\textbf{β-Arrestins Regulate Protease-activated Receptor-1 Desensitization but Not Internalization or Down-regulation*}

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The widely expressed β-arrestin isoforms 1 and 2 bind phosphorylated G protein-coupled receptors (GPCRs) and mediate desensitization and internalization. Phosphorylation of protease-activated receptor-1 (PAR1), a GPCR for thrombin, is important for desensitization and internalization, however, the role of β-arrestins in signaling and trafficking of PAR1 remains unknown. To assess β-arrestin function we examined signaling and trafficking of PAR1 in mouse embryonic fibroblasts (MEFs) derived from β-arrestin (βarr) knockouts. Desensitization of PAR1 signaling was markedly impaired in MEFs lacking both βarr1 and βarr2 isoforms compared with wild-type cells. Strikingly, in cells lacking only βarr1 PAR1 desensitization was also significantly impaired compared with βarr2-lacking or wild-type cells. In wild-type MEFs, activated PAR1 was internalized through a dynamin- and clathrin-dependent pathway and degraded. Surprisingly, in cells lacking both βarr1 and βarr2 activated PAR1 was similarly internalized through a dynamin- and clathrin-dependent pathway and degraded, whereas the β2-adrenergic receptor (β2-AR) failed to internalize. A PAR1 cytoplasmic tail mutant defective in agonist-induced phosphorylation failed to internalize in both wild-type and β-arrestin knockout cells. Thus, PAR1 appears to utilize a distinct phosphorylation-dependent but β-arrestin-independent pathway for internalization through clathrin-coated pits. Together, these findings strongly suggest that the individual β-arrestin isoforms can differentially regulate GPCR desensitization and further reveal a novel mechanism by which GPCRs can internalize through a dynamin- and clathrin-dependent pathway that is independent of arrestins.

Protease-activated receptor-1 (PAR1), a G protein-coupled receptor (GPCR) for thrombin, is the prototype member of a family of protease-activated receptors. PAR1 couples to Gi, Gq, and G12/13 to elicit a variety of signaling events important for hemostasis, thrombosis, and embryonic development (1, 2). PAR1 is activated by thrombin through an unusual proteolytic mechanism. Thrombin, a serine protease, binds to and cleaves the extracellular amino terminus of PAR1 (3). The newly formed amino terminus of PAR1 then functions as a tethered peptide ligand by interacting with the receptor to trigger signaling (3–5). Despite PAR1’s irreversible proteolytic mechanism of activation and the generation of a tethered ligand that cannot diffuse away, signaling by the receptor is rapidly terminated at the plasma membrane. The molecular mechanisms responsible for termination of PAR1 signaling are not clearly understood.

The molecular mechanisms responsible for GPCR desensitization and resensitization have been extensively studied for the β2-adrenergic receptor (β2-AR) (6, 7). In this model GPCRs are initially desensitized by rapid phosphorylation of the activated form of the receptor by G protein-coupled kinases (GRKs). Phosphorylated receptor then binds arrestin, which impedes interaction with G proteins. Arrestin also facilitates GPCR internalization by interacting with clathrin and the adaptor protein complex-2 (AP-2), components of the endocytic machinery (8, 9). Once internalized into endosomes, receptor dissociates from the ligand, becomes dephosphorylated, and is then recycled back to the plasma membrane ready for activation again.

Phosphorylation of activated PAR1 initiates rapid desensitization and internalization from the plasma membrane. Overexpression of GRK3 and GRK5 enhances PAR1 phosphorylation and markedly inhibits PAR1 signaling (10, 11). Activation of PAR1 cytoplasmic tail mutants that were not phosphorylated signaled more robustly than wild-type PAR1 (12, 13). Moreover, these same mutants also failed to exhibit agonist-triggered internalization. Interestingly, a cytoplasmic tail mutant of PAR1 with limited alanine substitution for serines between residues 391 and 406 is phosphorylated but defective in its ability to uncouple from signaling (14). This same receptor mutant is internalized following activation like wild-type PAR1. Together, these findings suggest that phosphorylation of PAR1’s cytoplasmic tail is important for both rapid desensitization and internalization, however, distinct mechanisms may regulate these processes.

