTAGTCTGGTACGT-3′) containing a BamHI site and a consensus stop codon. The upstream and downstream regions of PI3K were separately subcloned in the zero-blunt TOPO vector and sequence was verified by dideoxy sequencing. After digestion with the restriction enzymes BamHI/SacI, a three-fragment ligation was carried with the mammalian expression vector pRK5, to generate the plasmid containing the PI3KΔPIK cDNA (Fig. 1 a). The catalytic activity of PI3KΔPIK was indistinguishable from wild-type PI3K (unpublished data). The plasmids containing cDNAs encoding βARK1, FLAG-β2AR, HA-β2AR, and p110γ have been described previously (Naga Prasad et al., 2001). The PTEN plasmid was gift from Dr. Christopher Kontos (Duke University Medical Center).

GST fusion protein expression and pulldown experiments

Plasmid DNAs were transformed in *Escherichia coli* BL21 cells. Overnight cultures were grown in LB medium supplemented with ampicillin (100 μg/ml), diluted to an A_{600} of 0.2 in the same medium, and grown for another 1 h at 37°C. Cultured cells were then induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. Cells were then pelleted, washed once with PBS, and resuspended in PBS containing 1 mM PMSF, 2 mg/ml lysozyme, and incubated for 15 min on ice. Cells were lysed by adding Triton X-100 1%. Solublized cells were incubated with DNase (300 units) for 15 min on ice and centrifuged at 13,000 rpm for 10 min. Glutathione-Sepharose beads were added to the supernatant and gently agitated at 4°C for 2 h. Beads were washed three times with ice-cold PBS containing 1% Triton X-100 followed by three washes with cold PBS without detergent. Protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories), and the integrity of the fusion protein was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining.

GST fusion proteins (1–1.5 μg) on beads were incubated in 0.5 ml of binding buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.2% Triton X-100) for 2 h at 25°C together with purified βARK1 protein (5 μg). The beads were spun and washed three times with binding buffer followed by three washes with binding buffer without detergent. The beads were resuspended in SDS gel loading buffer and resolved by gel electrophoresis, immunoblotting and detection was carried out as described later.

Immobilized purified βARK1 protein was prepared by incubating βARK1 monoclonal antibodies with protein G agarose beads for 1 h at 4°C, followed by the addition of purified βARK1 protein. Subsequently, 10 μg of purified Gβγ was added to either βARK1 immobilized beads (5 μg), or the GST–PIK fusion protein beads (5 μg), and gently rocked for 45 min at room temperature. Beads were spun down, washed in binding buffer ×2, and resolved by gel electrophoresis. The presence of Gβγ was detected by immunoblotting with an antibody directed against the Gβ subunit. Purified Gβγ and βARK1 were gifts from Dr. Robert Lefkowitz.

Membrane fractionation

Membrane fractions were prepared as previously described (Naga Prasad et al., 2001). Briefly, cells were scraped in 1 ml of buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 2 μg/ml each leupeptin and aprotinin, and disrupted by using Dounce homogenizer. Intact cells and nuclei were removed by centrifugation at 1,000 *g* for 5 min. The supernatant was subjected to centrifugation at 38,000 *g* for 25 min. The pellet was resuspended in lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM sodium orthvanadate, and 2 μg/ml each leupeptin and aprotinin) and used as membrane fraction.

Lipid kinase assay

PI3K assays were carried out as previously described (Naga Prasad et al., 2001). Briefly, cells were lysed in lysis buffer in presence of protease inhibitors and membrane and cytosolic fraction was prepared as described above. 500 μg of membrane or cytosolic fraction was used for immunoprecipitation with either the C5/1 monoclonal antibody directed against βARK1 (Choi et al., 1997) or the anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) in presence of 35 μl of protein G-agarose (Life technologies). The samples were centrifuged at 10,000 rpm for 1 min and sedimented beads were washed once with lysis buffer, thrice with PBS containing 1% NP40 and 100 μM sodium-orthovanadate, three times with 100 mM Tris.Cl, pH 7.4, containing 5 mM LiCl and 100 μM sodium-orthovanadate, twice with TNE (10 mM Tris.Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 100 μM sodium-orthovandate). The last traces of buffer were completely removed using the insulin syringe and the pelleted beads were resuspended in 50 μl fresh TNE. To the resuspended pellet 10 μl of 100 mM $MgCl_2$ and 10 μl of 2 mg/ml PtdIns (20 μg) sonicated in TE (10 mM Tris.Cl, pH 7.4, and 1 mM EDTA) were added. The reactions were started by adding 10 μl of 440 μM ATP, 10 μCi ^{32}p γ ATP, and were incubated at 23°C for 10 min with continuous agitation. The reactions were stopped with 20 μl 6N HCl. Extraction of the lipids were done by adding 160 μl of chloroform:methanol (1:1) and the samples were vortexed and centrifuged at room temperature to separate the phases. 30 μl of the lower organic phase was spotted on to the 200-μ silica-coated flexi-TLC plates (Selecto-flexible; Fischer Scientific) precoated with 1% potassium oxalate. The spots were allowed to dry and resolved chromatographically with 2N glacial acetic acid:1-propanol (1:1.87). The plates were dried after resolution, exposed, and the autoradiographic signals were quantitated using Bio-Rad PhosphoImager. Lipid Preparation: PtdIns (Sigma-Aldrich or Avanti) or PtdIns-4,5-P_2 (Echelon) was dissolved in chloroform at a concentration of 10 mg/ml. 50 μl of this stock was dried down in a stream of air in a 1.5-ml Eppendorf tube. 250 μl of TE was added to the Eppendorf to bring the concentration to 2 mg/ml. The lipids were suspended by sonicating them in an ice bath for 5–10 min. Sonicated lipids were then added to each reaction. PtdIns(4)P (Sigma-Aldrich) or PtdIns-3,4,5-P_3 (Echelon) was used as a standard. The lipid standards were run as a separate lane on the TLC plate to identify the migration of PIP or PIP_3 (Renkonen and Luukkonen, 1976). TLC plates were stained with iodine to identify the formation lipids products (Renkonen and Luukkonen, 1976).

