Different Mechanisms Regulate Lysophosphatidic Acid (LPA)-dependent Versus Phorbol Ester-dependent Internalization of the LPA1 Receptor*  

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Lysophosphatidic acid (LPA) stimulates cells by activation of five G-protein-coupled receptors, termed LPA1–5. The LPA1 receptor is the most widely expressed and is a major regulator of cell migration. In this study, we show that phorbol ester (PMA)-induced internalization of the LPA1 receptor requires clathrin AP-2 complexes, protein kinase C, and a distal dileucine motif (amino acids 352 and 353) in the cytoplasmic tail but not β-arrestin. Agonist-dependent internalization of LPA1, however, requires a cluster of serine residues (amino acids 341–347) located proximal to the dileucine motif, β-arrestin, and to a lesser extent clathrin AP-2. The serine cluster of LPA1 is required for β-arrestin2-GFP translocation to the plasma membrane and signal desensitization. In contrast, the dileucine motif (IL) is required for both basal and PMA-induced internalization. Evidence for the β-arrestin independence of PMA-induced internalization of LPA1 comes from the observations that β-arrestin2-GFP is not recruited to the plasma membrane upon PMA treatment and that LPA1 is readily internalized in β-arrestin1/2 knock-out mouse embryonic fibroblasts. These results indicate that distinct molecular mechanisms regulate agonist-dependent and PMA-dependent internalization of the LPA1 receptor.

Adaptor proteins that are involved in the clathrin-mediated internalization of GPCRs include clathrin, AP-2, and β-arrestins (2, 8, 9). β-Arrestins bind to many GPCRs (e.g. β2AR, LPA1, etc.) (2, 7) at specific serine/threonine residues, which have been preferentially phosphorylated by G-protein receptor kinases (2). Many GPCRs are also phosphorylated by second messenger kinases like PKA or PKC, which usually leads to desensitization or, in some cases, receptor degradation (10–12). Association of GPCRs with β-arrestins typically causes signal desensitization followed by receptor endocytosis (2, 8). Depending on the characteristics of agonist-dependent arrestin association, two classes of GPCRs have been proposed, class A and class B, which differ in their affinities for β-arrestin1 and -2 and in their spatiotemporal association (13).

Other adaptor proteins like clathrin AP-2 can directly mediate the internalization of some GPCRs (PAR-1 and thromboxane A2) (14–16). Several studies have shown that discrete amino acid motifs such as dileucine-based (IL/IL) and tyrosine-based (YXXβ) motifs, which are present in the cytoplasmic tails of GPCRs, mediate the direct interaction with clathrin and AP-2 to promote endocytosis (15, 17, 18).

We have been studying the trafficking of the most widely expressed GPCR for the serum phospholipid lysosphosphatidic acid (LPA), termed LPA1 (7, 19). LPA has growth factor-like properties and is involved in a variety of processes such as wound healing, cell proliferation and survival, neurite retraction, and cell migration (20). LPA stimulates these processes mainly through the activation of five distinct GPCRs, LPA1–5 (21, 22). We have previously shown that the LPA1 receptor utilizes a clathrin- and β-arrestin-dependent pathway for agonist-induced internalization (7, 19).

In this study, we investigated the amino acid sequences in the cytoplasmic tail of the LPA1 receptor that are involved in receptor internalization. Two distinct motifs are required for agonist-dependent versus agonist-independent internalization of the LPA1 receptor. A serine cluster (341SDRSASS347) in the C-terminal tail is required for β-arrestin association, signal attenuation, and subsequent endocytosis after LPA stimulation. A more distal dileucine motif (352IL353) is required for agonist-independent and phorbol ester-induced internalization of the LPA1 receptor. This type of internalization is independent of β-arrestin but requires the clathrin adaptor AP-2. This suggests that two distinct mechanisms regulate agonist-dependent and PMA-dependent internalization of the LPA1 receptor.
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MATERIALS AND METHODS

Antibodies and Reagents—Lyso-phosphatidic acid (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; LPA) was purchased from Avanti Polar Lipids (Alabaster, AL). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. Wild-type and mutant HA-tagged LPA₁ receptors were detected with mouse anti-HA antibodies (Covance, Berkeley, CA). myo-[³H]Inositol was purchased from American Radiolabeled Chemicals (St. Louis, MO); Gö 6976 and Gö 6983 were purchased from Calbiochem. Cy3 donkey anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch. The rabbit anti-AP-2 (µ2 subunit) antibody was kindly provided by Dr. Juan Bonifacino (NICHD, National Institutes of Health).

