A requirement for membrane cholesterol in the β-arrestin- and clathrin-dependent endocytosis of LPA_1 lysophosphatidic acid receptors

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Summary

Lysophosphatidic acid (LPA) stimulates heterotrimeric G protein signaling by activating three closely related receptors, termed LPA_1, LPA_2 and LPA_3. Here we show that in addition to promoting LPA_1 signaling, membrane cholesterol is essential for the association of LPA_1 with β-arrestin, which leads to signal attenuation and clathrin-dependent endocytosis of LPA_1. Reduction of clathrin heavy chain expression, using small interfering RNAs, inhibited LPA_1 endocytosis. LPA_1 endocytosis was also inhibited in β-arrestin 1 and 2-null mouse embryo fibroblasts (β-arrestin 1/2 KO MEFs), but was restored upon re-expression of wild-type β-arrestin 2. β-arrestin attenuates LPA signaling as LPA_1-dependent phosphoinositide hydrolysis was significantly elevated in β-arrestin 1/2 KO MEFs and was reduced to wild-type levels upon re-expression of wild-type β-arrestin. Interestingly, extraction of membrane cholesterol with methyl-β-cyclodextrin inhibited LPA_1 signaling, β-arrestin membrane recruitment and LPA_1 endocytosis. Cholesterol repletion restored all of these functions. However, neither the stimulation of phosphoinositide hydrolysis by the M_1 acetylcholine receptor nor its endocytosis was affected by cholesterol extraction. LPA treatment increased the detergent resistance of LPA_1 and this was inhibited by cholesterol extraction, suggesting that LPA_1 localizes to detergent-resistant membranes upon ligand stimulation. These data indicate that although LPA_1 is internalized by clathrin- and β-arrestin dependent endocytosis, membrane cholesterol is critical for LPA_1 signaling, membrane recruitment of β-arrestins and LPA_1 endocytosis.

Key words: LPA, β-arrestin, Lipid raft, Endocytosis, G-protein-coupled receptor

Introduction

Lysophosphatidic acid (LPA, 1-acyl-2-lyso-sn-glycero-3-phosphate) is an abundant serum mitogen that evokes growth-factor-like responses in many cell types through activation of G-protein-coupled receptors (GPCR) (Moolenaar, 1999). LPA signaling affects a variety of cellular functions including: growth stimulation (cell proliferation and cell survival) (Fang et al., 2000; Goetzl et al., 2000; van Corven et al., 1992), induction of cytoskeletal rearrangements via Rho GTPases (Ridley and Hall, 1992), stimulation of serum-responsive genes (Hill et al., 1995), neurite retraction (Jalink et al., 1994), promotion of tumor cell migration/invasion (Stam et al., 1998) and the secretion of peptide growth factors (Hu et al., 2001; Pustilnik et al., 1999; Schwartz et al., 2001). Most of the effects of LPA are mediated through the activation of three members of the endothelial differentiation gene superfamily of receptors: LPA_1, LPA_2 and LPA_3 (Hla et al., 2001; Moolenaar, 1999). Upon LPA binding, both LPA_1 and LPA_2 activate the G_i, G_q and $G_{12/13}$ families of heterotrimeric G proteins; LPA_3 only activates G_i and G_q (Ishii et al., 2000). In addition to these well-characterized GPCRs, LPA also stimulates the orphan receptor, GPR23/LPA_4 (Noguchi et al., 2003) and the non-GPCR target, peroxisome proliferator-activated receptor γ (McIntyre et al., 2003). Given the complexity of cellular responses to LPA signaling and the potential role of LPA receptor subtypes in various cancers (Mills and Moolenaar, 2003), it is important to understand the mechanisms that regulate the activity of individual LPA receptors.

Upon agonist stimulation, most GPCRs are rapidly internalized into cells through a variety of different endocytic pathways. This facilitates either receptor downregulation or receptor resensitization (Marchese et al., 2003). Agonist stimulation usually leads to the rapid phosphorylation of serine/threonine residues located within cytoplasmically exposed regions of GPCRs (Ferguson et al., 1995). This subsequently induces the binding of β-arrestin proteins, which results in signal attenuation and often targets the GPCR to clathrin-coated pits for endocytosis (Lefkowitz and Shenoy, 2005). Internalized GPCRs transit through the endosomal system and are either sorted to lysosomes for degradation or become dephosphorylated by membrane-associated phosphatases and are recycled back to the plasma membrane (Ferguson, 2001). In addition to clathrin-mediated endocytosis, many GPCRs utilize a variety of clathrin-independent

internalization mechanisms including cholesterol-dependent pathways such as caveolae (Chini and Parenti, 2004). Also, β-arrestins are not universally required for GPCR endocytosis as shown for the thrombin receptor, PAR1, whose association with β-arrestins is required for signal attenuation but not for its endocytosis (Paing et al., 2002). Thus, the mechanisms that regulate both signal attenuation and receptor endocytosis can vary from one GPCR to another.

We have previously shown that LPA_1 is probably internalized by clathrin-dependent endocytosis as dominant-negative mutants of dynamin 2 (K44A) and Rab 5 (S34N), which regulate clathrin-dependent trafficking, strongly inhibited LPA_1 endocytosis (Murph et al., 2003). However, a recent study showed that LPA stimulation of the phosphoinositide 3-kinase (PI3-K)/Akt pathway was dependent upon membrane cholesterol (Peres et al., 2003) suggesting a positive role for cholesterol-rich plasma membrane microdomains in LPA signaling. As cholesterol-enriched microdomains, such as caveolae, can also mediate receptor endocytosis, it is not clear what the relationship is between LPA signaling from cholesterol-rich microdomains and the endocytosis of LPA receptors. To address this question and to gain a better understanding about the regulation of LPA receptors, we investigated the role of membrane cholesterol, β-arrestins and clathrin in the signaling and endocytosis of the ubiquitously-expressed LPA_1 receptor.

Materials and Methods

Antibodies and reagents

Lysophosphatidic acid (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; LPA) was purchased from Avanti Polar Lipids (Alabaster, AL). Isoproterenol and cytochalasin D was obtained from Sigma Chemical Co. (St Louis, MO) and carbachol from Fluka Chemika-Biochemika. FLAG-tagged LPA_1 receptors were detected with mouse anti-FLAG antibodies (Sigma, St Louis, MO); HA-tagged β_2AR and HA-tagged M_1 muscarinic acetylcholine receptor (mAChR) were detected with mouse anti-HA antibodies (Covance, Berkeley, CA). Alexa 488-labeled transferrin (Alexa 488-Tfn), Alexa 594- and Alexa 488-conjugated goat anti-mouse were purchased from Molecular Probes (Eugene, OR). Monoclonal antibodies to clathrin heavy chain and monoclonal anti-actin antibodies were purchased from BD Transduction labs (San Jose, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Mouse anti-AP2 antibodies were purchased from Affinity Bioreagents (Golden, CO). FITC-labeled anti-CD59 was obtained from Chemicon (Temecula, CA). Methyl-β-cyclodextrin and water-soluble cholesterol complexes were purchased from Sigma. myo-[^{3}H]inositol was purchased from American Radiolabeled Chemicals (St Louis, MO).

Cell culture and DNA transfection

HeLa cells stably expressing the LPA_1 receptor (termed LPA_1/HeLa cells), native HeLa cells, wild-type (WT) MEF and β-arrestin 1/2 KO MEF cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin (Media Tech, Herndon, VA) and 1 mM sodium pyruvate (Biosource International, Camarillo, CA) at 37°C with 5% CO_2. Cells were grown on glass coverslips (for immunolocalization) and transfected in six-well dishes, or were grown in 24-well dishes (for *myo*-[^{3}H]inositol labeling) using Lipofectin or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's directions. Plasmids encoding HA-tagged β_2AR, β-arrestin 1-GFP, β-arrestin-2-GFP, HA-tagged M_1 mAchR were transiently transfected at 1.0 μg/well (in six-well plates) and have been previously described (Paing et al., 2002; Scott et al., 2002).

siRNA-mediated reduction of clathrin

siRNA oligonucleotides to clathrin were purchased from Dharmacon (Lafayette, CO) and have been described previously (Motley et al., 2003). LPA_1/HeLa cells were transiently transfected with 300 pmol (10 cm dish) or 100 pmol (24-well plate) of siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection medium was replaced with complete medium (without penicillin/streptomycin) 5 hours later and the cells were incubated for 16 hours. The cells were transfected a second time as above and the medium was then replaced with serum-free medium (SFM) and incubated for an additional 16 hours before experimentation.

Indirect immunofluorescence

Cells were treated as described in the figure legends, 24-48 hours after transfection. Cells were then fixed in 2% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes and rinsed with 10% fetal bovine serum (FBS) containing 0.02% azide in PBS (PBS-serum). Fixed cells were incubated with primary antibodies diluted in PBS-serum containing 0.2% saponin for 45 minutes and then washed (three times, 5 minutes each) with PBS-serum. The cells were then incubated in fluorescently labeled secondary antibodies diluted in PBS-serum containing 0.2% saponin for 45 minutes, washed three times with PBS-serum, washed once with PBS and mounted on glass slides as previously described (Murph et al., 2003).

For Alexa 594-Tfn and FITC-labeled anti-CD59 internalization, LPA_1/HeLa cells were briefly rinsed three times with 0.5% bovine serum albumin (BSA) in SFM and incubated in the same medium for 30 minutes at 37°C. The cells were then incubated with Alexa 594-conjugated human transferrin (50 μg/ml) or FITC-conjugated anti-CD59 (1 μg/ml) for 30 minutes at 37°C in the presence or absence of 10 μM LPA. Antibodies bound to the cell surface were removed by rinsing the cells with 0.5% acetic acid, 0.5 M NaCl, pH 3.0 solution (for Alexa 488-Tfn) or 100 mM glycine, 20 mM magnesium acetate, 50 mM KCl, pH 2.2 (for FITC anti-CD59) (Naslavsky et al., 2004). Cells were rinsed in complete medium, fixed and processed for fluorescence microscopy. For assessing uptake in the presence of methyl-β-cyclodextrin (5 mM) or nystatin (50 μg/ml), cells were pre-treated with DMEM supplemented with 0.5% BSA, with or without drugs, for 60 minutes prior to antibody and/or LPA addition. All images were acquired using an Olympus BX40 epifluorescence microscope equipped with a 60× Planapo lens and photomicrographs were prepared using an Olympus MagnaFire SP digital camera (Olympus America, Melville, NY). Images were processed with Adobe Photoshop 6.0 software.

