Agonist-stimulated β-Adrenergic Receptor Internalization Requires Dynamic Cytoskeletal Actin Turnover*

Received for publication, October 20, 2005, and in revised form, January 18, 2006 Published, JBC Papers in Press, February 6, 2006, DOI 10.1074/jbc.M511435200

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Stimulation of β-adrenergic receptors (BARs) leads to sequential recruitment of β-arrestin, AP-2 adaptor protein, clathrin, and dynamin to the receptor complex, resulting in endocytosis. Whether a dynamic actin cytoskeleton is required for βAR endocytosis is not known. In this study, we have used β1- and β2ARs, two ubiquitously expressed members of the βAR family, to comprehensively evaluate the requirement of the actin cytoskeleton in receptor internalization. The integrity of the actin cytoskeleton was manipulated with the chemical agent that disrupts the actin cytoskeleton and mutants of the actin-binding protein cofilin that mimics a constitutive phosphorylated state and leads to normal agonist-stimulated βAR endocytosis. Finally, treatment with jasplakinolide, an inhibitor of actin turnover, resulted in dose-dependent inhibition of βAR internalization, suggesting that turnover of actin filaments at the receptor complex is required for endocytosis. Taken together, these data demonstrate that intact and functional dynamic actin cytoskeleton is required for normal βAR internalization.

β-adrenergic receptors (BARs) belong to the large family of seven-transmembrane receptors that are important regulators of cardiac function. In the heart, BARs bind catecholamines and transduce intracellular signals of appropriate magnitude and specificity through tightly regulated mechanisms of receptor activation, desensitization, and internalization (1). βAR signaling and the actin cytoskeleton are important for normal cardiac function, and alterations in either system have been implicated in the development of cardiac hypertrophy and failure (2). Agonist-mediated βAR internalization is a complex process that is mediated by many proteins, including β-arrestin, clathrin, and dynamin (1); however, whether the actin cytoskeleton is essential for βAR internalization is controversial (3).

Actin is a critical component of the cytoskeletal network, undergoes dynamic regulation in response to extracellular stimuli, and has been implicated in vesicular trafficking, including endocytosis (4). In budding yeast, actin assembly and disassembly are tightly coupled to vesicle budding and fusion events (5). Furthermore, actin rearrangements in yeast are thought to be essential for receptor endocytosis, since disruption of these rearrangements by either genetic or chemical methods inhibits receptor internalization (6). Although studies have shown transient recruitment of actin and regulators of actin polymerization (like N-WASP and Arp2/3) to clathrin-coated vesicles after detachment from the plasma membrane (7), the functional role of intact actin architecture in agonist-mediated receptor internalization in mammalian cells remains unclear. A number of studies both support and refute the involvement of actin cytoskeleton in receptor-mediated endocytosis (3).

The ambiguity in the requirement of actin cytoskeleton for receptor internalization stems from conflicting data obtained by using agents that chemically disrupt actin assembly/disassembly dynamics. For example, both latrunculin A and cytochalasin D disrupt actin cytoskeleton, but marked attenuation in internalization of endothelin or epidermal growth factor receptors is seen only with latrunculin A and not with cytochalasin D (8). In contrast, cytochalasin D blocks agonist-induced internalization of α1B-adrenergic receptor (9), underscoring the complexity of actin dynamics. Considering these contrasting results with chemical agents, we sought to investigate the role of cortical actin cytoskeleton in agonist-mediated βAR internalization by using a chemical agent that disrupts the actin cytoskeleton and mutants of the actin-binding protein cofilin known to alter the integrity of the actin cytoskeleton.