Internalization and lysosomal sorting of activated PAR1 is also important for termination of receptor signaling. Activated PAR1 is internalized through a dynamin- and clathrin-dependent pathway, like many recycling receptors (15, 16). Once internalized, PAR1 is sorted away from recycling receptors and protein complex-2; MEF, mouse embryonic fibroblasts; βarr, β-arrestin; MDC, monodansylcadaverine; AT1A-R, angiotensin type 1A receptor.
targeted to lysosomes for degradation; an event critical for termination of receptor signaling (17–19). In transfected fibroblasts, a mutant PAR1 able to internalize and recycle to the cell surface signaled persistently after activation by thrombin (19). This prolonged signaling is apparently due to recycling and continued signaling by receptors that return to the plasma membrane with their tethered ligands intact. Thus, phosphorylation of activated PAR1 promotes rapid desensitization at the plasma membrane while internalization and lysosomal sorting prevents the receptor from recycling and continuing to signal on the cell surface.

The β-arrestin isoforms 1 and 2 are widely expressed and bind to phosphorylated GPCRs to mediate desensitization and internalization (20). Phosphorylation of PAR1 is important for desensitization and internalization, however, the role of arrestins in signaling and trafficking of PAR1 is not known. In this study, we investigate the function of arrestin in PAR1 signaling and trafficking through the use of mouse embryonic fibroblasts (MEFs) derived from β-arrestin (βarr) knockouts (21). Our findings strongly suggest that βarr1 functions as the predominant regulator of PAR1 desensitization. Moreover, these studies reveal a novel mechanism by which GPCRs can internalize through a dynamin- and clathrin-dependent pathway that is independent of β-arrestins.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Agonist peptide SFLLRN was synthesized as the carboxyl amide and purified by reverse-phase high pressure liquid chromatography (UNC Peptide Facility, Chapel Hill, NC). Isoproterenol, monodansylcavenderine (MDC), and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO).

Monoclonal M1 and M2 anti-FLAG antibodies were from Sigma (St. Louis, MO). Anti-hemagglutinin (HA) mouse monoclonal antibody (HA.11) was from Covance (Richmond, CA). Rabbit polyclonal anti-β-arrestin antibody A1CT has previously been described (21). Anti-PAR1 1809 rabbit polyclonal antibody was generously provided by S. R. Coughlin (University of California, San Francisco, CA) (22). Anti-clathrin rabbit polyclonal antibody protein was a gift from F. Brodsky (University of California, San Francisco, CA) (23). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies for an additional hour. Images were collected using a Nikon Eclipse TE2000-U microscope fitted with a PlanApo 60× oil objective. Fluorescent images, 24-well dishes and incubated with 1 μg/ml M1 anti-FLAG antibody for 1 h at 4°C. Cells were washed, warmed to 37°C, and incubated with DMEM/HEPES/BSA for various times. Antibody that remained bound to the cell surface was removed by washing three times with PBS (Ca2+ - and Mg2+ -free) containing 0.05% EDTA; M1 anti-FLAG antibody was then removed by washing twice with 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, and 3% BSA, and the amount of accumulated antibody was measured by ELISA as previously described (13).

**Immunoblotting—**To detect β-arrestin expression, equivalent amounts of total cell lysates were resolved by SDS-PAGE, transferred, and blotted with a FLAG rabbit polyclonal anti-β-arrestin antibody and imaged by autoradiography. PAR1 protein was measured as follows. Cells plated in 6-well dishes (Falcon) were lysed in 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 200 μM sodium orthovanadate containing protease inhibitors. Protein concentrations were determined using BCA Protein Assay Reagent (Pierce, Rockford, IL), and equivalent amounts of lysates were used for immunoprecipitation with M2 anti-FLAG antibody. Immunoprecipitates were resolved by SDS-PAGE, transferred, and immunoblotted with anti-PAR1 1809 antibody. Immunoblots were developed with ECL-Plus (Amersham Biosciences, Inc., Arlington, IL), imaged by autoradiography, and quantitated by a Bio-Rad Fluor-S MultiImager (Richmond, CA).

**Immunofluorescence Microscopy—**Transfected cells grown on coverslips (22 × 22 mm) and transiently transfected with a total 0.5 μg of wild-type or mutant β-arrestin expression, equivalent amount of accumulated antibody was measured by ELISA as previously described (26).