Plasmids—HA-LPA₁ plasmid was generated by PCR, using a previously described FLAG-LPA₁ plasmid as template (19). HA-tagged truncation mutant receptors, as described in the figure legends, were generated using the Gene-Tailor site-directed mutagenesis kit (Invitrogen), according to manufacturer’s instructions, with the HA-LPA₁ wild-type plasmid as template. All plasmids were subjected to DNA sequencing to confirm their sequences.

β-Arrestin GFP plasmids were kindly provided by Dr. Stefano Marullo.

Cell Culture and DNA Transfection—HeLa cells stably expressing HA-tagged LPA₁ receptor (termed LPA₁/HeLa cells), native HeLa cells, wild-type (WT) MEF, and β-arrestin1/2 KO MEF cells were maintained at 37 °C in 5% CO₂. These cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin (Media Tech, Herndon, VA), and 1 mM sodium pyruvate (BIOSOURCE) at 37 °C with 5% CO₂. Cells were grown on glass coverslips (for immunofluorescence) or grown in 24-well dishes (MatTek Corp., Ashland, MA). The cells were co-transfected with the plasmid DNA as described in the figure legends. For experiments with siRNA-mediated reduction of AP-2, LPA₁/HeLa cells were transfected with siAP-2 as described in the figure legends. The day before experimentation, the cells were serum-starved overnight. Cells were incubated with or without inhibitors as described in figure legends and were then transfected onto ice and incubated with mouse anti-HA primary antibodies (Covance) for 1 h to label cell surface HA-tagged LPA₁ (wild-type and mutant) receptors. Cells were then either left at 4 °C (total surface) or transferred to 37 °C and treated as described in the figure legends. After incubation at 37 °C, antibodies bound to the cell surface were removed by rinsing the cells with 100 mM glycine, 20 mM magnesium acetate, 50 mM KCl, pH 2.2 (acid wash) (24), for 90 s. For experiments with co-transfected HA-LPA₁ receptors and β-arrestin2 GFP, the surface antibodies were not removed by acid wash. Cells were rinsed and processed for immunofluorescence as described below.

Immunofluorescence—HA-LPA₁ receptors were detected using mouse anti-HA antibody (Covance). Immunofluorescence detection of AP-2 was done using a mouse anti-AP-2 antibody (AP-6) (Santa Cruz Biotechnology). For internalization assays, following anti-HA antibody incubations at 4 °C and treatments at 37 °C, the cells were then fixed in 2% formaldehyde in phosphate-buffered saline (PBS) for 10 min and rinsed with 10% fetal bovine serum containing 0.02% azide in PBS (PBS serum). For indirect immunofluorescence and AP-2 detection, following overnight serum starvation and subsequent treatments, cells were fixed and rinsed with PBS serum and were incubated with mouse anti-HA or anti-AP-2 antibodies diluted in PBS serum containing 0.2% saponin for 45 min. Following fixation and incubation with primary antibodies, the cells were washed three times with PBS serum and then incubated with fluorescently labeled donkey anti-mouse secondary antibodies (Jackson ImmunoResearch) diluted in PBS serum containing 0.2% saponin for 45 min, washed three times with PBS serum, washed once with PBS, and mounted on glass slides as described previously (19). Images were captured using a Hamamatsu digital camera mounted on a Leica Inverted microscope with a 100× oil immersion objective. Images were processed with Adobe Photoshop 7.0 software.

Quantification of Internalization—For internalization assays, images were taken using a Hamamatsu digital camera mounted on a Leica inverted microscope with a 100× oil immersion objective. The images were analyzed by Simple PCI software (Compix, Cranberry Township, PA), and total fluorescence for both internalized (vesicles/cell) and surface antibody levels were measured as described previously (25). Internalization (cell-associated fluorescence after acid wash) is expressed as a percentage of total fluorescence of initial surface-bound antibodies (4 °C) for both wild-type and mutant LPA₁ receptors.