Cofilin is a small 15–20-kDa protein that binds to actin and increases actin dynamics by depolymerizing actin filaments (10). Cofilin biochemically drives the equilibrium between actin filament and monomers toward actin monomers, resulting in actin depolymerization (10). Furthermore, cofilin activity is regulated by phosphorylation, wherein phosphorylation of cofilin by LIM kinase inhibits the ability of cofilin to depolymerize actin and dephosphorylation leads to its activation (11). In this study, we tested the role of cortical actin cytoskeleton in βAR internalization by disrupting actin cytoskeleton employing small molecule agent latrunculin B (LB) and using cofilin mutants that mimic constitutively active and inactive forms to clearly define the role of actin in agonist-mediated βAR internalization.

MATERIALS AND METHODS

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells were maintained and transfected with Fugene6 (Roche Applied Science) as previously described (28). Stable cell lines or transiently transfected cells (after 48 h of transfection) were serum-starved for 2 h prior to agonist stimulation. The plasmid encoding enhanced yellow fluorescence protein, a marker of transfection, was used to monitor transfection efficiency.
fluorescent protein (YFP)-tagged β2AR was previously described (14), and cDNAs for the wild type (WT) β2AR and the β2AR mutants lacking putative protein kinase A (PKA) and G-protein-coupled receptor kinase (GRK) phosphorylation sites have been previously described (13). HA-β2AR and GFP-β-arrestin 2 double stable cell line was a gift from Dr. Marc Caron (Duke University Medical Center). Plasmid encoding HA-tagged wild-type dynamin and a cell line with stable expression of FLAG-GRK was a gift from Dr. L. S. Barak (Duke University Medical Center, Durham, NC). For experiments employing LB (Calbiochem) or jasplakinolide (Calbiochem), cells were pretreated with vehicle alone (Me2SO) (panels 1–3) or LB at the indicated concentrations of 1 μM (panels 4–6), 5 μM (panels 7–9), and 10 μM (panels 10–12) for 1 h prior to fixing in 4% paraformaldehyde. The cells were stained with phalloidin-Texas Red for actin cytoskeleton, and β2AR was visualized by green fluorescence. Disruption of actin cytoskeleton by LB treatment did not affect the membrane distribution of β2ARs. β2AR was visualized by green fluorescence. Disruption of actin cytoskeleton by LB treatment did not affect the membrane distribution of β2ARs. β2AR was visualized by green fluorescence.

**Confocal Microscopy**—Transiently transfected or stably expressing cells were plated onto glass bottom Petri dishes (MatTek Corp.) as previously described (14). The cells were serum-starved for 2 h and pretreated with (1, 5, and 10 μM) LB, Jas, or vehicle for 1 h prior to agonist stimulation. Cells were stimulated with 10 μM isoproterenol (10 min for β2AR or 30 min for β1AR) or 100 nM vasopressin (30 min). Following stimulation, cells were fixed, stained with phalloidin-Texas Red, and visualized by confocal microscopy. Progressive disruption of actin cytoskeleton (visualized by phalloidin staining) with increasing concentrations of LB results in significant inhibition of agonist-mediated β2AR internalization. The experiments were independently repeated at least three times, and a minimum of 100 cells were visualized for each of the given conditions.

**Generation of Cofilin Constructs**—Total RNA from mouse brain was isolated by phenol and guanidine thiocyanate extraction using RNA-BEE (Tel-Test, Inc.) followed by isopropyl alcohol precipitation. Total RNA was used to isolate the cDNA encoding cofilin (GenBank™ accession number NM_007687) by reverse transcription-PCR using primers corresponding to the 5’ and 3’ coding sequence of mouse cofilin. The amplified cDNA corresponding to cofilin was subcloned into PCR-II vector (Invitrogen) and sequenced to check for authenticity. Specific oligonucleotide primers were used to generate the S3A and S3D cofilin mutant constructs that contained C-terminal Myc epitope tags. Each cofilin construct was subcloned into pcDNA3.1 vector (Invitrogen) and sequenced to verify site-directed mutagenesis.