Quantification of LPA_1 colocalization with internalized Alexa-Tfn

Stably-transfected LPA_1/HeLa cells or transiently-transfected HeLa cells expressing M_1 mAChRs were grown on glass coverslips and treated with MβCD and/or water-soluble cholesterol as described in the figure legends. The cells were then incubated with 50 μg/ml Alexa 594-Tfn for 30 minutes in the presence or absence of 10 μM LPA or 1 mM carbachol, respectively. The cells were rinsed with a mild acid wash as described above, fixed with 2% formaldehyde in PBS and processed for immunofluorescence localization of LPA_1 using M1 mouse anti-FLAG IgG or M_1 mAChR using mouse anti-HA IgG followed by Cy2 secondary antibodies. The extent of LPA_1 or M_1 colocalization with internalized Alexa 594-Tfn was determined by quantifying the extent of pixel colocalization of GPCR staining with Alexa 594-Tfn fluorescence using Metamorph Imaging software (Universal Imaging, West Chester, PA) as described (Murph et al., 2003; Volpicelli et al., 2001). The background was subtracted from

unprocessed images and the percentage of GPCR pixels that overlapped with Alexa-Tfn pixels was measured. The data is presented as the mean±s.e.m. of measurements from 20 cells per sample from a representative experiment that was performed three independent times with similar results.

Immunoblotting

Following 72 hours of siRNA treatment, cells were solubilized by addition of lysis buffer (1% NP-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 M sodium orthovanadate, 0.02% azide, 100 μg/ml leupeptin and 0.1 mM PMSF) and incubated on ice for 60 minutes. The samples (12 μg protein per lane) were then separated by 10% SDS-PAGE and transferred to nitrocellulose. Clathrin heavy chain was detected using mouse anti-clathrin antibodies and actin was detected using monoclonal anti-actin antibodies. The binding of primary antibodies was detected by using an enhanced chemifluorescence detection kit (Amersham Biosciences, Piscataway, NJ).

Phosphoinositide hydrolysis

LPA_1/HeLa cells or mouse embryo fibroblasts derived from wild-type or β-arrestin 1/2 null mice were plated at a density of 4.0×10^4 cells/well into 24-well plates and transfected with plasmids encoding wild-type LPA_1 or M1 mAChRs alone or in combination with plasmids encoding wild-type β-arrestin 2 using Lipofectamine 2000. Transient transfection of plasmids encoding M_1 mAChRs was performed by using Lipofectin reagent. At 24 hours post-transfection, cells were labeled overnight with myo-[^{3}H]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 (Paing et al., 2002).

Triton X-100 extraction of cells

LPA_1/HeLa cells were plated onto glass coverslips in 35 mm dishes at a density of 0.2×10^6 cells per plate. After allowing cells to attach for 24 hours, the medium was changed to serum-free medium and the cells were incubated overnight (~16 hours). The following day, the cells were treated as described in the figure legends and subsequently incubated with ice-cold 1% Triton X-100 in PBS on ice for 3 minutes prior to fixation with ice-cold 2% formaldehyde in PBS. LPA_1 or was localized using indirect immunofluorescence microscopy. To monitor the fate of surface LPA_1, LPA_1/HeLa cells were incubated on ice with mouse anti-FLAG antibodies for 30 minutes after LPA treatment to label only surface LPA_1. These cells were then extracted with 1% Triton X-100, fixed and processed for immunofluorescence localization as described above. Relative receptor expression was quantified by measuring receptor pixel intensity using MetaMorph imaging software and was normalized to DNA content, labeled with Hoescht dye.

Whole-cell ELISA quantification of surface LPA_1

LPA_1/HeLa cells were plated in 24-well dishes (Falcon) at a density of 0.4×10^5 cells per well and grown overnight. Cells were then transiently transfected with no siRNA or with 100 pmol/well of clathrin-specific siRNA (Motley et al., 2003) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After 24 hours, the cells were again transfected with 100 pmol/well of clathrin-specific siRNA or no siRNA. 24 hours later, the cells were incubated in the presence or absence of 10 μM LPA for 45 minutes and fixed in 2% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes and rinsed with 10% fetal bovine serum (FBS), containing 0.02% azide, in PBS (PBS-serum). Fixed cells were incubated with mouse anti-M1 FLAG primary antibody diluted in PBS-serum (250 μl/well) for 1 hour and then washed (three times, 5 minutes each) with PBS-serum. The cells were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Pierce Biotechnology, Rockford, IL) diluted in PBS-serum (250 μl/well) for 1 hour, washed three times with PBS-serum and washed three times with PBS. The cells were then incubated for 1 hour at 37°C with ABTS (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) (Pierce Biotechnology). A 200 μl aliquot was then removed from each well, transferred to a 96-well plate and the absorbance read at 405 nm (corrected for blank). Internalization is expressed as the percent difference in surface LPA_1 between unstimulated cells and agonist-stimulated cells. The data are the mean±s.e.m. of six replicates/siRNA sample combined from two independent experiments.

Cholesterol measurements

HeLa cells, stably expressing FLAG-tagged LPA_1, were seeded in six-well plates at a density of 0.5×10^6 cells per well, allowed to attach overnight and then incubated with serum-free DMEM for 24 hours prior to treatment. The cells were treated for 60 minutes, as described in the figure legend, rinsed twice with ice-cold PBS (pH 7.4) and then solubilized in ice-cold PBS (pH 7.4) containing 1% Triton X-100 and protease inhibitor cocktail (2 mM AEBSF, 1 mM EDTA, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin and 0.3 μM aprotinin). Total cellular cholesterol was quantified using an Amplex Red Cholesterol Assay Kit (Molecular Probes; Eugene, OR), as indicated by the manufacturer. Briefly, cholesterol esters in the cell extracts are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield H_2O_2, which is detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). In the presence of horseradish peroxidase (HRP), Amplex Red reacts with H_2O_2 to produce fluorescent resorufin. Fluorescence was measured with a fluorescence microplate reader using excitation at 560 nm and fluorescence detection at 590 nm and total cholesterol was calculated from a standard curve using purified cholesterol. Cellular cholesterol was normalized to total protein concentration, which was quantified by BCA Protein Assay (Pierce Biotechnology).

Statistical analysis

The data is expressed as the mean±s.e.m. from the indicated number of independent experiments performed in triplicate. Differences were analyzed by two-factor ANOVA followed by a Tukey's statistical significance test.

Results

LPA_1 is internalized by clathrin-mediated endocytosis

Our previous work suggested that LPA_1 was internalized by clathrin-dependent endocytosis based on the inhibitory effects of mutant dynamin 2 K44A and Rab 5 S34N (Murph et al., 2003). However dynamin K44A can also inhibit endocytosis from cholesterol-rich caveolae (Damke et al., 1994; Henley et al., 1998; Murph et al., 2003). To directly test whether LPA_1 used a clathrin-dependent pathway, we determined the effects of reducing the cellular abundance of the clathrin heavy chain, using small interfering RNAs (siRNAs), on the endocytosis of LPA_1. Fig. 1A shows the distribution of FLAG-tagged LPA_1 in stably transfected HeLa cells (LPA_1/HeLa cells). In untreated cells, LPA_1 is localized predominantly at the plasma membrane and to a lesser extent, at the Golgi complex, which probably represents newly synthesized LPA_1 en route to the plasma membrane. Treatment with 10 μM LPA results in a redistribution of LPA_1 to numerous punctate structures, which

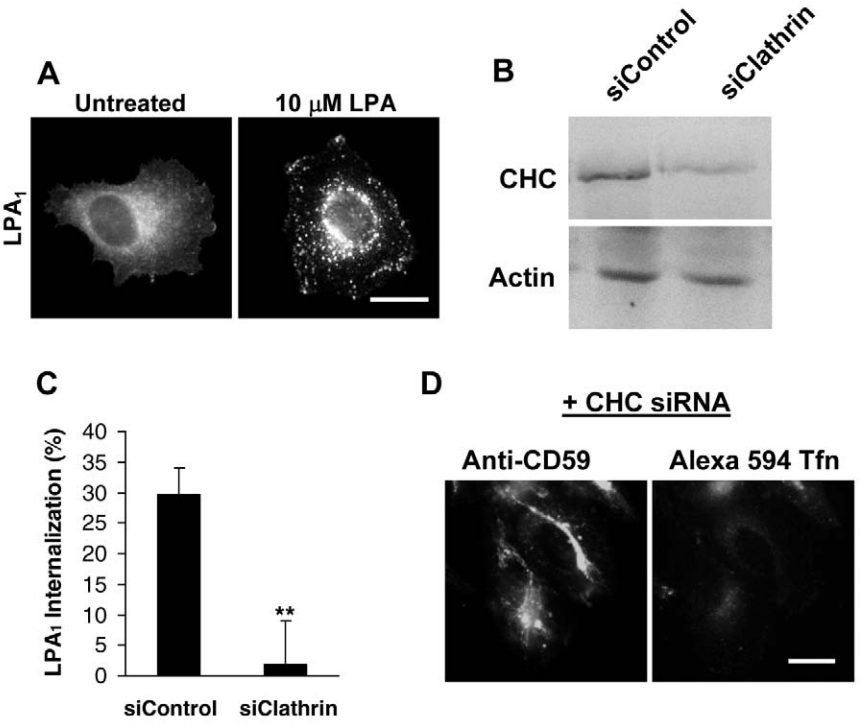


Fig. 1. siRNA-mediated reduction of clathrin inhibits agonist-induced endocytosis of LPA_1. (A) Stably-transfected LPA_1/HeLa cells were incubated in the presence or absence of 10 μM LPA for 30 minutes, fixed and processed for immunofluorescence detection of FLAG-tagged LPA_1 with M1 mouse anti-FLAG antibodies and fluorescently-labeled secondary antibodies. (B) Cell lysates were prepared from stably-transfected LPA_1/HeLa cells, which were either mock transfected (siControl) or transfected with clathrin siRNA (siClathrin) for 48 hours, separated by SDS-PAGE and immunoblotted for clathrin heavy chain (CHC) or actin. (C) Stably-transfected LPA_1/HeLa cells grown in 24-well plates were either mock transfected (siControl) or transfected with clathrin siRNA (siClathrin) for 48 hours prior to treatment with or without 10 μM LPA for 45 minutes. The cells were fixed and processed for whole-cell ELISA to quantify surface LPA_1 receptors as described in Materials and Methods. LPA_1 internalization is expressed as the percentage difference in surface LPA_1 between unstimulated cells and agonist-stimulated cells. The data are the mean±s.e.m. of six replicates/siRNA sample combined from two independent experiments. $^{**}P<0.01$ compared to levels in the siControl. (D) Stably transfected LPA_1/HeLa cells were treated with clathrin siRNA for 48 hours prior to incubation with FITC-labeled mouse anti-CD59 and Alexa 594-Tfn for 30 minutes and fluorescence visualization of anti-CD59 and Alexa 594-Tfn labeling. Bar, 10 μm.

