β-Arrestins are pleiotropic molecules that mediate signal desensitization, G-protein-independent signaling, scaffolding of signaling molecules, and chemotaxis. Protease-activated receptor-2 (PAR-2), a Gαq/11-coupled receptor, which has been proposed as a therapeutic target for inflammation and cancer, requires the scaffolding function of β-arrestins for chemotaxis. We hypothesized that PAR-2 can trigger specific responses by differential activation of two pathways, one through classic Gαq/Ca2+ signaling and one through β-arrestins, and we proposed that the latter involves scaffolding of proteins involved in cell migration and actin assembly. Here we demonstrate the following. (a) PAR-2 promotes β-arrestin-dependent dephosphorylation and activation of the actin filament-severing protein (cofilin) independently of Gαq/Ca2+ signaling. (b) PAR-2-evoked cofilin dephosphorylation requires both the activity of a recently identified cofilin-specific phosphatase (chronophin) and inhibition of LIM kinase (LIMK) activity. (c) β-Arrestins can interact with cofilin, LIMK, and chronophin and colocalize with them in membrane protrusions, suggesting that β-arrestins may spatially regulate their activities. These findings identify cofilin as a novel target of β-arrestin-dependent scaffolding and suggest that many PAR-2-induced processes may be independent of Gαq/11 protein coupling.

β-Arrestins were originally identified as terminators of G-protein-coupled receptor signaling and later as signaling scaffolds that regulate activation, subcellular localization, and signal duration of mitogen-activated protein kinases (MAPKs). Over the past 5 years, siRNA studies silencing individual β-arrestins have revealed both redundant and selective roles for either β-arrestin-1 or -2 in the regulation of additional signaling molecules such as phosphatidylinositol 3-kinase and RhoA (1–8) and for cellular processes such as chemotaxis, cytoskeletal reorganization, and epithelial permeability (9–15). Until recently, it was assumed that these β-arrestin scaffolds formed only after initial heterotrimeric G protein activation, but studies on several G-protein-coupled receptors have now demonstrated that β-arrestins can mediate signals independently of G-protein coupling (11, 16, 17), highlighting the importance of identifying the signaling cascades they regulate.

Protease-activated receptor-2 (PAR-2), which requires β-arrestins for desensitization, localization of ERK1/2, and cell migration, was one of the first G-protein-coupled receptors shown to utilize β-arrestin-dependent scaffolding (9, 10). PAR-2 is reported to trigger a wide variety of cellular responses (e.g. proliferation, chemotaxis, ion transport, epithelial barrier function, and tumor cell metastasis) in a cell type-specific manner (10, 18–25). It is activated by proteolytic cleavage of its N terminus, which unveils a tethered ligand (SLIGRL/SLIGKV, human/mouse) leading to Goq coupling, hydrolysis of phosphatidylinositol 4,5-bisphosphate, and mobilization of intracellular Ca2+ (25, 26). Most PAR-2 actions are thought to lie downstream of Ca2+ mobilization, although for some events, β-arrestin-dependent signaling can oppose the Goq/Ca2+ pathway (27). To date, the molecular mechanisms by which PAR-2 mediates processes such as cytoskeletal reorganization and chemotaxis have not been elucidated. Because cell migration requires the cell to extend pseudopodia at the leading edge while inhibiting extensions and dissolving stress fibers at the trailing edge (28–30) and β-arrestins are capable of spatially restricting activity of signaling molecules downstream of PAR-2 activation, we hypothesized that β-arrestins might contribute to the spatial and temporal control over actin assembly/disassembly through scaffolding of signaling molecules traditionally associated with chemotaxis. We focused on the actin filament-severing protein cofilin, the activity of which allows for rapid changes in cytoskeletal architecture, creation of new barbed ends for elongation, and recycling of ATP monomers (28, 30–33). Cofilin activation is controlled by opposing actions of LIMKs (which inactivate it by phosphorylation on Ser5) and cofilin-specific phosphatases (chronophin (CIN) and slingshot) that activate it (33–36) as well as by intracellular pH and phosphatidylinositol 4,5-bisphosphate levels (37). In the studies described here, we investigated the following: 1) whether PAR-2 promotes cofilin dephosphorylation and activation through a β-arrestin-dependent mechanism, 2) whether β-arrestins can scaffold and direct subcellular localization of cofilin, and 3) whether β-arrestin-dependent cofilin activation is independ-
ent of classic Goq signaling, and 4) whether upstream cofilin regulators CIN and LIMK are regulated by β-arrestins in response to PAR-2 activation.