**Immunofluorescence Microscopy—**Transfected cells grown on coverslips were incubated with either anti-PAR1 antibody or anti-HA antibody (β-AR expression) for 1 h at 4°C; under these conditions, >90% of the cell surface was labeled with antibody. Cells were washed to remove unbound antibody and exposed to agonist for various times at 37°C. Cells were fixed with 4% paraformaldehyde, permeabilized with 100 μg/ml saponin (20°C) for 30 s, and washed three times with PBS containing 1% nonfat dry milk and 150 mM sodium acetate, pH 7. Cells were then blocked in 1% nonfat dry milk/PBS for 15 min and incubated with Alexa-488-conjugated goat anti-mouse antibody and processed for fluorescence microscopy. Images were captured using an Olympus IX70 fluorescence microscope fitted with a PlanApo 60× oil objective and a Spot RT digital camera (Diagnostic Instruments). The final composite image was created in Adobe Photoshop 5.5. Confocal Microscopy—PAR1-expressing cells transiently transfected with wild-type or mutant K44A dynamin and wild-type and β-arrestin knockout MEFs transiently transfected with PAR1 wild-type or mutant C-tail/S/T–A were examined by confocal microscopy. Briefly, cells were incubated with anti-PAR1 antibody for 1 h at 4°C, washed, and treated in the absence or presence of agonist peptide SFLLRN for 10 min at 37°C. Cells were fixed, permeabilized, incubated with species-specific fluoresceine-conjugated secondary antibodies, processed as described above, and imaged by confocal microscopy. For clathrin co-localization studies, 488-nm-expressing cells were incubated with SFLLRN in the presence or absence of agonist peptide SFLLRN for 10 min at 37°C. Cells were fixed, permeabilized, incubated with species-specific fluoresceine-conjugated secondary antibodies for an additional hour. Images were collected using an Olympus Fluoview 300 laser scanning confocal imaging system (Melville, NY) configured with an Olympus IX70 fluorescence microscope fitted with a PlanApo 60× oil objective. Fluorescent images,
Results

Desensitization of PAR1 Signaling Is Impaired in β-Arrestin Knockout Cells—Desensitization of many GPCRs occurs by rapid phosphorylation of the activated form of the receptor and the subsequent binding of arrestins. Arrestin binding uncouples the receptor from signaling and facilitates receptor internalization. Termination of PAR1 signaling requires phosphorylation, and internalization of the receptor is phosphorylation-dependent (12, 13). However, the function of arrestins in the regulation of PAR1 signaling and trafficking remains unknown. To examine the role of arrestins in signaling and trafficking of PAR1, we used mouse embryonic fibroblasts (MEFs) derived from β-arrestin knockout (21). PAR1 containing an amino-terminal FLAG epitope was stably transfected into MEFs that lack expression of either one or both isoforms of β-arrestin. The genotype of the various transfected MEF cell lines is indicated above the lanes; note the complete loss of β-arrestin expression in cells derived from double knockout mice, lanes 5 and 6.

versus βarr2 expression in the various cell lines is due to the greater affinity of AICT antibody for βarr1 protein (21). Quantitative analyses of β-arrestin expression in the various cell lines indicate that the individual β-arrestin isoforms are expressed at relatively similar levels, validating comparisons between the cell lines (21). Thus, the expression of PAR1 in cells that lack either one or both β-arrestin isoforms provides an opportunity to examine arrestin function in signaling and trafficking of PAR1.

To assess the function of arrestin in the regulation of PAR1 signaling, we compared agonist-induced phosphoinositide hydrolysis in MEFs lacking β-arrestin expression to wild-type controls. In these experiments cells stably expressing similar amounts of FLAG-tagged PAR1 on the cell surface were labeled with [3H]inositol and incubated in the absence or presence of α-thrombin for various times at 37°C in media with lithium chloride. The accumulation of [3H]inositol phosphates was then measured. A, signaling by wild-type (WT1) and βarr1,2/−/− cells stably expressing PAR1, basal [3H]inositol phosphates were on average 1639 cpm/well (PAR1-WT1) and 613 cpm/well (PAR1-βarr1,2/−/−). B, signaling by PAR1-expressing cells that lack only βarr1 or βarr2 and wild-type controls, basal [3H]inositol phosphates were on average 1033 cpm/well (PAR1-WT2), 1104 cpm/well (PAR1-βarr1/−/−), and 2280 cpm/well (PAR1-βarr2/−/−). The data (mean ± S.E.; n = 3) shown are representative of three independent experiments and are expressed as fold increase [3H]inositol phosphates over basal. The initial surface expression of PAR1 in the individual cell lines was reported in Fig. 1. Note the failure of PAR1 to efficiently desensitize in cells that lack both isoforms of β-arrestin or βarr1 only.
phosphates following 30 min of agonist treatment (Fig. 2A, solid squares), a significantly greater accumulation of inositol phosphates than that observed with wild-type cells. Activation of PAR1 with agonist peptide SFLLRN, a full agonist for the receptor, also induced greater signaling in βarr1,2−/− cells compared with wild-type controls (data not shown). Signaling by endogenous PAR1 was similarly enhanced in β-arrestin double knockouts compared with wild-type control cells (data not shown). Thus, in the absence of β-arrestins, rapid termination of PAR1 signaling is markedly impaired.