**Membrane Fractionation and Radioligand Binding**—Plasma membrane and cytosolic fractions were prepared as previously described (14). Briefly, cells were homogenized in a buffer containing 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and aprotinin each. Cell debris was removed by centrifugation at 1,000 × g for 5 min. The supernatant was subjected to centrifugation at 37,000 × g for 25 min. The pellet plasma membrane was resuspended in binding buffer (75 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 5 mM EDTA). Early endosomal fraction was recovered by ultracentrifugation for 1 h at 300,000 × g of the cytosolic fraction following 37,000 × g centrifugation. Receptor binding with 25 μg of protein from...
the plasma membrane and early endosomal fraction was performed as described previously using [125I]cyanopindolol (250 pmol/liter) (14, 29).

**Immunoprecipitation and Immunoblotting**—The plasma membrane pellet was prepared as described above. Following 37,000 x g spin the pellet was resuspended in buffer containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Nonidet P-40, 20% glycerol, 10 mM NaF, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of aprotinin and leupeptin and phosphatase inhibitor mixture I and II (Sigma). β2ARs from the membrane fraction were immunoprecipitated using FLAG monoclonal antibody (Sigma). The immunoprecipitates were washed, and proteins were resolved in 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (Bio-Rad) membrane for immunoblotting. HA monoclonal antibody (Roche Applied Science) was used at 1:2000 dilution to detect dynamin. Similarly, cofilin was immunoprecipitated with Myc monoclonal antibody (Cell Signaling) from the membrane fractions and immunoblotted with Myc at a 1:1000 dilution. Blots were incubated with appropriate secondary antibody (1:2000) conjugated to horseradish peroxidase (Amersham Biosciences), and detection was carried out using enhanced chemiluminescence.

**Metabolic Labeling**—HEK 293 cells were transfected with 5 μg of FLAG-β2AR alone or along with 5 μg of WT or S3A or S3D cofilin or empty vector. The cells were incubated at 37 °C for 60 h and then placed in phosphate-free medium for 2 h at 37 °C prior to incubation in [32P]labeled medium (200 μCi) for 1 h. The cells were treated with 10 μM isoproterenol or vehicle for 3 min, washed three times with ice-cold 1× phosphate-buffered saline, and lysed for 1 h in 750 μl of radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% Nonidet P-40, 10 mM NaF, 10 mM sodium pyrophosphate, 0.1% SDS, 0.5% sodium deoxycholate, 100 μM sodium orthovanadate, 2 μg/ml each aprotinin and leupeptin). The cell lysate was cleared by centrifugation at 12,000 rpm for 20 min, and β2AR was immunoprecipitated from the supernatant with anti-FLAG M2 antibody (Sigma). The immunoprecipitates were washed in radioimmune precipitation buffer, the proteins were resolved on 12% SDS-polyacrylamide gels, and radiolabeled β2AR was detected by autoradiography.

**RESULTS**

**Disruption of Cortical Cytoskeleton by Latrunculin B Inhibits β2AR Internalization**—To examine the necessity for an intact actin cytoskeleton in agonist-mediated internalization of β2ARs, we first studied the effects of an actin-disrupting compound on receptors in the unstimulated state. HEK 293 cells were transfected with cDNA encoding β2AR-YFP and treated with 0–10 μM LB, and then the β2AR distribution was visualized by confocal microscopy. LB is a low molecular weight, membrane-permeable reagent that sequesters actin monomers and effectively disrupts cortical actin filaments (12). In the absence of LB, the cortical actin cytoskeleton is localized beneath plasma membrane as a discontinuous structure visualized by phalloidin-Texas Red staining that co-localizes with β2AR-YFP (Fig. 1A,
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panels 1, 2, and 3). Treatment of cells with LB resulted in a dose-dependent depolymerization of the cortical actin cytoskeleton with a maximal disruption observed at a concentration of 10 μM LB (Fig. 1A, panels 5, 8, and 11). Importantly, disruption of cortical actin organization by increasing concentrations of LB had no effect on the membrane distribution of βARs in the absence of agonist (Fig. 1A, panels 1, 4, 7, and 10). Next, we tested whether disruption of the actin cytoskeleton by LB impaired the agonist-mediated internalization of βARs. In the absence of LB, isoproterenol stimulation resulted in marked redistribution of the receptors into cellular aggregates (Fig. 1B, panels 1 and 3) without detectable changes in cortical actin organization (Fig. 1B, panels 2 and 3). We have previously observed similar internalization of βARs after isoproterenol stimulation with other cell types. As actin cytoskeleton depolymerized with increasing concentrations of LB (Fig. 1B, panels 5, 8, and 11), we observed a progressive impairment of agonist-mediated βAR internalization (Fig. 1B, panels 4, 7, and 10). Complete inhibition of βAR internalization was seen at 10 μM LB, with receptors forming aggregates at the plasma membrane without entering inside the cell (Fig. 1, panel 10). To show that LB treatment did not cause permanent cell toxicity, cells pretreated with LB were replenished with medium without LB for 12 h. Following the removal of LB, the cell shape, cytoskeleton architecture, and agonist-mediated βAR internalization were completely restored and were indistinguishable from cells that had no exposure to LB (data not shown). These studies demonstrate that an intact cortical cytoskeleton is required for agonist-mediated βAR internalization.