Confocal Microscopy for β-Arrestin2-GFP Translocation Assay—To observe β-arrestin2 GFP translocation to the plasma membrane in response to LPA or PMA stimulation, HeLa cells were plated onto 35-mm glass bottom microwell dishes (MatTek Corp., Ashland, MA). The cells were co-transfected with HA-tagged LPA₁R and β-arrestin2-GFP and, after 24 h post-transfection, were treated with either 10 µM LPA or 1 µM PMA as described in the figure legends. The cells were then processed for immunofluorescence as described earlier. Images were taken using a Zeiss laser-scanning confocal microscope (LSM-510).

siRNA-mediated Reduction of AP-2—siRNA oligonucleotides to the µ-subunit of adaptin were purchased from Dharmacon (Lafayette, CO) and have been described previously (26). LPA₁/HeLa cells were transiently transfected with 175 pmol (12-well plate) or 300 pmol (6-well plate) of siRNA to AP-2 (siAP-2) using Lipofectamine 2000 (Invitrogen). The transfection medium was replaced with complete medium (without penicillin/streptomycin) 5 h later, and the cells were trypsinized after an additional 2 h and plated in a 6-well plate with (immunofluorescence) or without (immunoblotting) coverslips and then incubated for 16 h. The cells were transfected a second time as above, and the medium was then replaced with serum-free medium and incubated for an additional 16 h before experimentation.
Immunoblotting—Following serum starvation overnight, cells were solubilized by addition of lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium orthovanadate, 0.02% azide, 100 mg/ml leupeptin and 0.1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 60 min. The samples were then separated by 10% SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membrane was incubated with mouse anti-HA (detection of HA-LPA1 receptor) (Covance) or rabbit anti-AP-2 (detection of AP-2 μ2 subunit) (Dr. Juan Bonifacino) antibodies. The binding of primary antibodies was detected by using the West Pico enhanced chemiluminescence detection kit (Pierce).

Phosphoinositide Hydrolysis—HeLa cells were plated at a density of 4 × 10^4 cells/well into 24-well plates and transfected with plasmids encoding wild-type or mutant HA-LPA1, using Exgen500 (Fermentas). At 24 h post-transfection, cells were labeled overnight with myo-[3H]inositol in inositol- and serum-free medium, treated as described in the figure legends, and then processed for analysis of phosphoinositide hydrolysis by anion exchange chromatography as described (7).

Statistical Analysis—The data are expressed as the mean ± S.E. from the indicated number of independent experiments. Differences were analyzed by two-factor analysis of variance followed by a Tukey’s statistical significance test.

RESULTS

Differential Sensitivity of LPA-Versus PMA-induced Internalization of the LPA1, Receptor (LPA1,R) to PKC Inhibitors—We were interested in defining the molecular determinants of the LPA1,R that mediate its endocytic trafficking. In addition to LPA, a previous study indicated that the phorbol ester, PMA, induced PKC-dependent phosphorylation, signal desensitization, and internalization of the LPA1,R in C9 rat hepatoma cells (27). To determine whether PMA also induced LPA1,R endocytosis in HeLa cells, we compared the effects of LPA and PMA on the distribution of LPA1,R by using indirect immunofluorescence microscopy after fixation (Fig. 1A). To label endosomes, these cells were incubated with Alexa-labeled transferrin (Tfn) prior to fixation. In untreated cells, LPA1,Rs were localized predominantly to the plasma membrane and Alexa-Tfn-labeled punctate endosomal compartments. Following either LPA or PMA treatment, LPA1,Rs co-localized...
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with Alexa-Tfn in endosomal compartments, which indicated that both of these stimuli induced LPA$_1$R endocytosis. Interestingly, these LPA$_1$R$^+$ and Alexa-Tfn$^-$ endosomes had a more clustered appearance following PMA treatment.

To quantify LPA$_1$R endocytosis, we took advantage of the N-terminal HA epitope tag on LPA$_1$R, which is exposed to the medium at the cell surface, and used an HA antibody co-internalization assay (Fig. 1B). Mouse anti-HA antibodies were first bound to cells expressing HA-tagged LPA$_1$Rs at 4 °C, and the cells were either left at 4 °C (total surface) or treated with vehicle, LPA, or PMA at 37 °C. During incubation at 37 °C, the HA antibody was co-internalized along with the LPA$_1$R. The remaining surface-bound antibodies were removed with a mild acid wash, whereas the co-internalized antibody bound to the LPA$_1$R receptor was retained. At 4 °C, the anti-HA antibodies bound to LPA$_1$Rs localized to the plasma membrane (Fig. 1B, left panel). When these cells were warmed to 37 °C in the absence of any stimuli and then rinsed with a mild acid wash, very little anti-HA antibody was internalized (Fig. 1B, untreated, 2nd panel from the left). However, incubation with either LPA or PMA markedly increased internalization of the anti-HA antibody, which localized to endosomal-like structures.