To determine whether the individual β-arrestin isoforms would differentially regulate PAR1 signaling, we examined agonist-induced phosphoinositide hydrolysis in MEFs in which the expression of only one β-arrestin isoform was abolished. Cells expressing similar amounts of surface PAR1 were treated in the absence or presence of α-thrombin for various times at 37 °C, and the accumulation of [3H]inositol phosphates was then measured. Wild-type cells showed −2-fold increase in inositol phosphate accumulation at 30 min following activation of PAR1 (Fig. 2B, open circles), consistent with wild-type cell signaling shown above. Cells that lack expression of only the βarr2 isoform showed at most a modest increase in signaling compared with wild-type control cells (Fig. 2B, solid circles). Thus, the remaining βarr1 appears to be sufficient to mediate PAR1 desensitization in βarr2−/− cells. Strikingly, PAR1 signaling was significantly more robust in cells in which only βarr1 expression was abolished; −9-fold increase in PI hydrolysis was measured following 30 min of PAR1 activation (Fig. 2B, solid squares). Similar results were obtained with other independently derived clones (data not shown). Thus, in cells that lack βarr1 but retain βarr2 expression, termination of PAR1 signaling is significantly impaired. These findings strongly suggest that βarr1 functions as the predominant regulator of PAR1 desensitization. Moreover, these findings provide the first example in which the individual isoforms of β-arrestin differentially regulate GPCR desensitization.

PAR1 Is Internalized via a β-Arrestin-independent Pathway—To investigate the role of arrestin in trafficking of PAR1, we compared agonist-induced internalization in cells that lack β-arrestins to wild-type controls by cell surface ELISA. Cells expressing similar amounts of FLAG-tagged PAR1 were incubated in the absence or presence of agonist peptide SFLLRN for various times at 37 °C. After agonist treatment, the amount of PAR1 remaining on the cell surface was measured and used as an index for receptor internalization. In wild-type cells, −50% of PAR1 was internalized from the cell surface following 30 min of agonist treatment (Fig. 3, A and B, open circles). These data are consistent with agonist-induced PAR1 internalization reported in other cell types (13, 16). In cells that lack expression of either βarr1 or βarr2, the rate of agonist-induced internalization was similar (Fig. 3A, solid circles and solid squares), suggesting that neither isoform of β-arrestin is solely required for receptor internalization. To our surprise, in cells that lack expression of both isoforms of β-arrestin, the rate of agonist-induced PAR1 internalization was remarkably similar to wild-type controls, with −50% of receptor internalized following 30 min of agonist treatment (Fig. 3B, open circles and solid squares). Similar results were observed in other independently derived clones (data not shown). Other experiments using antibody uptake assays to measure receptor internalization were consistent with these findings (data not shown). The ability of activated PAR1 to internalize in cells that lack both isoforms of β-arrestin strongly suggests that arrestins are not essential for PAR1 internalization.

Internalization of activated PAR1 assessed by immunofluorescence microscopy was consistent with a β-arrestin-independent pathway for receptor internalization. In these experiments, cells were incubated with anti-FLAG antibody for 1 h at 4 °C, so that only PAR1 on the cell surface bound antibody. Cells were then fixed, and the amount of PAR1 remaining on the cell surface was measured by ELISA and used as an index for receptor internalization. The data are expressed as a fraction of the total amount of antibody bound to the cell surface. These cell lines were the same used for analysis of PAR1 signaling (see Fig. 1 for PAR1 expression levels). The data (mean ± S.E.) are averages of three separate experiments performed in triplicate. A, PAR1 internalization in cells that lack only one β-arrestin isoform (βarr1 or βarr2) and wild-type controls. B, internalization of PAR1 in cells that lack expression of both β-arrestin isoforms and wild-type controls. The nonspecific antibody binding to untransfected βarr1,2−/− cells is shown (UT; X). Similar results were obtained with other independently derived clones expressing PAR1 (data not shown). Note that PAR1 was rapidly internalized following addition of agonist even in the absence of β-arrestin.