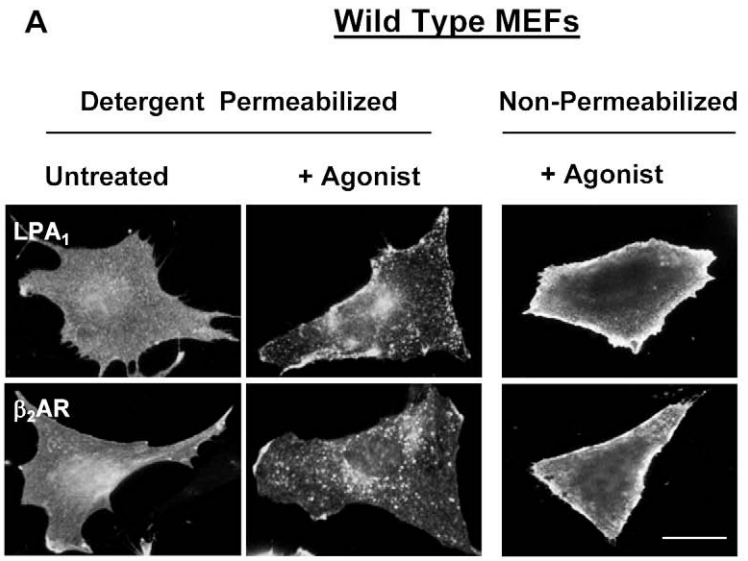
we have previously shown to colocalize with transferrin-receptor-positive endosomes (Murph et al., 2003).

To investigate the role of clathrin in LPA_1 endocytosis, we adapted a double-transfection procedure that was previously described (Motley et al., 2003) to knock down clathrin heavy chain amounts in HeLa cells to near undetectable levels. Using this procedure, we observed a 73% reduction in the abundance of clathrin relative to mock-transfected siControl cells (Fig. 1B) in LPA_1/HeLa cells. Treatment of cells with clathrin siRNA did not alter the abundance of actin. Endocytosis of LPA_1 was quantified by using a whole-cell ELISA, which measures the agonist-induced loss of cell surface LPA_1 (Kim and Benovic, 2002; Paing et al., 2002). In siControl cells, 10 μM LPA induced LPA_1 internalization (~30%) and this was strongly inhibited in siClathrin cells (~2%) (Fig. 1C). In siClathrin cells, both the agonist-stimulated internalization of β_2-adrenergic receptors (β_2ARs) (data not shown), which are known to use clathrin-dependent mechanisms (von Zastrow and Kobilka, 1992) and the constitutive endocytosis of Alexa transferrin was strongly inhibited (Fig. 1D).

As a negative control, we examined the effects of clathrin knockdown on the endocytosis of anti-CD59 antibodies bound to endogenous CD59, which is internalized via cholesterol-rich, detergent-resistant membranes and then merges with a clathrin-independent trafficking pathway that is regulated by the Arf6 GTPase as shown by Naslavsky et al. (Naslavsky et al., 2004). LPA_1/HeLa cells were transfected either with or without clathrin siRNAs and then incubated with FITC-labeled mouse anti-CD59 antibodies along with Alexa 594-Tfn for 30 minutes. The cells were acid-stripped to remove surface-bound anti-CD59 antibodies and Alexa 594-Tfn. In cells transfected with clathrin siRNAs, FITC-labeled anti-CD59 antibodies localized to pleomorphic tubulovesicular structures (Fig. 1D, Anti-CD59) similar to those described (Naslavsky et al., 2004). As expected, these same siRNA-treated cells did not internalize Alexa 594-Tfn (Fig. 1D, Alexa 594-Tfn). Taken together, these results indicate that LPA_1 is internalized by clathrin-mediated endocytosis.

β-arrestins are critical for LPA_1 signal attenuation and receptor endocytosis

Clathrin-mediated endocytosis of many GPCRs is also dependent upon their association with the multi-functional β-arrestins (Ferguson et al., 1996; Marchese et al., 2003). β-arrestin binding is initiated through the agonist-induced phosphorylation of cytoplasmic serine/threonine residues in the GPCR by G protein receptor kinases (GRKs) such as GRK2 (Pitcher et al., 1998). β-arrestin binding promotes both receptor desensitization, by preventing receptor-G protein coupling and clathrin-dependent endocytosis of the receptor. To determine whether β-arrestins are required for LPA_1 endocytosis, we compared agonist-stimulated internalization of LPA_1 and β_2AR in mouse embryo fibroblasts (MEFs) derived from either wild-type or β-arrestin 1 and 2 null mice (Kohout et al., 2001) (Fig. 2). Wild-type MEFs were transiently transfected with plasmids encoding either LPA_1 or β_2ARs and then incubated in the presence or absence of agonist. In the absence of agonist treatment, both receptors were primarily localized to the plasma membrane in a diffuse pattern (Fig. 2A, untreated). Upon agonist treatment for 30 minutes, both LPA_1 and β_2ARs redistributed to small punctate endosomal



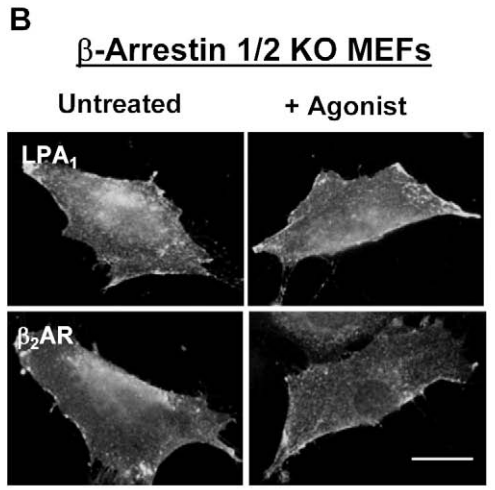


Fig. 2. Agonist-induced endocytosis of LPA$_1$ is inhibited in β-arrestin 1/2 double knockout mouse embryo fibroblasts. (A) Wild-type MEFs were transiently transfected with plasmids encoding either FLAG-tagged LPA$_1$ or HA-tagged β_2AR and then incubated in the presence or absence of agonist (10 μM LPA or 20 μM isoproterenol, respectively) for 30 minutes prior to indirect immunofluorescence localization of the receptor proteins either in the presence or absence of detergent permeabilization. (B) β-arrestin 1/2 double knockout MEFs were transiently transfected with plasmids encoding either FLAG-tagged LPA$_1$ or HA-tagged β_2ARs and incubated in the presence or absence of agonist, as above, prior to indirect immunofluorescence localization of the receptor proteins. Bar, 10 μm.

structures dispersed throughout the cell. The labeling of these structures was not observed in non-permeabilized cells, thus indicating that they were internal endosomal structures (Fig. 1A, non-permeabilized). In contrast to wild-type MEFs, agonist treatment of β-arrestin 1/2 KO MEFs expressing either LPA$_1$ or β_2ARs did not lead to their endocytosis (Fig. 2B, +agonist). Expression of wild-type β-arrestin-2-GFP in the knockout cells restored agonist-induced endocytosis of both LPA$_1$ (Fig. 3A, 30 minutes) and β_2ARs (data not shown), thus indicating that β-arrestins were required for the endocytosis of LPA$_1$ as well as β_2ARs.

Previous studies have shown that agonist stimulation of different GPCRs leads to the translocation of cytosolic β-arrestin proteins to the plasma membrane (Barak et al., 1997). For some GPCRs such as β_2ARs, this association with β-arrestins is transient and is not observed following receptor endocytosis, whereas other GPCRs, such as angiotensin AT1a receptors and vasopressin receptors, maintain a stable association with β-arrestins even on endosomes after endocytosis (Oakley et al., 1999). To determine whether LPA$_1$ formed a transient or stable association with β-arrestins, we examined the distribution of LPA$_1$ and β-arrestin-2-GFP after 0, 2 and 30 minutes of LPA treatment (10 μM) (Fig. 3A). In untreated cells, LPA$_1$ localized to the plasma membrane and β-arrestin-2-GFP localized in a diffuse cytoplasmic pattern (Fig. 3A, Untreated). After 2 minutes of LPA treatment, LPA$_1$ localized to small punctate structures, which partially colocalized with β-arrestin-2-GFP (Fig. 3A, inset, arrows). However, many punctate structures contained LPA$_1$ but did not contain β-arrestin-2-GFP, particularly in the larger and more pleotropic structures. Following 30 minutes of LPA treatment, LPA$_1$ localized to heterogeneously sized endosomal structures, but β-arrestin-2-GFP returned to the diffuse cytoplasmic pattern observed in untreated cells (Fig. 3A, 30 minutes). This suggested that β-arrestins dissociate from LPA$_1$ receptors at or near the cell surface and do not form a stable association with β-arrestin proteins, as defined by Oakley et al. (Oakley et al., 1997). Taken together, these data indicate that β-arrestins are critical for the endocytosis of LPA$_1$ and that LPA$_1$ only transiently associates with β-arrestins at the cell surface.