To confirm the endosomal localization of the internalized anti-HA-bound LPA$_1$Rs, we repeated these experiments and included Alexa-Tfn during the incubations at 37 °C (Fig. 1C). In these experiments, the internalized anti-HA-bound LPA$_1$Rs extensively co-localized with Alexa-Tfn-labeled endosomes. The results of these anti-HA antibody internalization experiments gave the same results as those obtained by indirect immunofluorescence after treatment and fixation. The bound anti-HA antibody did not alter the pattern of internalization or the endosomal localization of LPA$_1$Rs, thus validating this antibody internalization assay to monitor LPA$_1$R internalization.

We quantified the amount of anti-HA antibody that was internalized relative to what was initially bound at 4 °C by using Simple PCI image analysis software as published previously (7) (Fig. 1D). Cells incubated in the absence of LPA and PMA only internalized about 9% of the initial bound anti-HA antibody (Fig. 1D, untreated, white bar). This represents the basal internalization of LPA$_1$Rs. Incubation with LPA for 30 min induced ~38% internalization of anti-HA antibody-bound LPA$_1$Rs (Fig. 1D, LPA, white bars) and PMA induced ~28% internalization of anti-HA-bound LPA$_1$Rs (PMA, white bars).

To test the role of PKC in LPA$_1$R internalization, we examined the effects of the PKC inhibitors Gö 6976, which inhibits both conventional PKCs and PKD/PKC$_{\mu}$ (28), and Gö 6983, which inhibits conventional and novel PKC isoforms but not PKD/PKC$_{\mu}$ (28, 29). Preincubation with 5 mM Gö 6976 (Fig. 1D, PMA, black bar), but not Gö 6983 (Fig. 1D, PMA, gray bar), inhibited PMA-induced internalization of LPA$_1$Rs by ~70%. Neither inhibitor inhibited LPA-induced internalization (Fig. 1D, LPA). These results indicated that PKC, and perhaps PKD/PKC$_{\mu}$, was required for PMA-induced but not LPA-induced internalization. This also raised the possibility that different mechanisms regulated LPA$_1$R internalization in response to these two stimuli.
ysis but was indistinguishable from WT LPA1Rs in basal activation. However, successive truncation of the cytoplasmic tail led to a progressive increase in LPA-induced PI hydrolysis (Fig. 2B, see Δ353, Δ347, and Δ340). These results indicated that the region between residues 340 and 361 dampened LPA signaling and suggest that these are important for signal attenuation.

**The Serine-rich Region Is Critical for β-Arrestin Association**—Because the region between residues 340 and 361 dampened LPA1R signaling, we hypothesized that this region was critical for signal desensitization and therefore β-arrestin interaction. We next sought to determine whether the serine-rich region is required for β-arrestin interaction. We have previously showed that LPA1Rs transiently (i.e. following 2 min of LPA stimulation) associate with β-arrestins at the plasma membrane and that this association is required for signal attenuation (7). In cells expressing LPA1Rs and β-arrestin2-GFP, the latter protein translocates to the plasma membrane and co-localizes with LPA1Rs and clathrin AP-2 complexes following a 2-min LPA stimulation (7).

To investigate the role of the cytoplasmic tail in LPA1R/β-arrestin interactions, HeLa cells were co-transfected with different HA-tagged LPA1Rs and β-arrestin2-GFP, stimulated with LPA for 2 min, and then analyzed by confocal fluorescence microscopy (Fig. 3A). In cells expressing WT, Δ347, Δ353, or Δ361 LPA1Rs, β-arrestin2-GFP translocated to the plasma membrane and co-localized with LPA1Rs (Fig. 3A, see arrows), after brief LPA stimulation (Fig. 3A, WT). In contrast, β-arrestin2-GFP failed to translocate to the plasma membrane in cells expressing Δ340 LPA1Rs (Fig. 3A, Δ340). This suggested that the serine-rich region between aa 340 and 347 is critical for interaction of LPA1Rs and β-arrestin2, but that neither the di-leucine motif nor the PDZ binding domain was required for β-arrestin recruitment.