Fig. 3. Agonist-induced PAR1 internalization in wild-type and β-arrestin knockout MEFs. Cell lines stably expressing PAR1 were incubated with or without 50 μM SFLLRN for various times at 37 °C. Cells were then fixed, and the amount of PAR1 remaining on the cell surface was measured by ELISA and used as an index for receptor internalization. The data are expressed as a fraction of the total amount of antibody bound to the cell surface. These cell lines were the same used for analysis of PAR1 signaling (see Fig. 1 for PAR1 expression levels). The data are expressed as a fraction of the total amount of antibody bound to the cell surface. These cell lines were the same used for analysis of PAR1 signaling (see Fig. 1 for PAR1 expression levels). The data (mean ± S.E.) are averages of three separate experiments performed in triplicate. A, PAR1 internalization in cells that lack only one β-arrestin isoform (βarr1 or βarr2) and wild-type controls. B, internalization of PAR1 in cells that lack expression of both β-arrestin isoforms and wild-type controls. The nonspecific antibody binding to untransfected βarr1,2−/− cells is shown (UT; X). Similar results were obtained with other independently derived clones expressing PAR1 (data not shown). Note that PAR1 was rapidly internalized following addition of agonist even in the absence of β-arrestin.
restin isofoms (21). Therefore, we examined β2-AR internalization in our PAR1 stably transfected cells to exclude the possibility of differences that might arise during clonal selection. HA-tagged β2-AR was transiently transfected into wild-type and βarr1,2−/− cells, and internalization was assessed by immunofluorescence microscopy. In wild-type cells expressing both isoforms of β-arrestin, isopropenol induced substantial internalization of β2-AR into endocytic vesicles at 15 min (Fig. 4B, e and f). In contrast, agonist failed to induce β2-AR internalization in the same βarr1,2−/− cells that showed robust PAR1 internalization (Fig. 4B, compare g and h). These findings are consistent with a role for β-arrestins in internalization of β2-AR. Thus, the failure of β2-AR to internalize in βarr1,2−/− cells that showed robust PAR1 internalization suggests that these receptors utilize distinct mechanisms for internalization.

The constitutive internalization of PAR1 maintains an intracellular pool of receptors, which functions in part to replenish the cell surface with new receptors after thrombin stimulation (17, 27). The molecular mechanisms responsible for constitutive trafficking of PAR1 are not known (28). We therefore examined whether β-arrestins mediated constitutive internalization of PAR1 in transfected MEFs. Cells expressing PAR1 were incubated with anti-FLAG antibody, washed, and warmed to 37 °C for various times to permit constitutive internalization of PAR1. After incubations, antibody was stripped from the cell surface, and internalized antibody was measured by ELISA as previously reported (13). In wild-type cells expressing both isoforms of β-arrestin, ~15% of antibody initially bound to PAR1 at the cell surface was internalized at 60 min (Fig. 5A, A, B, and C, open circles). In cells that lack expression of only one β-arrestin isoform, a substantial fraction of antibody was also internalized after 60 min of incubation (Fig. 5B, solid squares). Together these data suggest that, like agonist-dependent internalization, agonist-independent constitutive internalization of PAR1 is mediated through a β-arrestin-independent pathway.
Phosphorylation in both wild-type and PAR1 C-tail:S/T wild-type MEFs transiently transfected with wild-type PAR1 or mutant PAR1 C-tail:S/T→A were treated in the absence (a and c) or presence (b and d) of 50 μM SFLLRN for 10 min at 37 °C. Cells were then fixed, immunostained for PAR1, processed, and imaged by confocal microscopy. B. βarr1,2−/− MEFs transfected with wild-type PAR1 or mutant PAR1 C-tail:S/T→A were treated in the absence (e and g) or presence (f and h) of 50 μM SFLLRN for 10 min at 37 °C and processed as described above. Note the presence of PAR1-containing endosomes in agonist-treated cells transfected with wild-type PAR1 but the absence of such endosomes in agonist-treated cells transfected with mutant PAR1 C-tail:S/T→A. The imaged cells are representative of many cells examined in two independent experiments. The scale bar denotes 10 μm.

Agonist-induced PAR1 Internalization Requires Phosphorylation in Both Wild-type and β-Arrestin Knockout Cells—Activated PAR1 is rapidly phosphorylated and internalized from the plasma membrane (12, 13). A previously described mutant PAR1 expressing both isoforms of β-arrestin, agonist peptide caused a substantial decrease in the amount of PAR1 protein: ~60–70% of PAR1 was degraded at 90 min consistent with that reported for other cell types (18) (Fig. 7, a and b, WT1 and WT2). In cells that lack either one or both isoforms of β-arrestin, agonist induced a similar ~60–70% decrease in PAR1 protein at 90 min (Fig. 7, A and B). Taken together, these findings strongly suggest that activated PAR1 is internalized, sorted to lysosomes, and degraded via a β-arrestin-independent pathway.