Since confocal microscopy revealed that LB inhibited βAR internalization after isoproterenol treatment, we employed radioiodinated ligand binding to receptors in cellular fractions from plasma membranes and early endosomes as an independent measure of alterations in βAR internalization in the presence of LB. HEK 293 cells were transfected with cDNA encoding FLAG-β2AR and treated with isoproterenol or vehicle alone, and βARs in the plasma membrane and early endosome fractions were quantified. Early endosomal fraction was validated by Western immunoblotting for Rab5, a well-known marker of early endosomal compartments (Fig. 2B). These results indicate that an intact actin cytoskeleton is required for agonist-mediated βAR internalization.

Intact Cortical Actin Cytoskeleton Is Required for Clathrin- and/or Caveolin-mediated Internalization Pathways—Agnost-treated βARs utilize two pathways for internalization, through either clathrin-coated pits or caveolae (13–15). To examine whether intact actin cortical cytoskeleton is required for clathrin- or caveolae-mediated receptor internalization, we employed βAR phosphorylation mutants that we have recently shown selectively internalize via either clathrin- or caveolae-dependent processes (13). In that study, we showed that βAR mutants that lack the PKA phosphorylation sites (PKAβAR) utilize clathrin-dependent pathways for agonist-mediated internalization, whereas βAR mutants that lack G-protein-coupled receptor kinase phosphorylation sites (GRKβAR) use a caveolae-dependent pathway for internalization (13). HEK 293 cells stably expressing FLAG-tagged WT β2AR, PKAβAR, or GRKβAR were pretreated with LB and stimulated with isoproterenol. In the absence of LB, cells treated with isopropenol showed marked redistribution of WT β2AR, PKAβ2AR, or GRKβ2AR into intracellular aggregates (Fig. 2, panels 1–3). In contrast, pretreatment of cells with LB caused a complete inhibition of receptor internalization as visualized by receptor aggregates on the plasma membrane for the WT β2AR and both βAR mutants (Fig. 2, panels 4–6). These results indicate that an intact actin cytoskeleton is required for both caveolin- and clathrin-mediated βAR endocytosis, since disruption of the actin cytoskeleton completely blocks receptor internalization through either pathway.

Depolymerization of Cortical Actin Cytoskeleton Does Not Impair β-Arrestin Translocation or Dynamin Recruitment to the Ligand-activated β2AR—The internalization of BAR in response to agonist stimulation requires the sequential recruitment of β-arrestin to the activated receptor complex and dynamin to the early vesicle that forms at the plasma membrane. Since disruption of the actin cytoskeleton inhibits proper receptor internalization after agonist exposure, we examined whether a mechanism for the inhibition of receptor internalization could be the impaired recruitment of β-arrestin and dynamin to the receptor complex. HEK 293 cells stably expressing β2AR and GFP-β-arrestin 2 were pretreated with LB, and agonist-mediated β-arrestin translocation to the receptor complex was examined by confocal microscopy. The translocation of β-arrestin from the cytosol to the plasma membrane after isoproterenol stimulation was similar in the absence or presence of LB (Fig. 3), demonstrating that LB-mediated disruption of the cortical actin cytoskeleton does not impact β-arrestin translocation to the receptor complex. We next investigated whether the disruption in the actin cytoskeleton affected the recruitment of dynamin to the agonist-occupied receptor complex. HEK 293 cells were co-transfected