We next quantified the percentage of cells where β-arrestin2-GFP co-localized with LPA1Rs following brief LPA stimulation (Fig. 3B). In the cells expressing WT, Δ353, and Δ361 LPA1Rs, β-arrestin2-GFP co-localized with LPA1Rs in ∼90% of the cells. In contrast, β-arrestin2 GFP marginally co-localized with LPA1Rs in only ∼10% of the cells expressing the Δ340 LPA1R. β-Arrestin2-GFP co-localized with the Δ347 LPA1R in 70% of the cells. These results suggest that the serine-rich region between aa 340 and 347 is required for β-arrestin interaction with the LPA1Rs.

**Different Motifs in the Cytoplasmic Tail of the LPA1 Receptor Are Required for LPA- Versus PMA-induced Endocytosis**—We next sought to examine the role of the different amino acid motifs in the cytoplasmic tail of LPA1R in either LPA-induced or PMA-induced endocytosis. HeLa cells were transiently transfected with different HA-tagged LPA1 receptors and tested for their ability to co-localize anti-HA antibody following stimulation with either 10 μM LPA or 1 μM PMA for 30 min (Fig. 4).

Following LPA treatment, WT, Δ347, Δ353, and Δ361 LPA1Rs internalized anti-HA antibody into endosomal compartments, which suggested that these mutant receptors retained the ability to undergo LPA-induced internalization (Fig. 4A, upper panels). In contrast, cells expressing Δ340 LPA1Rs did not internalize anti-HA antibodies, which suggested that this receptor was not internalized in response to LPA. Because Δ340 LPA1Rs lack the serine-rich domain, which is retained in all of the other receptor mutants, these results indicate that the serine-rich domain (aa, 340–347) is essential for LPA-induced endocytosis.

In contrast to LPA-induced internalization, neither the Δ340 nor Δ347 LPA1R mutants were internalized in response to PMA stimulation; only WT, Δ353, and Δ361 LPA1Rs were internalized upon PMA treatment (Fig. 4A, bottom panels). This indicated that the di-leucine motif (aa 352 and 353) was critical for PMA-induced internalization but not for LPA-induced internalization. MetaMorph quantification of anti-HA internalization indicated that both Δ340 and Δ347 LPA1Rs displayed reduced basal endocytosis in addition to the inhibition of LPA-induced or PMA-induced endocytosis, respectively (Fig. 4B). Taken together, these results indicated that the serine-rich domain in the cytoplasmic tail, which is important for

**Figure 3.** The serine-rich domain in the cytoplasmic tail of LPA1R is required for β-arrestin2-GFP translocation to the plasma membrane. A, HeLa cells were co-transfected with WT or mutant HA/LPA1R and β-arrestin2-GFP plasmids as described under "Materials and Methods.” Surface receptors were labeled with mouse anti-HA antibodies at 4 °C for 1 h. The cells were washed and then incubated in the absence (data not shown) or presence of 10 μM LPA for 2 min at 37 °C. The cells were fixed and processed for confocal fluorescence microscopy as described under "Materials and Methods.” Arrows point to regions of co-localization. A schematic diagram of the cytoplasmic tail is shown above the micrographs with the deletion mutants indicated. B, β-Arrestin2-GFP translocation to the plasma membrane after 2 min of LPA or PMA stimulation was quantified by scoring 50 cells for each receptor construct for co-localization with LPA1Rs. The data from four separate experiments were averaged and reported as the mean ± S.E. **, p < 0.01 in comparison to WT LPA1R-expressing cells.
β-arrestin interaction, mediates LPA-induced endocytosis of LPA1Rs but that a novel di-leucine motif, and perhaps also the serine-rich domain, is responsible for basal and PMA-induced internalization.

PMA-induced Internalization of the LPA1 Receptor Is β-arrestin-independent—The results of the previous experiment suggested that PMA-induced internalization might be independent of β-arrestins because Δ347 LPA1Rs, which can recruit β-arrestin to the plasma membrane and are able to undergo LPA-induced internalization, were not internalized in response to PMA.

To test this hypothesis, we asked whether PMA treatment induced β-arrestin2-GFP translocation to the plasma membrane using confocal fluorescence microscopy (Fig. 5). In untreated cells, as shown previously, LPA1Rs localized predominantly to the plasma membrane distribution, whereas β-arrestin2-GFP had a diffuse cytoplasmic distribution. After LPA treatment for 2 min, both the LPA1Rs and β-arrestin2-GFP co-localized to punctate structures at the plasma membrane (Fig. 5A, 2 min/LPA). Prolonged exposure to LPA for 30 min led to the endosomal localization of LPA1Rs and β-arrestin2-GFP localized to the cytoplasm (Fig. 5A, 30 min/LPA).