PAR1 Is Internalized via a Dynamin- and Clathrin-dependent Pathway in Both Wild-type and β-Arrestin Knockout Cells—β-Arrestins recruit GPCRs to clathrin-coated pits by interacting with clathrin and the adaptor protein complex-2 (8, 9). GPCRs are then internalized by the actions of dynamin, a GTPase that facilitates detachment of clathrin-coated pits from the plasma membrane (29, 30). A mutant K44A dynamin defective in GTPase activity can block endogenous dynamin function and inhibit clathrin-dependent endocytosis in many cell types (31, 32). To determine whether PAR1 internalization was dynamin-dependent in transfected MEFs, we examined whether a mutant K44A dynamin would block agonist-induced PAR1 internalization. In these experiments, cells expressing PAR1 were transiently transfected with wild-type and mutant K44A dynamin tagged with GFP. Cells incubated with anti-FLAG antibody to label surface PAR1 were treated in the presence of agonist peptide SFLLRN for 90 min at 37 °C. Cell lysates were prepared, and the amount of receptor protein remaining was assessed by immunoblotting with anti-PAR1 antibody. In wild-type cells expressing both isoforms of β-arrestin, agonist peptide caused a substantial decrease in the amount of PAR1 protein: ~60–70% of PAR1 was degraded at 90 min consistent with that reported for other cell types (18) (Fig. 7, A and B, WT1 and WT2). In cells that lack either one or both isoforms of β-arrestin, agonist induced a similar ~60–70% decrease in PAR1 protein at 90 min (Fig. 7, A and B). Taken together, these findings strongly suggest that activated PAR1 is internalized, sorted to lysosomes, and degraded via a β-arrestin-independent pathway.

Agonist-induced PAR1 internalization requires phosphorylation in both wild-type and β-arrestin knockout cells. A, wild-type MEFs transiently transfected with wild-type PAR1 or mutant PAR1 C-tail:S/T→A were treated in the absence (a) or presence (b) of SFLLRN for 10 min at 37 °C. Cells were then fixed, immunostained for PAR1, processed, and imaged by confocal microscopy. B. βarr1,2−/− MEFs transfected with wild-type PAR1 or mutant PAR1 C-tail:S/T→A were treated in the absence (e) or presence (f) of SFLLRN for 10 min at 37 °C and processed as described above. Note the presence of PAR1-containing endosomes in agonist-treated cells transfected with wild-type PAR1 but the absence of such endosomes in agonist-treated cells transfected with mutant PAR1 C-tail:S/T→A. The imaged cells are representative of many cells examined in two independent experiments. The scale bar denotes 10 μm.

Fig. 6. Agonist-induced PAR1 internalization requires phosphorylation in both wild-type and β-arrestin knockout cells. A, wild-type MEFs transiently transfected with wild-type PAR1 or mutant PAR1 C-tail:S/T→A were treated in the absence (a and c) or presence (b and d) of 50 μM SFLLRN for 10 min at 37 °C. Cells were then fixed, immunostained for PAR1, processed, and imaged by confocal microscopy. B. βarr1,2−/− MEFs transfected with wild-type PAR1 or mutant PAR1 C-tail:S/T→A were treated in the absence (e and g) or presence (f and h) of 50 μM SFLLRN for 10 min at 37 °C and processed as described above. Note the presence of PAR1-containing endosomes in agonist-treated cells transfected with wild-type PAR1 but the absence of such endosomes in agonist-treated cells transfected with mutant PAR1 C-tail:S/T→A. The imaged cells are representative of many cells examined in two independent experiments. The scale bar denotes 10 μm.

Fig. 7. Agonist-induced degradation of PAR1 in wild-type and β-arrestin knockout cells. A, cells stably expressing FLAG-tagged PAR1 were incubated in the absence (−) or presence (+) of 50 μM SFLLRN for 90 min at 37 °C. PAR1 was immunoprecipitated from equivalent amount of cell lysates and analyzed by immunoblotting with anti-PAR1 antibody. Immunoblots were developed and quantitated using a Fluor-S Multimager. PAR1 was detected as a prominent ~68 kDa in lysates from transfected cells, this band was absent in lysates prepared from untransfected (UT) cells. B, data are expressed as fraction of total PAR1 detected in untreated control cells. The values shown are the averages (mean ± S.E.) of three independent experiments. Note the exposure to agonist caused substantial degradation of PAR1 even in the absence of β-arrestins.
absence or presence of SFLLRN for 10 min at 37°C, processed, and imaged by confocal microscopy. In wild-type cells transfected with dynamin (Dyn2-wt), agonist triggered substantial redistribution of PAR1 into endocytic vesicles at 10 min (Fig. 8B, white arrow). In contrast, agonist failed to trigger PAR1 internalization in both wild-type and βarr1.2−/− cells transfected with mutant K44A dynamin (Dyn2-K44A) (Fig. 8, A and B, SFLLRN). In both wild-type and βarr1.2−/− cells PAR1 internalization was evident in adjacent cells not expressing mutant K44A dynamin (Fig. 8A and B, SFLLRN, open arrow). Thus the ability of mutant K44A dynamin to block PAR1 internalization suggests that the receptor utilizes a dynamin-dependent pathway in these cells, consistent with previous reports (16).