FIGURE 3. Disruption of the actin cytoskeleton prevents neither β-arrestin nor dynamin recruitment to the receptor complex. A, HEK 293 cells stably expressing HA-β2AR and GFP-β-arrestin 2 were stimulated in the presence and absence of LB. The cells were fixed with ice-cold methanol and β-arrestin was visualized by green fluorescence. In the absence of isoproterenol (ISO) stimulation, β-arrestin is cytosolic in distribution (panels 1 and 3). Stimulation with isoproterenol results in β-arrestin translocation to the membrane even in the presence of LB (panels 2 and 4). B, HEK 293 cells were co-transfected with FLAG-β2AR and HA-dynamin. The cells were pretreated with LB and stimulated with isoproterenol for 0, 5, and 7 min. β2ARs were immunoprecipitated (IP) from the membrane fraction using FLAG monoclonal antibody and immunoblotted (IB) for HA to monitor co-immunoprecipitating dynamin. Lower panels, immunoblotting shows similar levels of dynamin (HA) and β2AR (FLAG), C, summary data of four independent experiments representing recruitment of dynamin to the receptor complex.
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with FLAG-β2AR and HA-dynamin, pretreated with LB or vehicle alone, and then stimulated with isoproterenol for 0, 5, and 7 min. Association of dynamin with immunoprecipitated β2ARs showed that isoproterenol stimulation resulted in significant recruitment of dynamin to the receptor complex that was equal in the absence or presence of LB (Fig. 3, B and C). Taken together, these studies demonstrate that the mechanism by which LB blocks β2AR internalization is distinct from the events that are responsible for the recruitment of β-arrestin and dynamin to the activated receptor complex.

Cofilin Blocks Agonist-dependent β2AR Internalization—As an alternative independent approach to investigate the requirement of intact actin cytoskeleton, we examined the effects of cofilin on β2AR internalization. Cofilin is a phosphoprotein that is activated after dephosphorylation, promoting breakdown of actin filaments (10). Previous work has demonstrated that mutations of serine to alanine (S3A) or serine to aspartate (S3D) in cofilin leads to constitutively active or inactive phosphorylation, promoting breakdown of actin filaments (10). Previous work has demonstrated that mutations of serine to alanine (S3A) or serine to aspartate (S3D) in cofilin leads to constitutively active or inactive phosphorylation, promoting breakdown of actin filaments (10). Previous work has demonstrated that mutations of serine to alanine (S3A) or serine to aspartate (S3D) in cofilin leads to constitutively active or inactive phosphorylation, promoting breakdown of actin filaments (10). Previous work has demonstrated that mutations of serine to alanine (S3A) or serine to aspartate (S3D) in cofilin leads to constitutively active or inactive phosphorylation, promoting breakdown of actin filaments (10). Previous work has demonstrated that mutations of serine to alanine (S3A) or serine to aspartate (S3D) in cofilin leads to constitutively active or inactive phosphorylation, promoting breakdown of actin filaments (10).

In contrast, isoproterenol stimulation in the presence of S3D cofilin resulted in marked redistribution of receptors into cellular aggregates (Fig. 4C, panels 3 and 4). Interestingly, WT cofilin, which has a cytosolic distribution in unstimulated cells, translocated to the membrane upon isoproterenol treatment (Fig. 4A, panels 2 and 4) that was validated by cellular fractionation studies (Fig. 4D). The constitutively activated cofilin mutant (S3A) was localized at the cell membrane in both resting and stimulated cells, whereas in contrast, the inactivated cofilin mutant (S3D) had a cytosolic distribution under both basal and stimulated conditions (Fig. 4, B and C).