Immunoblotting and detection

Immunoblotting and detection of βARK1, FLAG-PIK, PI3KΔPIK-HA, FLAG-β_2AR, HA-β_2AR, β-adaptin, and clathrin were blotted as described previously (Laporte et al., 1999, 2000). Immunoprecipitating antibodies were added to 500 μg of cell lysate and the immune complexes were washed and resuspended in gel-loading buffer. Blots were incubated with antibodies recognizing β-adaptin, clathrin heavy chain (BD Transduction Laboratories), HA (Roche Molecular Biochemicals), and FLAG (Sigma-Aldrich) at a 1:2,000 dilution and the βARK1 monoclonal antibody at a 1:10,000 dilution. 50 μg of cell lysates were resolved by a 10% SDS-PAGE gel and transferred to PVDF membrane, phospho-PKB primary antibody was used at 1:1,000 dilution. Detection was carried out using enhanced chemiluminescence (Amersham Pharmacia Biotech). Densitometric analysis was carried out using Bio-Rad Flouro-S Multiimage software.

Determination of β2AR sequestration in HEK 293 cells by [^{125}I]-cyanopindolol binding

β_2AR sequestration was performed as previously described (Naga Prasad et al., 2001). HEK 293 cells were transfected with plasmids containing β_2AR (250 ng) or β_2AR (250 ng) and PIK domain (4 μg) cDNAs. Total binding was determined in the presence of 175 pM [^{125}I]-cyanopindolol (CYP) alone, 175 pM [^{125}I]-CYP plus 100 nM CGP12177 was used to determine internalized receptors and nonspecific binding was determined using 175 pM [^{125}I]-CYP plus 1 μM propranolol (Menard et al., 1997). Sequestration was calculated as the ratio of (specific receptor binding of [^{125}I]-CYP in the presence of CGP12177 and/or specific receptor binding of [^{125}I]-CYP in the absence of CGP12177).

Confocal microscopy in living and fixed cells

Confocal microscopy was carried out as previously described (Naga Prasad et al., 2001). HEK 293 cells were transfected with the plasmids containing cDNAs encoding either the β_2AR-YFP (2 μg) or β_2AR-YFP (2 μg) and PIK domain (4 μg) or β_2AR-YFP (2 μg) and PTEN (2 μg). Cells were plated onto glass-bottom dishes for observation in the confocal microscope. Live cells were treated with isoproterenol (10 μM) and images were collected sequentially over a time course of 0–10 min. For dual staining of β_2AR-HA or FLAG-PIK, cells were fixed in 4% paraformaldehyde in PBS for 30 min after 10 min of 10 μM isoproterenol stimulation. Cells were permeabilized with 0.1% Triton X-100 in PBS for 20 min, incubated in 1% BSA in PBS for 1 h. Cells were washed with PBS and incubated with anti-HA or anti-FLAG monoclonal antibody (1:250) with 1% BSA in PBS for 1 h. Cells were washed and incubated with goat anti–mouse IgG conjugated with Texas red (1:500; Molecular Probes) for 1 h. Samples were visualized using single sequential line excitation filters at 488 and 568 nm and emission filter sets at 505–550 nm for GFP detection and 585 for Texas red detection.

Treatment of cells with phospholipids

Modification of a previously described method was used to vary the concentration of D-3 phospholipids in living cells (Jones et al., 1999). Briefly, one of the synthetic phospholipids DiC16 PtdIns-3-P, DiC16 PtdIns-4,5-P_2, or DiCPtdIns-3,4,5-P_3 (AVANTI), was mixed with phosphatidylcholine and phosphoinositol (Sigma-Aldrich) at a 1:100:100 ratio and dried under N_2. Phospholipids were then re-suspended in 10 mM Hepes, pH 7.4, containing 1 mM EDTA and sonicated. Cells were treated with Saponin (0.04 mg/ml) in serum-free medium along with the vesicles containing the phospholipids at given concentration of PtdIns-3-P or PtdIns-4,5-P_2 or PtdIns-3,4,5-P_3 for 10 min at 25°C. Cells were then treated with isoproterenol (10 μM) for 5 min at 37°C, then lysed for immunoprecipitation experiments with a buffer containing 0.8% Triton X-100, 20 mM tris-HCl, pH 7.4, 300 mM NaCl, 1 mM EDTA, 20% glycerol, 0.1 PMSF, 10 μg/ml leupeptin and aprotinin.

Transferrin uptake

Transferrin uptake was carried out as described previously (van Dam and Stoorvogel, 2002). Briefly, HEK 293 cells were transfected with GFP-PIK (4 μg). 24 h after transfection, the cells were split into six glass-bottom petri dishes (Mat Tek Corporation). The following day the cells were serum starved for 1 h before transferrin treatment. Transferrin–Texas red conjugate was added to the cells at a final concentration of 33 μg/ml and incubated at 37°C for 30 min. After 30 min the cells were washed with PBS and fixed in 4% paraformaldehyde. Confocal microscopy was carried out on these cells as described earlier above.

Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using an unpaired Student's *t* test and analysis of variance where appropriate. Results for the β_2AR sequestration by CYP binding was analyzed using Graph-pad prism.

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