Dynamin functions in detachment of clathrin-coated pits from the plasma membrane and can also mediate detachment of caveolae in some cell types (33, 34). To determine whether PAR1 internalized through a clathrin-dependent pathway, we examined colocalization of activated PAR1 with clathrin in both wild-type and βarr1.2−/− cells expressing mutant K44A dynamin (Dyn2-K44A) (Fig. 8A and B, SFLLRN). These images are representative of many cells examined in two separate experiments. The scale bar represents 20 µm. B, inhibition of agonist-induced PAR1 internalization by clathrin inhibitors. Wild-type and βarr1.2−/− cells were either left untreated (none) or pretreated with 400 µM MDC or 0.4 M sucrose for 10 min at 37°C. Cells were then incubated in the absence or presence of 50 µM SFLLRN for 20 min at 37°C, and the amount of PAR1 remaining on the cell surface was measured by ELISA. The data (mean ± S.E.) are expressed as a fraction of the total amount of antibody bound to untransfected control cells and are representative of three separate experiments performed in triplicate. Note the marked inhibition of agonist-induced PAR1 internalization in MDC- and sucrose-treated cells.

Par1 internalization (35), whereas monodansylcadaverine (MDC) appears to interfere with invagination of clathrin-coated pits (36, 37). Both wild-type and βarr1.2−/− cells were pretreated in the absence or presence of inhibitors MDC or sucrose for 10 min at 37°C. Cells were then treated with or without agonist peptide
SFFLRN for 20 min at 37 °C, and the amount of PAR1 remaining on the cell surface was then measured by ELISA. In wild-type and βarr1,2−/− cells, agonist-induced PAR1 internalization was markedly reduced in the presence of either MDC or sucrose compared with untreated controls (Fig. 9B). By contrast, neither of these inhibitors significantly altered agonist-triggered increases in phosphoinositide hydrolysis in both wild-type and β-arrestin knockout cells (data not shown). Together these findings strongly suggest that upon activation PAR1 is recruited to clathrin-coated pits and internalized through a dynamin-dependent pathway in both wild-type and β-arrestin knockout cells.

**DISCUSSION**

PAR1’s proteolytic mechanism of activation is clearly distinct from that of most GPCRs and raises questions regarding the molecular mechanisms responsible for termination of PAR1 signaling. Phosphorylation of activated PAR1 is important for both rapid termination of receptor signaling and internalization from the plasma membrane (12, 13). Many phosphorylated GPCRs bind arrestins, which uncouple the receptor from signaling and facilitate receptor internalization (20). The role of arrestins in the regulation of PAR1 signaling and trafficking has not previously been determined. Prior studies have used heterologous overexpression of wild-type and dominant-negative forms of arrestin to assess function; however, such studies are often complicated by the expression of endogenous protein. Our recent generation of mouse embryonic fibroblasts (MEFs) derived from β-arrestin knockouts offered an opportunity to assess function in cells in which the expression of the individual arrestin isoforms was eliminated genetically by gene knockout (21). We therefore assessed signaling and trafficking of PAR1 in cells that lack either one or both isoforms of β-arrestin.

PAR1 is endogenously expressed in MEFs, and thrombin signaling is completely abolished in MEFs derived from PAR1 knockout mice, thus PAR1 is the predominant mediator of thrombin signaling in these cells (38). In cells lacking both β-arrestin isoforms (βarr1,2−/−) endogenous PAR1 signaling was significantly more robust than wild-type controls. Consistent with these results, in transfected MEFs expressing similar amounts of PAR1, the rate of PAR1 desensitization was significantly slowed, resulting in a greater accumulation of inositol phosphates in β-arrestin knockouts compared with wild-type control cells. Thus, in the absence of β-arrestins, desensitization of PAR1 signaling is significantly impaired. To our knowledge these findings are the first to demonstrate a role for β-arrestins in regulation of PAR1 signaling. Moreover, in cells that lack only βarr1, the rate of PAR1 desensitization was markedly impaired compared with βarr2 lacking cells and wild-type controls. These results strongly suggest that the βarr1 isoform functions as the predominant regulator of PAR1 desensitization. These findings contrast with our recent report in which both β-arrestin isoforms were found to be equally effective in regulating desensitization of β2-AR and AT1A-R using these same knockout cells (21). Thus, these studies provide the first example in which the β-arrestin isoforms can differentially regulate GPCR desensitization.