As mentioned above, β-arrestin binding to activated GPCRs leads to signal attenuation (Lefkowitz and Whalen, 2004). We next investigated whether β-arrestins were important for the desensitization of LPA$_1$. We examined the ability of LPA$_1$ receptors, which activate G_i, G_q and $G_{12/13}$ signaling pathways (Fukushima et al., 1998), to promote phosphoinositide (PI) hydrolysis, via G_q stimulation of phospholipase C, in the wild type and β-arrestin 1/2 KO MEFs (Fig. 3B). LPA$_1$-transfected wild type and β-arrestin 1/2 KO MEFs were labeled with [^{3}H]inositol and the accumulation of inositol phosphates was determined in untreated cells and cells treated with 10 μM LPA for 60 minutes at 37°C. LPA treatment increased the accumulation of [^{3}H]inositol phosphates in wild-type MEFs by ~2.5-fold. However, stimulation of LPA$_1$-transfected β-arrestin 1/2 KO MEFs led to a 4.3-fold increase in inositol phosphate accumulation, suggesting that β-arrestins are important for attenuation of LPA signaling. To further test this, we determined the effects of re-expression of wild-type β-arrestin 2 on inositol phosphate accumulation in LPA$_1$-transfected β-arrestin 1/2 KO MEFs (Fig. 3B). Co-transfection of wild-type β-arrestin 2 and LPA$_1$ in the β-arrestin 1/2 KO MEFs reduced the magnitude of LPA-induced inositol phosphate

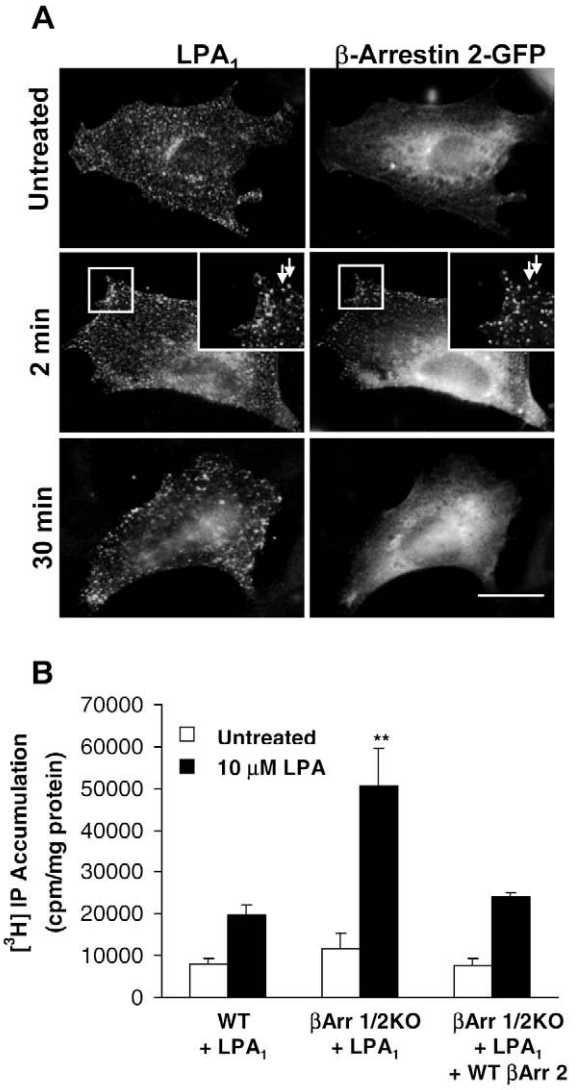


Fig. 3. Re-expression of wild-type β-arrestin 2 GFP in β-arrestin knockout MEFs restores LPA_1 signal attenuation and receptor endocytosis. (A) β-arrestin 1/2 double knockout MEFs were transiently transfected with plasmids encoding LPA_1 and wild-type β-arrestin-2-GFP. Cells were then incubated with 10 μM LPA for 0, 2, or 30 minutes prior to fixation and indirect immunofluorescence. The inset shows a magnified image of the boxed region and the arrows indicate punctate structures that co-label for both LPA_1 and β-arrestin-2-GFP. Note that the recruitment of β-arrestin2-GFP to these punctate structures is transient, observable after 2 minutes of LPA treatment but not after 30 minutes of LPA treatment. (B) MEFs derived from wild type (WT) or β-arrestin 1/2 null (βArr 1/2KO) mice were transfected with plasmid encoding wild-type LPA_1 receptors; β-arrestin 1/2 null MEFs were also co-transfected with plasmids encoding LPA_1 and wild-type β-arrestin 2 (βArr 1/2KO + LPA_1 + WT βArr2). Cells were then labeled with $[^3H]$myo-inositol overnight in serum-free medium and incubated for 1 hour in the absence (Untreated) or presence of 10 μM LPA prior to analysis of phosphoinositide hydrolysis, as described in Materials and Methods. The radioactivity recovered in the different samples was normalized to total cellular protein and the data are presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated three times. $^{**}P<0.01$, comparison of LPA-stimulated phosphoinositide hydrolysis in β-arrestin 1/2 KO MEFs to that observed in WT MEFs. Bar, 10 μm.

accumulation to 3.1-fold, which was similar to that observed in LPA_1-transfected WT MEFs (2.5-fold). Taken together, these observations strongly support the notion that β-arrestin association with LPA_1 receptors is important for signal attenuation and for clathrin-mediated receptor internalization.

Membrane cholesterol is required for LPA_1 signaling and receptor endocytosis

Having established that the agonist-induced endocytosis of LPA_1 was mediated by a β-arrestin- and clathrin-dependent pathway, we next investigated the role of membrane cholesterol in LPA_1 signaling and trafficking. As mentioned, membrane cholesterol has been shown to be important for LPA stimulation of PI3-kinase/Akt signaling (Peres et al., 2003). To address this question, we first examined the effects of cholesterol extraction with methyl-β-cyclodextrin (MβCD) and the effects of cholesterol disruption with the cholesterol-binding drug, nystatin, on LPA stimulation of phosphoinositide hydrolysis, which is stimulated by Gq signaling. As a first step, we measured the effects of these cholesterol-perturbing drugs and the effects of water-soluble cholesterol:MβCD complexes on the cellular abundance of cholesterol in LPA_1/HeLa cells by using a quantitative cholesterol measurement assay (see Materials and Methods) (Table 1). Control LPA_1/HeLa cells contained 17.10±0.13 μg cholesterol/mg protein and treatment with 5 mM MβCD for 1 hour reduced cellular cholesterol by 62% to 6.52±0.05 μg cholesterol/mg protein. Addition of 10 mM cholesterol, as a water-soluble MβCD complex, for 1 hour after MβCD extraction, increased cellular cholesterol levels to approximately twice that observed in control cells (34.2±0.78 μg cholesterol/mg protein). In contrast, treatment of LPA_1/HeLa cells with 50 μg/ml nystatin for 1 hour slightly elevated the amount of cellular cholesterol (20.8±1.5 μg cholesterol/mg protein) and addition of water-soluble cholesterol to nystatin-treated cells increased cellular cholesterol amounts by approximately 2.5-fold relative to control LPA_1/HeLa cells (42.9±1.3 μg cholesterol/mg protein). This is consistent with the notion that nystatin merely binds sterols but does not extract them from cells.

We next examined the effects of these cholesterol-perturbing drugs on LPA_1 stimulation of phosphoinositide hydrolysis,

Table 1. Effects of cholesterol perturbing agents on cellular cholesterol abundance

	Cholesterol content (μg/mg protein)*
Control	17.1±0.13
5 mM MβCD	6.5±0.05
5 mM MβCD + 10 mM cholesterol/MβCD	34.2±0.78
50 μg/ml Nystatin	20.8±1.5
50 μg/ml Nystatin + 10 mM cholesterol/MβCD	42.9±1.3

*Stably transfected LPA_1/HeLa cells were left untreated (Control) or were incubated with 5 mM MβCD for 1 hour, 50 μg/ml nystatin for 1 hour, 5 mM MβCD for 1 hour followed by 10 mM cholesterol/MβCD for 1 hour or 50 μg/ml nystatin for 1 hour followed by 10 mM cholesterol/MβCD for 1 hour. Cells were then solubilized and total cellular cholesterol content was quantified as described in the Materials and Methods. The amount of cellular cholesterol was normalized to total cellular protein and is presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated twice.

which is promoted by Gq stimulation of phospholipase C. LPA stimulation of native HeLa cells (Fig. 4A, HeLa) resulted in a small 1.9-fold increase in accumulation of [^{3}H]inositol phosphates, whereas stimulation of LPA$_1$/HeLa cells resulted in a large increase in PI hydrolysis (~14-fold) (Fig. 4A, lane 1). Treatment of LPA$_1$/HeLa cells with 5 mM MβCD reduced LPA stimulated PI hydrolysis to 3.9-fold (72% inhibition) (Fig. 4A, lane 2). Treatment of LPA$_1$/HeLa cells with 50 μg/ml nystatin did not significantly affect agonist-dependent PI hydrolysis (Fig. 4A, lane 4). Addition of water-soluble cholesterol:MβCD complexes, which contained 10 mM cholesterol, to MβCD-treated or nystatin-treated cells greatly increased the extent of basal accumulation of labeled inositol phosphates (Fig. 4A, lanes 3 and 5). Addition of water-soluble cholesterol led to a greater increase in the basal level of inositol phosphate accumulation as compared to that observed in LPA-stimulated samples, which consequently decreased the fold induction of PI hydrolysis by LPA. This could be due to enhanced G_q signaling that is independent of LPA$_1$ receptors. These results suggested that the presence of membrane cholesterol was important for LPA$_1$ stimulation of PI hydrolysis.