MATERIALS AND METHODS

All chemicals were from Sigma unless otherwise stated. Antibodies and final dilutions for Western blot (WB), immunoprecipitation (IP) and immunofluorescence (IF) were as follows: from Chemicon, rabbit anti-phospho (Ser3)-cofilin (1:100 for IF) and mouse anti-cofilin (4 μg/ml for IP); from BD Pharmaningen, mouse monoclonal antibodies to cofilin (1:1000 for WB and 1:250 for IF) and β-arrestin-1 (1:1000 for WB); from Dr. Robert J. Lefkowitz (Duke University Medical Center), affinity-purified rabbit antibody to β-arrestin-1 + 2 (A1CT; 1:500 for WB and 1:200 for IF) or β-arrestin-2 (A2CT, which recognizes β-arrestin-1 weakly as well; 1:500 for WB); from Cell Signaling Technology Inc., rabbit phospho-LIMK1/2 (1:500 for WB) and rabbit anti-phospho (Ser3)-cofilin (1:1000 for WB); from Santa Cruz Biotechnology, goat anti-actin (1:2000 for WB), mouse monoclonal anti-LIMK C10 (2 μg/ml for IP), rabbit anti-Myc A14 (1.5 μg/ml for IP, 2:00 for IF, and 1:1000 for WB), anti-Myc 9E10 (1:1000 for WB), and rabbit anti-Gaα11 (1:1000 for WB); from Molecular Probes, Alexa488, Alexa595, and Alexa680-conjugated secondary antibodies (1:1000 for IF and 1:45,000 for WB); from Rockland, IRDye800-conjugated secondary antibodies (1:1000 for IF and 1:250 for IF) and mouse anti-cofilin (4

Transfections and Cell Lines

MEFs from wild type (MEFwt) and β-arrestin-1/2−/− knock-out mice (MEFβarrDKO) were gifts from Dr. Robert Lefkowitz (Duke University Medical Center) and have been described previously (38). MDA-MB-468 cells were purchased from ATCC and grown in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum. FLAG- and GFP-tagged β-arrestin-1 and -2 were a gift from Dr. Robert Lefkowitz (Duke University Medical Center) and have been described previously (39, 40). Dominant negative CIN and Myc-tagged CIN constructs have been performed previously (36). Transient transfections were performed on 70–80% confluent cells using Lipofectamine 2000 (Invitrogen), and experiments were performed between 24 and 48 h of transfection.

Protein Analysis and Western Blotting

5 × 10^5 cells/60-mm dish (grown for 24 h) were serum-starved for 2 h, treated with 100 nM 2fAP for 0–90 min at 37 °C, lysed in 0.25 ml of lysis buffer (phosphate-buffered saline, pH 7.6, 1% Triton X-100, 1 mM EGTA, 2 mM NaF, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μM each aprotinin, leupeptin, and benzamidine). 25 μg of cleared lysate protein was analyzed by SDS-PAGE (15% for cofilin, 10% for LIMK, and 12.5% for β-arrestin) followed by Western blotting. Blots were imaged using the LICOR Odyssey imaging system, and LICOR software was used to calculate integrated intensities of bands. Images of Western blots were assembled using Adobe Photoshop 5.0 and imported into Canvas. Some gels were spliced to eliminate blank lanes or lanes containing samples unrelated to the figure.

siRNA Transfections

Two sets of oligos were used to knock down β-arrestins. The first set were chemically synthesized double-stranded siRNAs (β-arrestin-1, 5'-AAAGCCUUCUGCCGGAUAAU-3'; β-arrestin-2, 5'-AAGGACGCCAAGUGUUGUG-3'; β-arrestin-1 and -2, 5'-ACCUGCGCCUUCCGGCUAUG-3'; control (non-targeting sequence), 5'-AUCUCUCGGAGCUUCGUACGGU-3') with 19-nucleotide duplex RNA and 2-nucleotide 3’ DT overhangs purchased from City of Hope Beckman Research Center (Duarte, CA) and Sigma Genosys in deprotected and desalted form. Specificity of these siRNA sequences for each β-arrestin has been validated previously (10, 16, 17, 40–43). A second set of siRNA oligos for each β-arrestin-1 and -2 and siRNA to human Gaq were obtained from Santa Cruz Biotechnology, and those sequences are proprietary. Each knockdown experiment described herein was confirmed by quantitative Western blot analysis (LICOR Odyssey infrared imaging system) using an internal loading control (ERK1/2, tubulin, or actin). Specificity of β-arrestin siRNA was further confirmed using antibodies that recognize both β-arrestin-1 and -2 (A1CT and A2CT), simultaneously demonstrating knockdown of one β-arrestin while levels of the other are unaffected. Cells (40–50% confluent) were transfected with 20 nmol of siRNA/10-cm dish or 100 pmol of siRNA/30-mm well using Genesilencer (Gene Therapy Systems), Lipofectamine 2000 (Invitrogen), or N-ter (Sigma) according to the manufacturers’ instructions. Assays were performed 48 h after siRNA transfection.