In contrast to LPA stimulation, treatment with PMA for 2 min did not lead to the recruitment of β-arrestin2-GFP to the plasma membrane (Fig. 5A, 2 min/PMA). Prolonged PMA treatment also led to the endosomal localization of LPA1Rs, whereas β-arrestin2-GFP remained in the cytoplasm (Fig. 5B, 30 min/PMA). We quantified the percentage of cells in which β-arrestin2-GFP was translocated from the cytosol to the plasma membrane after brief LPA or PMA stimulation (Fig. 5B). These results confirmed that β-arrestin2-GFP is recruited to the plasma membrane by LPA1Rs upon LPA treatment but not after PMA treatment. To further investigate whether PMA-induced internalization of LPA1Rs was β-arrestin-independent, we compared the internalization of WT LPA1Rs, after stimulation by LPA or PMA, in MEFs derived from β-arrestin1/2 double knock-out mice (Fig. 6).

We transiently transfected MEFs derived from wild-type mice (MEF WT) and those derived from β-arrestin1/2 KO mice (MEF KO) with WT and HA-tagged LPA1Rs and tested for their ability to internalize anti-HA antibody as before (Fig. 6). Cell surface LPA1Rs were readily labeled at 4 °C with anti-HA antibodies suggesting that LPA1Rs are transported to the plasma membrane in both MEF WT and MEF KO cells (Fig. 6, untreated, left panels). In the absence of stimuli, little to no anti-HA antibody was internalized in either MEF WT or MEF KO cells. Both LPA and PMA stimulation for 30 min induced LPA1R internalization in MEF WT cells. LPA-induced internal-
ization of LPA₁₇Rs was blocked in MEF KO cells (Fig. 6), as we have previously shown (7). Remarkably, PMA stimulation was able to induce robust LPA₁₇R internalization in MEF KO cells. Taken together, these results indicate that PMA-induced internalization of LPA₁₇Rs is independent of β-arrestins.

Basal and PMA-induced Endocytosis of LPA₁₇Rs Is Sensitive to siRNA Depletion of Clathrin AP-2—Several studies have shown that tyrosine- and dileucine-based motifs can interact with the clathrin adaptor protein AP-2 and mediate internalization of receptors (32). We have shown that PMA-induced internalization of the LPA₁₇Rs is β-arrestin-independent but requires a dileucine-based motif in the cytoplasmic tail (Figs. 4–6). Therefore, we asked whether clathrin AP-2 was an alternate adaptor protein for PMA-induced internalization of LPA₁₇Rs. We used siRNA to reduce AP-2 protein levels in HeLa cells and to determine its effects on LPA₁₇R internalization. Treatment of cells with siAP-2 but not siControl RNA reduced the abundance of AP-2 by more than 70% (Fig. 7B). Immunofluorescence labeling confirmed the reduction of AP-2 in cells (Fig. 7A). We tested the effects of siAP-2 or siControl RNA on anti-HA antibody internalization (Fig. 7C). Unstimulated cells exhibited a 16% basal internalization of LPA₁₇R, which was inhibited by ~85% after siAP-2 treatment (Fig. 7C, untreated). Likewise, cells treated with PMA showed an ~28% internalization, which was also inhibited by ~86% following siAP-2 treatment. LPA stimulation led to ~33% internalization, and siAP-2 treatment led to a partial inhibition of LPA₁₇R internalization (~50% inhibition). These results indicated that both basal and PMA-induced internalization of LPA₁₇Rs was strongly inhibited by depletion of AP-2, whereas LPA-induced internalization was only partially inhibited.

**DISCUSSION**

The endocytosis and intracellular trafficking of GPCRs is an important mechanism by which cells modulate their responsiveness toward a variety of extracellular stimuli. Studies of a variety of GPCRs have identified specific amino acid sequences in the cytoplasmically exposed domains of GPCRs that are critical for receptor endocytosis (33, 34).

In this study, we investigated role of sequences within the cytoplasmic tail of the lysophosphatidic acid receptor, LPA₁₇R, on its basal, LPA-induced, and PMA-induced endocytosis. LPA-induced internalization and signal attenuation was dependent upon β-arrestins and upon a serine-rich domain in the cytoplasmic tail. This domain was also essential for β-arrestin interaction with LPA₁₇Rs. In contrast, both basal and PMA-induced endocytosis were dependent upon clathrin AP-2 adaptors and a di-leucine sequence, distal to the serine-rich domain. Unlike LPA stimulation, PMA stimulation did not induce the recruitment of β-arrestins from the cytoplasm to the plasma membrane. Furthermore, PMA-induced internalization was not dependent upon β-arrestins. These findings indicate that distinct mechanisms regulate basal and PMA-induced versus LPA-induced endocytosis of LPA₁₇Rs.