As a control, we examined the effects of MβCD on the stimulation of PI hydrolysis by an unrelated G_q-coupled receptor, the M$_1$ muscarinic acetylcholine receptor (M$_1$ mAChR). For these experiments, HeLa cells were transiently transfected with plasmids encoding either wild-type LPA$_1$ or M$_1$ mAChRs. Whereas 5 mM MβCD inhibited LPA$_1$-mediated PI hydrolysis (Fig. 4B, compare lanes 3 and 4) as expected, it did not significantly reduce the extent of PI hydrolysis in response to agonist stimulation (1 mM carbachol) of M$_1$ mAChR-expressing cells (Fig. 4B, compare lanes 5 and 6). Immunofluorescence microscopy indicated that the transfection efficiencies of the LPA$_1$ and M$_1$ mAChR plasmids were comparable and were approximately 40% (data not shown). These results indicated that the reduction of LPA$_1$-mediated PI hydrolysis by MβCD was not due to inhibition of either $G\alpha_q$ or phospholipase C activities, but rather was due to a specific inhibition of LPA$_1$ function. Taken together, these results suggest that the presence of plasma membrane cholesterol is critical for LPA$_1$-dependent signaling to phospholipase C (Fig. 4).

Next, we investigated whether membrane cholesterol was also important for LPA$_1$ endocytosis. We compared the effects of MβCD on the agonist-induced endocytosis of LPA$_1$ and M$_1$ mAChRs, which are also internalized by clathrin- and β-arrestin-dependent mechanisms (Vogler et al., 1999). Stably-transfected LPA$_1$/HeLa cells or transiently-transfected HeLa cells expressing M$_1$ mAChRs were pre-incubated in the presence or absence of 5 mM MβCD for 1 hour. These cells were then incubated with the respective agonists and Alexa 594-Tfn for 30 minutes. In untreated cells, LPA$_1$ and M$_1$ mAChRs localized to the plasma membrane, whereas Alexa 594-Tfn labeled pleomorphic endosomal structures (Fig. 5A, untreated). Agonist stimulation induced the endocytosis of

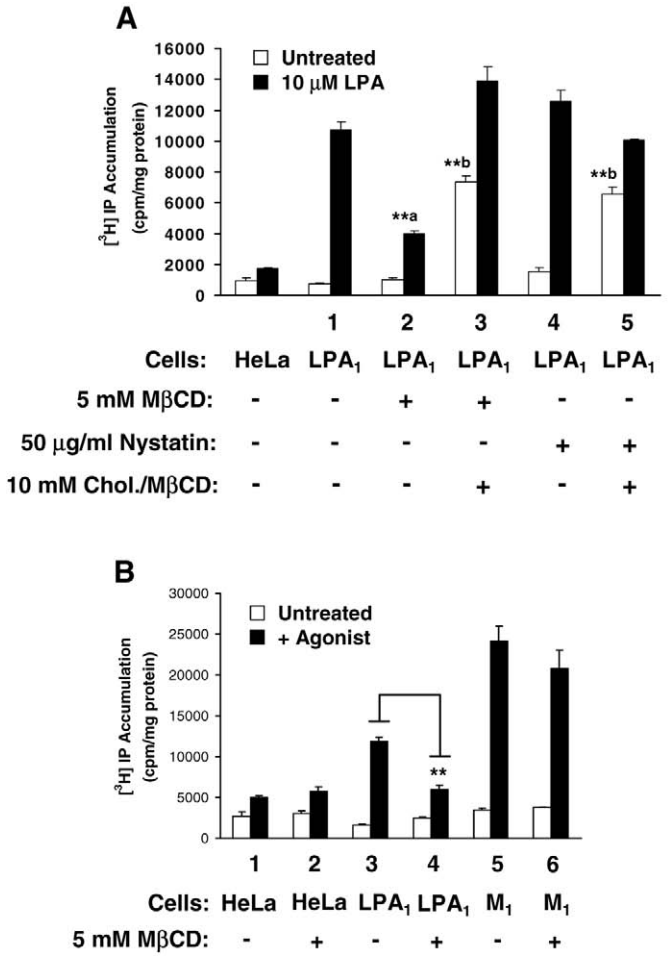


Fig. 4. Stimulation of phosphoinositide hydrolysis by LPA$_1$ receptors is inhibited by cholesterol extraction with methyl-β-cyclodextrin. (A) Either native HeLa cells or stably transfected LPA$_1$/HeLa cells were labeled overnight with [^{3}H] myo-inositol and then either left untreated (HeLa and lane 1) or pre-incubated with 5 mM MβCD for 1 hour (lane 2), 50 μg/ml nystatin for 1 hour (lane 4), 5 mM MβCD for 1 hour followed by 10 mM cholesterol/MβCD complexes for 60 minutes (lane 3), or 50 μg/ml nystatin for 1 hour followed by 10 mM cholesterol/MβCD complexes for 60 minutes (lane 5) prior to an additional 1 hour treatment with 10 μM LPA. Cells were then solubilized and the total accumulation of labeled inositol phosphates was determined. The radioactivity recovered in the different samples was normalized to total cellular protein and the data are presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated four times. **a, P<0.01, comparison of LPA-stimulated phosphoinositide hydrolysis in MβCD-treated LPA$_1$/HeLa cells to that observed in non-MβCD-treated LPA$_1$/HeLa cells. **b, P<0.01, comparing phosphoinositide hydrolysis in MβCD-treated or nystatin-treated LPA$_1$/HeLa cells that were incubated with water-soluble cholesterol to that observed in unstimulated LPA$_1$/HeLa cells. (B) HeLa cells were transiently transfected with plasmids encoding either vector alone (lanes 1 and 2), LPA$_1$ (lanes 3 and 4), or M$_1$ mAChRs (lanes 5 and 6). The cells were incubated in the absence (–) or presence (+) of 5 mM MβCD for 1 hour prior to a subsequent 1 hour incubation with agonist (10 μM LPA or 1 mM carbachol). After solubilization, the radioactively-labeled inositol phosphates were isolated as described. The radioactivity recovered in the different samples was normalized to total cellular protein and the data are presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated three times. **P<0.01, comparison of LPA-stimulated phosphoinositide hydrolysis in MβCD-treated LPA$_1$-transfected HeLa cells to that observed in non-MβCD-treated cells.

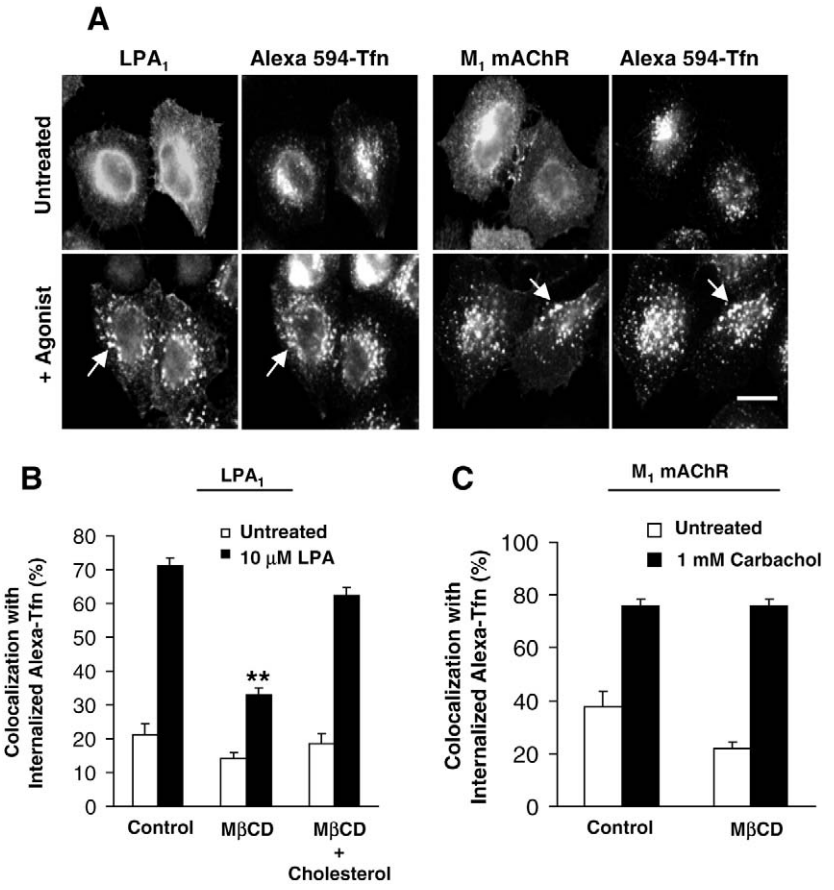


Fig. 5. Cholesterol extraction inhibits the agonist-induced endocytosis of LPA_1 but not M_1 mAChRs. (A) HeLa cells were transiently transfected with plasmids encoding either LPA_1 or M_1 mAChRs and were incubated in the absence (Untreated) or presence of agonist (10 μM LPA or 1 mM carbachol, respectively) and 50 μg/ml Alexa 594-Tfn for 30 minutes and then processed for indirect immunofluorescence localization of the transfected receptors. Bar, 10 μm. (B and C) HeLa cells were transfected as described above and were pre-incubated for 1 hour in the absence (Control) or presence of 5 mM MβCD or MβCD and 10 mM cholesterol/MβCD complexes prior to a subsequent incubation in the presence or absence of 10 μM LPA and 50 μg/ml Alexa 594-Tfn. The cells were fixed and processed for immunofluorescence localization of the transfected receptors. The extent of colocalization between LPA_1 (B) or M_1 mAChRs (C) and the internalized Alexa 594-Tfn was quantified using Metamorph image analysis as described in Materials and Methods. The data are expressed as the mean±s.e.m. of 20 cells/condition from a representative experiment that was performed three times with similar results. $^{**}P<0.01$ compared with control, LPA-treated cells.

both receptors into endosomal structures that colocalized with internalized Alexa 594-Tfn (Fig. 5A, +agonist). To quantify GPCR endocytosis, we measured the extent of GPCR (either LPA_1 or M_1) colocalization with the internalized Alexa 594-Tfn using Metamorph image analysis (Murph et al., 2003; Volpicelli et al., 2001). In control cells, treatment with 10 μM LPA increased LPA_1 and Alexa 594-Tfn colocalization by 3.3-fold relative to untreated cells (Fig. 5B, Control). Pre-incubation with 5 mM MβCD reduced this agonist-induced colocalization by more than 50% (Fig. 5B, compare black bars in control cells to cells treated with 5 mM MβCD). Addition of 10 mM water-soluble cholesterol restored LPA_1 and Alexa 594-Tfn colocalization to near control levels (Fig. 5B, MβCD + cholesterol). In contrast to LPA_1, agonist treatment (1 mM carbachol, 30 minutes) stimulated a twofold increase in M_1 mAChR colocalization with Alexa 594-Tfn, which was not inhibited by MβCD (Fig. 5C). These data suggested that plasma membrane cholesterol is important for both LPA_1 endocytosis and LPA_1 signaling.