In addition to regulating GPCR desensitization, β-arrestins can facilitate GPCR internalization. Arrestins bind phosphorylated GPCRs and interact with clathrin and the adaptor protein complex-2 (AP-2) to promote receptor internalization (8, 9). PAR1 requires phosphorylation for internalization through a dynamin- and clathrin-dependent pathway (13, 16), raising the possibility that arrestin might function in this process. In contrast, we found that activated PAR1 is internalized through a dynamin- and clathrin-dependent pathway even when both β-arrestin isoforms are absent. Constitutive internalization of PAR1 was also intact in β-arrestin knockout cells. Interestingly, PAR1 internalization required phosphorylation even in the absence of β-arrestins. Together, these findings are consistent with a distinct phosphorylation-dependent but arrestin-independent pathway for PAR1 internalization. By contrast, the β2-AR failed to internalize in these same β-arrestin knockout cells. The β2-AR is known to bind arrestin and internalize through a classic dynamin- and clathrin-dependent pathway (39). Thus, the failure of β2-AR to internalize in β-arrestin knockout cells that show robust PAR1 internalization suggests that these receptors have distinct requirements for internalization through clathrin-coated pits. Together, these studies provide strong evidence for a novel mechanism by which GPCRs can internalize through a dynamin- and clathrin-dependent pathway that is independent of arrestins.

The relative contributions of receptor uncoupling via phosphorylation and arrestin binding versus receptor internalization to the rapid termination of PAR1 signaling remain poorly understood. In this study we demonstrate that, in the absence of β-arrestins, rapid desensitization of PAR1 signaling is markedly impaired while internalization remains intact. Thus, internalization is not required for rapid desensitization of PAR1 signaling. In many cases, arrestins can mediate both desensitization and internalization of GPCRs (21); our observations, however, are consistent with a distinct arrestin-independent mechanism for PAR1 internalization. This is also consistent with a recent report in which termination of PAR1 signaling and internalization were separated by mutation of phosphorylation sites within PAR1’s cytoplasmic tail (14). Similar mutants have been reported for the m2 muscarinic acetylcholine receptor (40). In cells lacking arrestins PAR1 signaling was eventually slowed; this may be due to internalization and lysosomal sorting of activated PAR1. Indeed, a mutant PAR1 that internalized and recycled back to the cell surface signaled persistently following activation by thrombin (19). These studies strongly suggest that internalization and lysosomal sorting are critical for termination of PAR1 signaling (18, 19). Thus, β-arrestin is required for rapid desensitization of PAR1 signaling, whereas internalization and lysosomal sorting appear to contribute to termination of PAR1 signaling observed at later times.

Internalization of GPCRs through clathrin-coated pits is a multistep process involving numerous proteins. β-Arrestins bind to clathrin and the adaptor protein complex-2 (AP-2) and thereby link phosphorylated GPCRs to the endocytic machinery (8, 9). It is becoming increasingly clear that the individual β-arrestin isoforms can differentially regulate GPCR internalization. The β2-AR preferentially utilizes βarr2 for sequestration through clathrin-coated pits, whereas both β-arrestin isoforms were equally effective for internalization of the AT1A-R (21). Interestingly, upon recruitment to activated receptors, βarr1 undergoes dephosphorylation at a carboxyl-terminal serine residue (41). This is thought to be critical for interaction with clathrin but not for uncoupling the receptor from signaling. In this study we demonstrate that βarr1 is required for rapid PAR1 desensitization but not for internalization through clathrin-coated pits. Thus, it is possible that upon PAR1 activation βarr1 is recruited to the receptor but fails to undergo dephosphorylation and is, therefore, unable to promote receptor interaction with clathrin. This possibility remains to be tested. In addition to arrestins, AP-2 can function as an adaptor to recruit receptor tyrosine kinases and other membrane proteins to clathrin-coated pits (42); whether AP-2 functions in PAR1 internalization is not known.
In summary, mouse embryonic fibroblasts derived from β-arrestin knockouts provided an opportunity to assess arrestin function in PAR1 signaling and trafficking. These studies show that βarr1 functions as the predominant regulator of PAR1 desensitization and strongly suggest that the individual β-arrestin isoforms can differentially regulate GPCR desensitization. Moreover, these studies reveal a novel arrestin-independent mechanism for PAR1 internalization from the plasma membrane. The challenge now is to elucidate the mechanisms by which activated PAR1 is recruited to clathrin-coated pits and internalized from the plasma membrane; an event critical for termination of receptor signaling (18, 19).

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