**Molecular Determinants of Agonist-induced LPA₁₇R Endocytosis**—Inspection of the cytoplasmic tail of LPA₁₇Rs indicated the presence of several distinct amino acid motifs as follows: a di-cysteine palmitoylation motif, a serine-rich domain, a di-leucine motif, and a terminal PDZ-binding domain (Fig. 2A). Analysis of a panel of LPA₁₇R truncation mutants indicated that LPA-induced internalization of LPA₁₇Rs required the serine-rich domain but not the di-leucine motif or the PDZ-binding domain (Fig. 4). The serine-rich domain was also required for LPA-induced recruitment of β-arrestin to the plasma membrane (Fig. 3). Interestingly, progressive truncation of the LPA₁₇R from the C-terminal PDZ binding domain toward the

*Figure 6. PMA-induced internalization of LPA₁₇R is β-arrestin-independent. Wild-type (MEF WT) or β-arrestin1/2 double knock-out cells (MEF KO) were transfected with WT HA-LPA₁₇R plasmid, and surface receptors were labeled with mouse anti-HA antibodies at 4 °C for 1 h. The cells were washed and were either left at 4 °C or transferred to 37 °C in the presence or absence of 10 µM LPA or 1 µM PMA for 30 min at 37 °C. Any remaining surface-bound antibodies were removed by mild acid wash, and the cells were fixed and processed for immunofluorescence staining as described under “Materials and Methods.”*
serine-rich domain led to a corresponding increase in the magnitude of LPA-induced PI hydrolysis (Fig. 2B). We hypothesize that efficient β-arrestin interaction with LPA1Rs requires both the serine-rich domain and additional amino acids distal to this region. This serine-rich domain (SRSASSH47) bears a resemblance to the serine clusters found in GPCRs that form stable interactions with β-arrestins, which endure even upon delivery to endosomes (30). However, the interaction between LPA1Rs and β-arrestins appears to be transient as β-arrestins are recruited to the plasma membrane by LPA1Rs following a 2-min stimulation with LPA but return to the cytoplasm by 30 min, a time when LPA1Rs localize to endosomes. These results are consistent with the well-established model for ligand-induced GPCR internalization where G protein receptor kinases phosphorylate specific serine or threonine residues within the third intracellular loop or cytoplasmic tail of GPCRs, which in turn promotes the binding of β-arrestins (35). β-Arrestin binding attenuates G protein signaling and targets the GPCR to clathrin-coated pits for endocytosis.

We have previously shown that both clathrin and β-arrestins are critical for agonist-induced LPA1R endocytosis (7). β-Arrestin binds directly to clathrin heavy chain and to clathrin AP-2 (8). siRNA knockdown of clathrin heavy chain inhibited LPA1R internalization by more than 90% (7). However, we found that siRNA knockdown of clathrin AP-2 only inhibited agonist-induced LPA1R internalization by ~50% (Fig. 7). Previous studies have shown that only a subset of all clathrin-coated pits contain AP-2 (26). Alternative adaptor proteins such as AP-180 and CALM also mediate clathrin-coated pit assembly (36). Thus, our results indicating only a partial inhibition of LPA1R internalization upon knockdown of AP-2 is consistent with a partial depletion of clathrin-coated pits. We hypothesize that the remaining AP-2-independent clathrin-coated pits mediate the agonist-induced LPA1R internalization by virtue of the β-arrestin-clathrin interaction.

PMA Stimulation of LPA1R Endocytosis—Avendano-Vazquez et al., (2005) previously showed that, in addition to LPA, PMA stimulated the phosphorylation and endocytosis of LPA1Rs in rat hepatocytes (27). We confirmed these observations in HeLa cells, where PMA also stimulated the endocytosis of LPA1Rs. Similar to Avendano-Vazquez et al. (27), we found that bisindolylmaleimide I also inhibited PMA-induced internalization of LPA1Rs.3 We further investigated the role of PKC by using the inhibitors Gö 6976, which inhibits conventional PKC isoforms and inhibits PKD/PKCμ, but not by Gö 6983, which inhibits conventional and novel PKC members but not PKD/PKCμ (29). Surprisingly, we found that PMA-induced internalization was inhibited by Gö 6976 but not by Gö 6983 (Fig. 1D). This suggests that PKD/PKCμ may be important for PMA-induced LPA1R internalization; however, its precise role in LPA1R trafficking will require additional studies. Interestingly, PKD/PKCμ is activated by LPA signaling in intestinal epithelial cells via a pertussis toxin-sensitive pathway (37). PKD/PKCμ is known to regulate trans-Golgi network-to-plasma membrane transport, by promoting the fission of transport vesicles, and is implicated in the membrane recycling of integrins (38, 39).