LPA_1 localizes to detergent-resistant membrane microdomains upon agonist stimulation

Although the data above indicates that membrane cholesterol is essential for LPA_1 endocytosis, the data in Fig. 1-3 indicate that LPA_1 is internalized by clathrin- and β-arrestin-dependent mechanisms, which are distinct from cholesterol-dependent endocytic pathways (Nichols, 2003). To further investigate this apparent difference, we examined whether LPA_1 localized to detergent-resistant membrane domains, which are enriched in both cholesterol and glycosphingolipids (Razani et al., 2002). We examined the effects of LPA stimulation on the resistance of LPA_1 to extraction with Triton X-100. LPA_1/HeLa cells were incubated with 10 μM LPA for different times before extraction with ice-cold 1% Triton X-100 and indirect immunofluorescence (Fig. 6A). LPA_1 staining in unstimulated cells was greatly reduced following Triton X-100 extraction (Fig. 6A, 0 minute). In contrast, the cell-associated LPA_1 staining, which remained after detergent extraction, was increased with time of agonist stimulation (Fig. 6A). We quantified the detergent-resistant LPA_1 staining associated with the cells by measuring the pixel intensity of LPA_1-specific fluorescence using MetaMorph image analysis (see materials and methods) and normalizing this value to DNA content as assessed by Hoescht dye labeling (Fig. 6B). This analysis showed that detergent extraction of unstimulated cells reduced the level of cell-associated LPA_1 staining to 5% of that observed in untreated and non-extracted cells. Cell-associated LPA_1 staining progressively increased with time of LPA treatment such that after 30 minutes of LPA stimulation approximately 38% of LPA_1 immunoreactive staining remained after detergent extraction, relative to control cells (Fig. 6B, solid circles). Extraction of membrane cholesterol with 5 mM MβCD prior to LPA stimulation blocked the LPA-induced increase in detergent resistance of LPA_1 (Fig. 6B, open triangles). As detergent resistance of proteins can also be increased by their association with the actin cytoskeleton, we examined the effects of inhibiting actin polymerization with cytochalasin D (5 μM) on

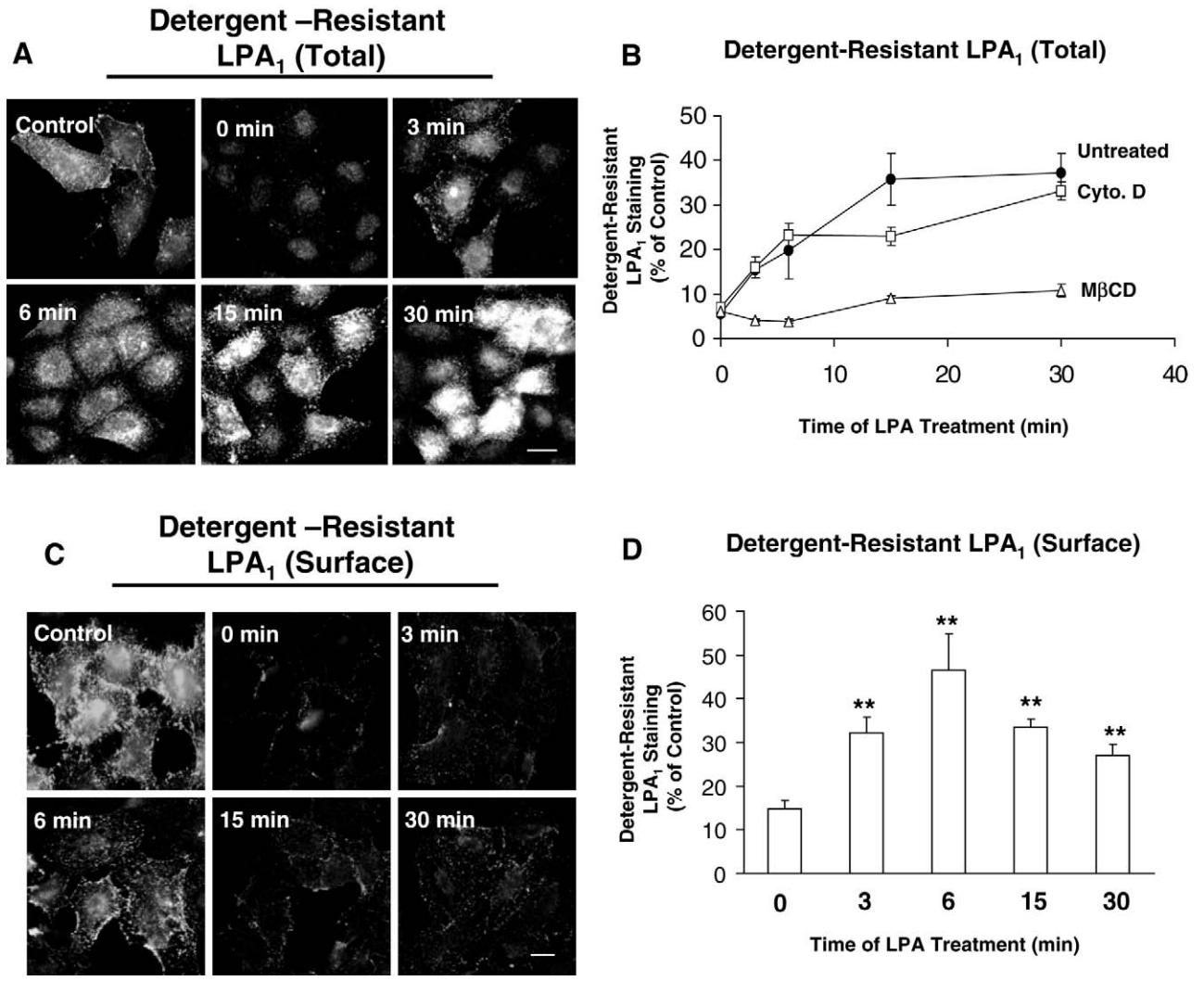


Fig. 6. LPA$_1$ receptors localize to detergent-resistant cellular domains upon agonist stimulation. (A) LPA$_1$/HeLa cells were incubated with 10 μM LPA for different times and subsequently treated with 1% cold Triton X-100 on ice for 3 minutes, fixed and processed for indirect immunofluorescence localization of LPA$_1$. (B) Quantitative analysis of receptor expression after detergent extraction was performed by MetaMorph image analysis as described in Materials and Methods. Cells were either untreated, treated with 5 μM cytochalasin D (Cyto. D) for 30 minutes, or treated with 5 mM MβCD for 1 hour, prior to incubation with 10 μM LPA for the indicated times. The LPA$_1$ labeling in detergent-extracted cells was normalized to the amount of LPA$_1$ labeling observed in non-agonist-treated cells, which had not been subjected to detergent extraction. The data are presented as the mean±s.e.m. of five to six cells per time point and are from a representative experiment that was repeated twice with similar results. (C) LPA$_1$/HeLa cells were incubated with 10 μM LPA for different times and incubated with mouse anti-FLAG antibody on ice for 30 minutes prior to extraction with ice-cold 1% Triton X-100, to label surface LPA$_1$ receptors. Cells were then processed for indirect immunofluorescence localization of surface LPA$_1$. (D) Quantitative analysis of surface LPA$_1$ receptor expression after detergent extraction was performed by MetaMorph image analysis as described in Materials and Methods. The LPA$_1$ labeling in detergent-extracted cells was normalized to the amount of LPA$_1$ labeling observed in non-agonist treated cells, which had not been subjected to detergent extraction. The data are presented as the mean±s.e.m. of five to six cells per time point and are from a representative experiment that was repeated twice with similar results. $^{**}P<0.01$, comparison of the amount of detergent-resistant surface LPA$_1$ staining after the indicated time of agonist treatment with that observed in unstimulated cells. Bar, 10 μm.

the detergent resistance of LPA$_1$ (Fig. 6B, open squares). We observed no noticeable difference between the detergent resistance of LPA$_1$ in cells pre-treated with cytochalasin D and untreated cells, after brief exposure to LPA (i.e. 0 to 8 minutes). A slight delay in the rate of increase of LPA$_1$ detergent resistance was observed between 10 and 20 minutes of LPA treatment in cells that were pre-treated with cytochalasin D, but the extent of detergent resistance was the same in both untreated and cytochalasin D-treated cells after 30 minutes of LPA stimulation. These data indicate that LPA treatment promotes the association of LPA$_1$ with detergent resistant membranes and that this process is inhibited by cholesterol extraction.

Given that LPA treatment for 30 minutes promotes the endocytosis of LPA$_1$ into transferrin receptor$^+$ endosomes, it is likely that some of the detergent resistant LPA$_1$ receptors observed after longer LPA treatment reside in endosomes.