Since β2AR phosphorylation is necessary for receptor internalization following isoproterenol stimulation, we examined whether overexpression of Cofilin mutants altered the level of β2AR phosphorylation after agonist stimulation. HEK-293 cells expressing β2AR and cofilin or cofilin mutants were metabolically labeled with 32P and stimulated with isoproterenol. FLAG-β2ARs were immunoprecipitated and resolved on an SDS-polyacrylamide gel, and phosphorylation was monitored by autoradiography. Neither WT cofilin nor the cofilin mutants inhibited isoproterenol-stimulated phosphorylation of β2AR (Fig. 4E). Interestingly, isoproterenol-stimulated phosphorylation of the β2AR was more dramatic in the presence of WT cofilin or the cofilin mutants. Furthermore, WT cofilin or the cofilin mutants did not inhibit the phosphorylation of either β2AR.
that lacked GRK phosphorylation sites or β2AR in the presence of the PKA inhibitor, H-89 (data not shown). These studies demonstrate the critical requirement of intact actin cytoskeleton for receptor internalization and that intact actin cytoskeleton is not required for the phosphorylation of the receptor.

**Intact Cortical Actin Cytoskeleton Is Required for V2R Internalization**—To determine whether the internalization of other GPCRs requires an intact cortical actin cytoskeleton, we evaluated the agonist-induced internalization of vasopressin receptor, which utilizes a clathrin-mediated pathway (17). Confocal microscopy was performed on HEK 293 cells that were transfected with vasopressin receptor tagged with GFP (V2R-GFP) and stimulated with 100 nM vasopressin. In the absence of LB, there was a marked redistribution of receptors, indicating that intact actin cytoskeleton is necessary for receptor internalization (Fig. 5A, panels 1 and 2). In contrast, LB pretreatment completely disrupted the actin architecture (Fig. 5A, panels 3 and 4). These findings suggest that the actin cytoskeleton is a necessary component for the internalization of GPCRs.

**Disruption of Cortical Cytoskeleton in Other Cell Types Blocks β2AR Internalization**—We next addressed whether the effects of LB on β2AR internalization were specific to HEK293 cells or could be recapitulated in other cell types. U2 OS (osteosarcoma epithelial) cells were transfected with cDNA encoding β2AR-YFP, and receptor internalization was examined in the absence or presence of pretreatment with LB. U2 OS cells that were not pretreated with LB showed a defined actin architecture (Fig. 5B, panels 1 and 3), and β2ARs underwent effective internalization after treatment with isoproterenol (Fig. 5B, panels 1 and 3). In contrast, LB pretreatment completely disrupted the actin architecture (Fig. 5B, panels 5 and 6), and β2ARs did not effectively internalize after agonist exposure (Fig. 5B, panels 4 and 6). Furthermore, treatment of β2AR-transfected COS 7 cells with LB demonstrated findings similar to those with HEK 293 cells (data not shown).

**Dynamic Cytoskeletal Actin Turnover Is Required for β2AR Endocytosis**—Since disruption of actin cytoskeleton markedly blocked receptor internalization, we examined whether actin stabilization would also alter β2AR trafficking. HEK 293 cells were transfected with YFP-tagged β2ARs and pretreated with 1, 5, and 10 μM jasplakinolide, an actin-stabilizing compound that effectively inhibits active actin turnover and jasplakinolide compete for the same binding site on F-actin (18). Previous studies have shown that jasplakinolide-treated actin filaments resist labeling with phalloidin as jasplakinolide and phalloidin compete for the same binding site on F-actin (18). We observed that increasing jasplakinolide concentration resulted in decreased jasplakinolide staining and was interpreted as stabilization of F-actin structure (Fig. 6, panels 2, 5, 8, and 11). Interestingly, increasing doses of jasplakinolide resulted in progressive and complete inhibition of receptor internalization (Fig. 6, panels 1, 4, 7, and 10), indicating that factors altering either cytoskeletal actin depolymerization or stabilization attenuate receptor internalization.