Phorbol esters stimulate the production of LPA in cervical and ovarian cancer cells (40). Thus, it is a formal possibility that PMA-induced internalization of LPA1Rs is simply because of the stimulation of LPA secretion by cells, which in turn is expected to bind to LPA1Rs and stimulate internalization. However, if this scenario were true, then one would expect that PMA-induced internalization exhibits the same requirements as LPA-induced internalization (e.g. β-arrestin dependence, amino acid motifs, and sensitivity to AP-2 depletion). Because our data indicate that there are distinct differences in the requirements for LPA- versus PMA-stimulated internalization of LPA1Rs (Figs. 4–7), we believe that LPA and PMA promote LPA1R internalization through distinct mechanisms. Several studies have shown a role for PKC in heterologous desensitization and internalization of GPCRs such as purinergic P2Y1 receptors, sphingosine 1-phosphate binding S1P1 receptors, and gastrin-releasing peptide receptors (10, 41–43). In the case of S1P1 receptors, which belong to the lysophospholipid-binding family of GPCRs, both G-protein receptor kinase-dependent and PKC-dependent mechanisms regulate the agonist-dependent and agonist-independent endocytosis of these receptors, respectively (10). Our studies show a second member of the lysophospholipid-binding family of GPCRs, the LPA1 receptor, also utilizes distinct mechanisms for agonist-dependent and agonist-independent internalization.

Molecular Determinants of Basal and PMA-induced LPA1R Endocytosis—Surprisingly, we found that both basal and PMA-induced endocytosis of LPA1Rs required the distal di-leucine motif (Fig. 4). We also found that PMA did not promote β-arrestin recruitment to the plasma membrane and that PMA stimulated LPA1R endocytosis in β-arrestin1/2 double knock-out MEFs (Figs. 5 and 6). Thus, we conclude that PMA-induced endocytosis of LPA1Rs is independent of β-arrestins. Many receptors that utilize di-leucine-based internalization motifs directly bind to clathrin AP-2 adaptor complexes (15, 44). GPCRs such as the human cytomegalovirus-US28 receptor, which uses a di-leucine internalization motif, and α1β-adrenergic receptors, which does not use a di-leucine motif, directly bind to AP-2 adaptors during their endocytosis (23, 45). In addition, the thrombin receptor PAR1 has been shown to bind to AP-2 during thrombin-activated endocytosis; however, the interaction with AP-2 is mediated by a tyrosine-based endocytic motif (15).

We hypothesized that clathrin AP-2 may serve as an alternate adaptor to facilitate PMA-induced endocytosis of LPA1Rs. Indeed, depletion of AP-2 through siRNA knockdown strongly inhibited PMA-induced endocytosis of LPA1Rs (Fig. 7). Surprisingly, AP-2 depletion strongly inhibited basal endocytosis of LPA1Rs as well, suggesting that basal and PMA-induced endocytosis may utilize similar endocytic mechanisms.

Why do LPA1Rs utilize two different adaptor complexes for endocytosis? During LPA-stimulated internalization, β-arrestin binding serves to attenuate receptor signaling and secondarily promotes clathrin-dependent endocytosis. However, during basal or PMA-induced endocytosis, β-arrestin does not engage LPA1Rs. Because β-arrestin binding is preceded by
G-protein receptor kinase phosphorylation of agonist-bound receptors, it is likely that basal and PMA-stimulated endocytosis is more efficiently facilitated through the direct interaction between the di-leucine motif of LPA₁Rs and clathrin AP-2.

We have shown here that distinct amino acid motifs within the cytoplasmic tail of LPA₁Rs as well as distinct clathrin adaptor proteins are responsible for agonist-dependent and independent endocytosis. These observations reveal an additional layer of regulation for LPA₁Rs. Future studies should provide details about the functional significance that these two endocytic mechanisms may provide to LPA receptor signaling.

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