Studies have shown that transferrin receptor$^+$ endosomes are enriched in cholesterol (Hao et al., 2002). To investigate the effects of agonist stimulation on the detergent resistance of surface LPA_1 receptors, we labeled surface LPA_1 with mouse anti-FLAG antibodies on ice prior to detergent extraction (Fig. 6C,D). The LPA_1 expressed in LPA_1/HeLa cells contains an N-terminal FLAG epitope tag that is accessible to the extracellular medium. In the absence of detergent, mouse anti-FLAG antibodies labeled only the cell surface in control cells (Fig. 6C, Control). Triton X-100 extraction removed most of the surface-bound antibody (only 15% of control, un-extracted cells remained) (Fig. 6C,D, 0 minute). The detergent resistance of surface LPA_1 increased with time of agonist treatment up to 45% of control levels after 6 minutes and then declined after 15 minutes and 30 minutes of LPA treatment (Fig. 6C,D). These results suggest that the LPA_1 receptor associates with detergent-resistant membranes following agonist stimulation, both at the cell surface and following endocytosis in cholesterol-rich endosomes.

Membrane cholesterol is required for the plasma membrane recruitment of cytosolic β-arrestins by activated LPA_1

As β-arrestins are required for the clathrin-mediated endocytosis of LPA_1, we examined whether membrane cholesterol was important for the association of β-arrestins with LPA_1 or with β_2AR, as a control. Preliminary experiments showed that, in HeLa cells that were transiently transfected with plasmid encoding either LPA_1 or β_2ARs along with β-arrestin -2-GFP, both LPA_1 and β_2ARs transiently recruited cytosolic β-arrestin-2-GFP to punctate plasma membrane structures after 2 minutes of agonist stimulation (Fig. 8A,B, Control). Double-labeling experiments showed that β-arrestin-2-GFP extensively colocalized with the plasma membrane clathrin adaptor, AP2 (Fig. 7), suggesting that brief LPA stimulation led to the recruitment of β-arrestin-2-GFP to cell surface clathrin-coated pits. After 30 minutes of agonist stimulation, both LPA_1 and β_2ARs localized to endosomal structures, but β-arrestin 2 GFP returned to a cytosolic distribution (data not shown). This is consistent with published reports showing that β_2ARs transiently associate with β-arrestins (Oakley et al., 1999).

We next examined the effects of cholesterol extraction on the surface recruitment of β-arrestin-2-GFP by LPA_1 and β_2AR after 2 minutes of agonist stimulation (Fig. 8). β-arrestin-2-GFP localized in a diffuse cytoplasmic pattern in unstimulated cells and both LPA_1 and β_2AR were localized to the plasma membrane (data not shown). After 2 minutes of LPA treatment, β-arrestin-2-GFP colocalized with LPA_1 in punctate spots at the cell surface (Fig. 8A, Control, left panels), which also colocalized with AP2 (see Fig. 7). Similarly, after 2 minutes of isoproterenol treatment of β_2AR-expressing cells, β-arrestin-2-GFP localized to punctate spots at the cell surface (Fig. 8B, Control, right panels). Pre-incubation with 5 mM MβCD for 60 minutes completely inhibited the recruitment of β-arrestin-2-GFP to the cell surface by LPA_1 and β-arrestin-2-GFP remained in a diffuse cytosolic distribution (Fig. 8A, MβCD, see inset). Addition of 10 mM water-soluble cholesterol restored the ability of LPA_1 to recruit β-arrestin-2-GFP to the cell surface in MβCD-treated cells (Fig. 8A, MβCD-cholesterol). In contrast, incubation with MβCD did not inhibit β-arrestin-2-GFP recruitment to punctate surface spots by β_2ARs (Fig. 8B, MβCD, see inset). Addition of water-soluble cholesterol did not alter the surface recruitment of β-arrestin 2-GFP by β_2ARs (Fig. 8B, MβCD-cholesterol).

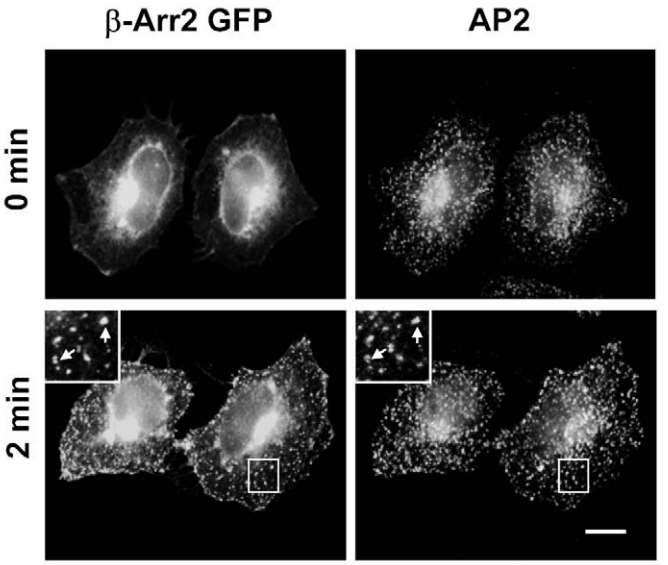


Fig. 7. LPA stimulation leads to the colocalization of β-arrestin-2-GFP with clathrin AP2 adaptors. LPA_1/HeLa cells were transiently-transfected with plasmids encoding β-arrestin-2-GFP and incubated with 10 μM LPA for either 0 minute or 2 minutes prior to fixation. Endogenous clathrin AP2 was localized in permeabilized cells using mouse anti-AP2 antibodies. The inset shows a high magnification image of the boxed region and the arrows indicate structures where β-arrestin-2-GFP colocalized with AP2. Bar, 10 μm.

To quantify these effects, we determined the percentage of cells that showed β-arrestin recruitment to the plasma membrane after 2 minutes of agonist stimulation of either LPA_1 or β_2AR (Fig. 8C). In control cells and in cholesterol repleted cells (i.e. MβCD-cholesterol), β-arrestin-2-GFP was recruited to the cell surface in ~80% of cells expressing either LPA_1 or β_2AR. Only 4% of LPA_1-expressing cells showed surface recruitment of β-arrestin-2-GFP in MβCD-treated cells. In contrast, approximately 50% of β_2AR-expressing cells exhibited surface recruitment of β-arrestin-2-GFP. Taken together, these results indicate that LPA_1 is much more dependent upon plasma membrane cholesterol for the recruitment of β-arrestin than β_2ARs. This also provides a possible link between membrane cholesterol and clathrin-dependent endocytosis of LPA_1 as β-arrestin is critical for endocytosis of LPA_1.

Discussion

All members of the GPCR superfamily share the ability to rapidly respond to agonist stimulation and then to undergo desensitization (Lefkowitz and Shenoy, 2005). Many of these GPCRs are also rapidly internalized into cells via one of several distinct endocytic pathways. However, the mechanisms that regulate GPCR desensitization and determine the specific endocytic pathway used for internalization vary from receptor