*Receptor Internalization Requires Dynamic Actin Cytoskeleton*

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**FIGURE 5.** Universal requirement of intact actin cytoskeleton for receptor internalization. A, pretreatment of cells with LB prevents vasopressin receptor internalization. HEK 293 cells were transfected with V2R-GFP and were pretreated with 10 μM LB. In the absence of LB pretreatment, stimulation of cells with 100 nM vasopressin for 30 min results in internalization of receptors as visualized by green fluorescence (panels 1 and 3). Intact actin cytoskeleton is visualized by phalloidin-Texas Red staining (panels 2 and 3). LB pretreatment prior to vasopressin stimulation results in complete disruption of actin cytoskeleton as visualized by loss of phalloidin-Texas Red staining (panels 4 and 6). LB pretreatment results in reduced jasplakinolide staining as visualized by loss of phalloidin-Texas Red staining (panels 5 and 8). LB pretreatment of U2S sarcoma cells prevents agonist-mediated β2AR internalization. U2S sarcoma cells were transfected with β2AR-YFP and stimulated with isoproterenol in the presence and absence of LB. In the absence of LB pretreatment, isoproterenol stimulation triggers receptor internalization as visualized by cellular aggregates (panels 1 and 3). Intact elaborate actin cytoskeleton is visualized by phalloidin-Texas Red staining (panels 2 and 3). LB pretreatment results in disruption of actin cytoskeleton as monitored by phalloidin-Texas Red staining associated with inhibition of receptor internalization (panels 4 and 6).
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**FIGURE 6. Stabilization of actin turnover by jasplakinolide (JAS) prevents isoproterenol-induced internalization of β2AR.** HEK 293 cells transiently expressing β2AR-YFP were pretreated with jasplakinolide at various concentrations for 1 h (0 μM: panels 1, 2, and 3; 1 μM: panels 4, 5, and 6; 5 μM: panels 7, 8, and 9; and 10 μM: panels 10, 11, and 12). Cells were stimulated with isoproterenol for 10 min, fixed, and stained with phalloidin-Texas Red. Receptor internalization was visualized by confocal microscopy. Green, β2ARs; red, actin.

**DISCUSSION**

The activation and internalization of GPCRs is critical to normal cellular physiology, and alterations in these processes underlie the pathophysiology of several disease states, including heart failure. Although the cytoskeleton has long been implicated in the process of vesicle budding and trafficking, the role of actin in receptor internalization, especially in mammalian cells, remains poorly characterized. In our current study, we demonstrate the requirement for an intact actin cytoskeleton in β2AR internalization by using both biochemical and molecular tools. Administration of either LB, which effectively sequesters actin monomers and disrupts cortical actin filaments (12, 19), or jasplakinolide, which shifts the equilibrium from actin monomers to actin filaments (18), results in complete inhibition of receptor internalization. The requirement of cytoskeletal actin for receptor internalization is further validated by using cofilin, which effectively shifts the equilibrium of actin from filaments to monomers.

Our results demonstrate that disruption of the actin cytoskeleton did not alter β2AR membrane distribution in unstimulated cells but markedly inhibited β2AR internalization in response to agonist. This is consistent with a previous study of a non-GPCR, the transferrin receptor, where disruption of the actin cytoskeleton significantly inhibited constitutive internalization (20). Previous studies have shown that inhibition of constitutive endocytosis of transferrin receptors by disruption of the actin cytoskeleton is dependent on the cell type and growth conditions. Our findings demonstrate a dose-dependent disruption of the actin cytoskeleton by LB, which results in complete inhibition of agonist-mediated β2AR internalization that is independent of cell type.