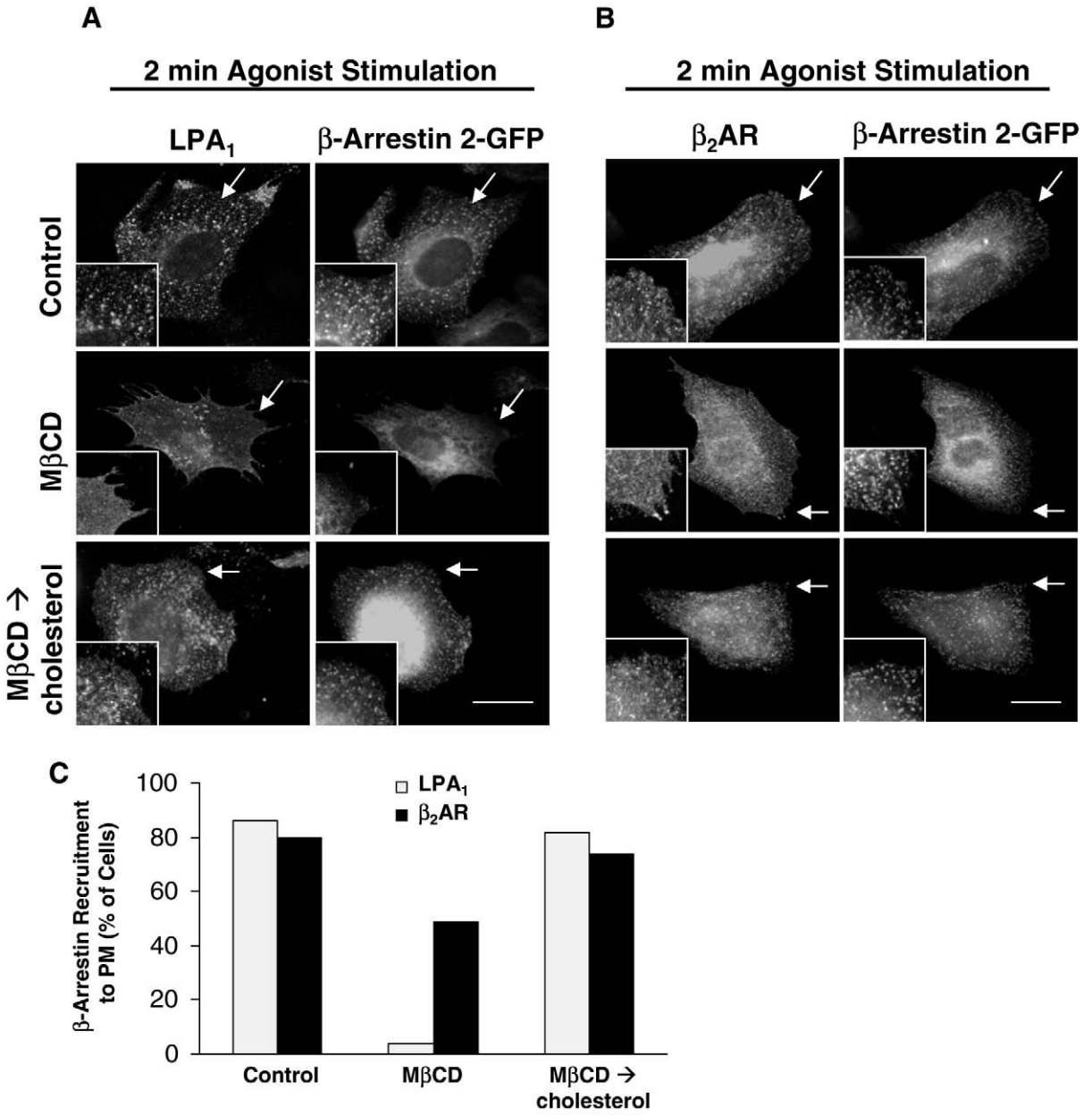


Fig. 8. MβCD extraction prevents recruitment of β-arrestin-2-GFP to the plasma membrane by LPA$_1$ but not by β_2AR. (A,B) HeLa cells were transiently transfected with plasmid encoding either LPA$_1$ (A) or β_2AR (B) along with β-arrestin-2-GFP. The cells were then left untreated, pre-treated with 5 mM MβCD for 1 hour, or treated sequentially with 5 mM MβCD for 1 hour and 10 mM water-soluble cholesterol for 1 hour prior to incubation with 10 μM LPA for 2 minutes. The cells were then fixed and processed for indirect immunofluorescence microscopy. The inset shows a magnified image of the region of the cell indicated by the arrow. (C) The percentage of cells exhibiting recruitment of β-arrestin-2-GFP to punctate plasma membrane spots after 2 minutes agonist stimulation was determined by scoring 100 cells per condition for cells expressing LPA$_1$ and β_2AR. Bar, 10 μm.

to receptor. In this study, we found that LPA$_1$ receptors are internalized by a clathrin- and β-arrestin-dependent pathway, but that they also require plasma membrane cholesterol for receptor signaling and for their subsequent clathrin-dependent endocytosis. Our results indicate that the key requirement of membrane cholesterol for LPA$_1$ endocytosis is for the association of LPA$_1$ with β-arrestin, which promotes both signal attenuation and clathrin-dependent endocytosis of the receptor.

Caveolae and other detergent-resistant membrane domains are cholesterol- and glycosphinoglipid-rich, are sites of active signal transduction and have been implicated in the activation of heterotrimeric G proteins, Ras signaling and eNOS signaling (Razani et al., 2002). Several lines of evidence suggest that LPA$_1$Rs associate with cholesterol-rich, detergent-resistant membranes and that this is important for LPA$_1$-dependent signaling. First, cholesterol extraction with MβCD strongly inhibited LPA$_1$ induction of phosphoinositide hydrolysis, via $G\alpha_q$-mediated stimulation of phospholipase C (Fig. 4). $G\alpha_q$ has been shown to be enriched in caveolae (Huang et al., 1997; Oh and Schnitzer, 2001), which supports the notion that LPA$_1$

stimulates PI hydrolysis by associating with $G\alpha_q$ in detergent-resistant membranes. Re-addition of cholesterol to MβCD-treated cells increased both the basal and LPA-stimulated levels of PI hydrolysis (Fig. 4A). The fact that MβCD extraction did not affect PI hydrolysis by the M_1 mAChR suggests that cholesterol depletion does not impair either $G\alpha_q$ or phospholipase C activity per se, but that LPA_1 stimulation of PI hydrolysis is particularly sensitive to cholesterol depletion (Fig. 4C). Two possible explanations for the difference between LPA_1 and M_1 mAChRs are that either LPA_1 exclusively couples to $G\alpha_q$ that is localized to detergent-resistant membrane domains or that membrane cholesterol is required for the physical association of LPA_1 with $G\alpha_q$. Second, we found that LPA stimulation enhanced the resistance of both surface and total LPA_1 to extraction with TX-100 detergent (Fig. 6). Resistance to detergent extraction is a common property of proteins that are associated with caveolae and other cholesterol-rich membrane regions (Razani et al., 2002). The detergent-resistance of surface LPA_1 increased during the first 6 minutes of LPA treatment and then declined. This is consistent with a transient association of LPA_1 with detergent-resistant microdomains prior to β-arrestin- and clathrin-dependent endocytosis. Disruption of the actin cytoskeleton with 5 μM cytochalasin D did not alter the agonist-induced detergent resistance of LPA_1 suggesting that the increased detergent resistance of LPA_1 was not due to its association with the actin cytoskeleton. However, cholesterol extraction completely prevented the agonist-induced detergent resistance of LPA_1, which is consistent with the notion that LPA_1 associates with cholesterol-rich membrane microdomains.

Interestingly, total detergent-resistant LPA_1 staining increased with time of LPA treatment even after longer periods of agonist stimulation (Fig. 6B, 30 minutes). We have previously shown that about 35-40% of surface LPA_1 receptors are internalized into transferrin receptor$^+$ endosomes after 30 minutes of LPA treatment (Murph et al., 2003). We hypothesize that some of the detergent-resistant LPA_1 staining observed after longer LPA treatment resides in transferrin receptor$^+$ endosomes, which are known to be enriched in cholesterol (Hao et al., 2002). Finally, a recent study showed that LPA stimulation of phosphoinositide 3-kinase and the downstream effector kinase, Akt, was inhibited by MβCD treatment in Vero cells (Peres et al., 2003). Collectively, these data suggest that LPA_1 association with cholesterol-rich plasma membrane regions is critical for LPA-induced signaling through $G\alpha_q$. Given that a pool of $G\alpha_q$ is present in cholesterol-rich caveolae, we hypothesize that the localization of LPA_1 to detergent-resistant membranes is important for their association with the pool of $G\alpha_q$ that is localized to these domains.

In support of a role for cholesterol in LPA_1 endocytosis, we found that cholesterol extraction inhibited LPA_1 association with β-arrestin and the subsequent clathrin-dependent endocytosis of the receptor. Many different GPCRs interact with β-arrestins, which is important for the proper regulation of receptor function (Lefkowitz and Whalen, 2004). Phosphorylation of specific serine/threonine residues in either the cytoplasmic tail or the third intracellular loop, by G protein receptor kinases, leads to the recruitment of β-arrestin proteins, which in turn block G protein/receptor coupling (desensitization) and also promote clathrin-dependent endocytosis (Ferguson et al., 1996; Ferguson et al., 1995). Our data show that wild-type LPA_1 receptors transiently recruit β-arrestin-2-GFP to discrete AP2$^+$ structures at the cell surface, in an agonist-stimulated fashion (Fig. 3), but do not colocalize on endosomes with β-arrestin-2-GFP (Figs 3 and 8). β-arrestins promote clathrin-dependent endocytosis of GPCRs by localizing receptors to clathrin coated pits through an interaction of β-arrestins with both clathrin heavy chain and the μ_2 subunit of the AP-2 clathrin adaptor complex (Goodman et al., 1996; Laporte et al., 1999). Using MEFs derived from β-arrestin 1 and 2 double-knockout mice, we showed that both signal attenuation and endocytosis of LPA_1 is dependent upon β-arrestin (Figs 2 and 3). Using an RNA interference approach to reduce the cellular abundance of clathrin heavy chain, we showed that knockdown of clathrin inhibited the internalization of LPA_1, transferrin receptors, but not the internalization of the GPI-anchored protein, CD59, which localize to cholesterol-rich membrane regions (Fig. 1). Taken together, these data indicate that LPA_1 receptors are internalized by β-arrestin- and clathrin-dependent endocytosis.

The most significant finding of these studies was that cholesterol extraction inhibited β-arrestin recruitment to the plasma membrane by LPA_1 and the subsequent endocytosis of these receptors (Figs 5 and 8) and that re-addition of cholesterol to MβCD treated cells restored both of these functions. As β-arrestin binding to LPA_1 precedes receptor endocytosis, we hypothesize that cholesterol is required for the association of LPA_1 with β-arrestins and that it is the lack of β-arrestin binding that leads to the inhibition of LPA_1 endocytosis. This is a novel and previously unappreciated role for membrane cholesterol in the recruitment of β-arrestins. We speculate that other GPCRs that localize to caveolae may also associate with β-arrestin in a cholesterol-dependent manner. β_2ARs localize to caveolae in cardiomyocytes in the absence of agonist but move out of caveolae and into clathrin-coated pits upon ligand binding (Ostrom et al., 2001; Rybin et al., 2000). As β_2AR endocytosis requires β-arrestin binding, it is probable that β-arrestin also binds to these receptors in caveolae. Whether the association of β-arrestin with β_2ARs in cardiomyocytes is cholesterol-dependent remains to be determined.

In contrast to LPA_1, cholesterol extraction did not inhibit the endocytosis of M_1 mAChRs (Fig. 5), which also follow a β-arrestin- and clathrin-dependent pathway (Vogler et al., 1999) nor did cholesterol extraction inhibit the association of β_2ARs with β-arrestins. This suggests that the cholesterol dependence of β-arrestin recruitment is a unique property of LPA_1. Cholesterol may be important either for the direct recruitment and binding of β-arrestins to LPA_1Rs or for the recruitment of kinases such as GRKs that phosphorylate agonist-stimulated LPA_1. Indeed, GRK4 and GRK6 are palmitoylated, which is required for their membrane association (Premont et al., 1996; Stoffel et al., 1994), and palmitoylation has been shown to target many proteins to cholesterol-rich membranes including SNARES (Salaun et al., 2005), flotillins (Neumann-Giesen et al., 2004) and RGS16 (Hiol et al., 2003). Recent work has shown that both LPA stimulation and activation of protein kinase C with phorbol esters promotes LPA_1 phosphorylation (Avendano-Vazquez et al., 2005).

Are there physiological contexts where changes in cellular cholesterol modulate LPA signaling? One intriguing example may be prostate cancer cells, whose growth is potently stimulated by LPA. Cholesterol is elevated in these cells and

contributes to their enhanced growth (Heemers et al., 2001; Mills and Moolenaar, 2003; Zhuang et al., 2002), perhaps by augmenting LPA signaling. Future studies should provide the answer to this and other questions about this novel process.

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