Previous studies using various chemical agents that disrupt actin cytoskeleton have provided conflicting results. Although latrunculin A and cytochalasin D disrupt actin cytoskeleton, cytochalasin D does not inhibit agonist-mediated internalization of endothelin A or gastrin-releasing peptide (8). In contrast, latrunculin A inhibits internalization of endothelin A or gastrin-releasing peptide receptors. Consistent with these studies, we show in our current investigation that depolymerization of the actin cytoskeleton by LB results in prevention of β2AR internalization. The variability in the effect of these different small molecules on internalization could be due to the method through which these agents depolymerize actin. Latrunculin A and B bring about depolymerization by shifting the equilibrium to monomeric actin, whereas cytochalasin D preferably inhibits actin turnover in stress fibers, indicating that stress fibers may not play a critical role in agonist-mediated internalization. Since solely relying on chemical agents to disrupt actin can be problematic, we comprehensively examined the requirement of cortical actin cytoskeleton by using cofilin mutants that are well recognized to disrupt actin structure. Our finding that constitutively active but not inactive cofilin prevents βAR internalization is consistent with our hypothesis that an intact actin architecture is necessary for proper receptor endocytosis. This observation that βAR endocytosis requires dynamic actin cytoskeleton is consistent with our recent finding that phosphorylation of tropomyosin, a molecule known to stabilize actin filaments, is required for β2AR internalization (21). Importantly, the use of a phosphorylation-deficient mutant of tropomyosin results in inhibition of agonist-mediated β2AR internalization, showing that dynamic actin cytoskeleton is required for receptor internalization.

Agonist stimulation of β2AR triggers phosphorylation of the receptor and results in the recruitment of β-arrestin. β-Arrestin recruits AP-2 adaptor protein and clathrin to the receptor complex, initiating the clathrin-mediated endocytosis (1). Our findings demonstrate that β-arrestin translocation to the receptor complex was not affected by disruption of cortical actin and suggest that the intact actin cytoskeleton may not be necessary for β-arrestin recruitment to the receptor complex. In fact, β-arrestin translocation and the formation of clathrin-coated pits (CCPs) can be observed as early as 3 min after stimulation even in cells in which the actin cytoskeleton has been completely depolymerized. Previous studies have shown that pretreatment of cells with LB markedly reduces the size of preexisting CCPs that are formed by the fusion of individual CCPs after agonist stimulation (15). This is consistent with our observation that receptor aggregates form at the plasma membrane following agonist stimulation.

Dynamins are a critical protein that has been implicated in catalyzing a GTP-dependent endocytic vesicle formation from the plasma membrane (22, 23). Importantly, translocation of dynamin to the agonist-occupied receptor was not prevented by the disruption of the cortical actin cytoskeleton, consistent with the findings that CCPs may recruit dynamin as a priming step in the process of endocytosis (24, 25). Since dynamin has been shown to directly interact with G-actin or other actin accessory proteins (26), the recruitment of dynamin to CCPs may provide the necessary mechanism linking the CCP to the actin cytoskeleton (27). Indeed, our findings suggest that the requirement of the cortical actin cytoskeleton in agonist-stimulated GPCR endocytosis occurs after recruitment of dynamin, since we observed localized receptor aggregates at the plasma membrane. Moreover, recent studies have suggested that both dynamin and actin together could function as mechanoenzymes during the later stages of endocytosis, leading to the complete detachment of the CCP from the plasma membrane and its movement inside the cell (4, 25). Our studies are consistent with this finding, since the complete depolymerization of the actin filaments by either a chemical agent like LB or cofilin results in inhibition of the receptor internalization. Interestingly, treatment of cells with jasplakinolide, a chemical agent that stabilizes actin (18), also inhibits receptor internalization, suggesting a need of dynamic assembly/disassembly of the actin net-
work to bring about efficient internalization. Taken together, our studies demonstrate the necessity for a functional and dynamic actin cytoskeleton for proper βAR internalization.

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