Co-immunoprecipitations and Protein Binding Assays

Cofilin/β-Arrestin Interactions—Cleared lysates from confluent 15-cm dishes of untransfected MDA-MB-468 cells (lysis buffer: phosphate-buffered saline supplemented with 1% Nonidet P-40 and protease/phosphatase inhibitors) were incubated with 5 μg of rabbit anti-cofilin (Chemicon) prebound to Protein A-agarose for 4 h, analyzed by 15% SDS-PAGE, and blotted with mouse β-arrestin antibody. Alternatively MDA-MB-468 cells were co-transfected with GFP-tagged β-arrestin-1 or -2 and FLAG-cofilin. Cofilin was immunoprecipitated with anti-FLAG followed by SDS-PAGE and Western blotting with anti-FLAG or anti-GFP.
**β-Arenergic and Cofilin Regulation**

*CIN/β-Arenergic Interactions*—MDA-MB-468 cells, transfected with both FLAG-β-arrestin and Myc-CIN, Myc-CIN or FLAG-β-arrestin alone, or a vector control were treated with 2μM for 0–90 min and lysed (lysis buffer: 50 mM Hepes, 150 mM NaCl, 10 mM MgCl₂, 5% glycerol, and 1% Nonidet P-40 supplemented with protease inhibitors). Cleared lysates were immunoprecipitated with antibody to FLAG or Myc (A14) for 2 h followed by 12% SDS-PAGE and Western blotting with anti-β-arrestin-1, anti-Myc (9E10), or anti-CIN.

*LIMK/β-Arenergic Interactions*—FLAG-β-arrestin-1 and -2 were overexpressed in NIH3T3 cells and immunoprecipitated with anti-FLAG (M2)-agarose. Washed beads from transfected or untransfected cells were mixed with recombinant His-tagged LIMK (amino acids 285–639) for 2 h followed by 12% SDS-PAGE and Western blotting with anti-β-arrestin-1, anti-Myc (9E10), or anti-CIN.

**Assay of LIMK Activity**

Cleared cell lysates (LIMK lysis buffer: Hepes, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM MgCl₂, 1 mM MnCl₂, and protease inhibitors) were incubated with anti-LIMK for 8 h at 4 °C. Phosphatase inhibitors were added (1 mM NaF and NaVO₄) to cleared lysates just before addition of antibody. Washed beads were incubated in LIMK lysis buffer supplemented with 5 μM cold ATP, 5 μCi of [γ-32P]ATP, and 2 μg of recombinant GST-cofilin at 30 °C for 45 min. Reactions were terminated by transfer of the reaction mixture to 2X Laemmli sample buffer followed by SDS-PAGE, Coomassie staining, and autoradiography. GST-cofilin bands were excised, and incorporated radiolabel was determined by scintillation counting.

**Cofilin Dephosphorylation Assays**

Immobilized GST-cofilin was phosphorylated with constitutively active His₆-LIMK (100 ng of LIMK and 500 ng of GST-cofilin) in LIMK reaction buffer (25 mM Tris, pH 7.2, 0.5 mM EGTA, 250 μM NaVO₄, 0.25 mM diithiothreitol, 0.05% β-mercaptoethanol, 0.015% Brij 35, 250 μM cold ATP, 1 μCi of [γ-32P]ATP) for 30 min at 30 °C. GST-cofilin was pelleted, washed, incubated with either lysis buffer alone or lysates from MDA-MB-468 cells treated with 100 μM 2μAP for 0–45 min, and then analyzed by SDS-PAGE followed by autoradiography. Bands were excised and counted in a Beckman scintillation counter.

**Ca²⁺ Mobilization Assays**

Cells were incubated in physiological salt solution (PSS: 137 mM NaCl, 4.7 mM KCl, 0.56 mM MgCl₂, 2 mM CaCl₂, 1.0 mM Na₂HPO₄, 10 mM Hepes, 2.0 mM l-glutamine, and 5.5 mM d-glucose, pH 7.4) containing 0.1% bovine serum albumin and 5 μM Fura-2/AM for 20 min at 37 °C. Cells were washed and mounted in a microincubator containing 1 ml of PSS-bovine serum albumin at 37 °C on the stage of a Nikon inverted microscope (40× objective). Agonists were directly added to the bath. The Ca²⁺ ionophore, ionomycin, was added at the end of all experiments to determine maximum [Ca²⁺]. Fluorescence was detected in individual cells using a Nikon charge-coupled device camera and a video microscopy acquisition program (Metafluor). Fluorescence was measured at 340 and 380 nm excitation and 510 nm emission. The ratio of the fluorescence at the two excitation wavelengths, which is proportional to the [Ca²⁺], was determined, and intracellular [Ca²⁺] was calculated using the Grynkiewicz equation (48). Average [Ca²⁺], values for a range of 10–12 cells were calculated and presented as a single trace.
PAR-2 Promotes Cofilin Dephosphorylation and Filament Severing Activity—Cofilin can be activated by dephosphorylation on Ser3; thus, we investigated whether cofilin phosphorylation was altered in response to the PAR-2 activation using the specific PAR-2 activating peptide 2fAP (49, 50), inactive reverse 2fAP, or trypsin and probing cell lysates with a phospho (Ser3)-specific cofilin antibody. In the human breast cancer cell line (MDA-MB-468), we observed a robust decrease in phosphorylation as early as 30 s after PAR-2 activation (Fig. 1, A–C). This effect was dose-dependent (Fig. 1B) and was observed in another breast cancer cell line, MDA-MB-231 (not shown). Maximum cofilin dephosphorylation (66% for 2fAP and 75% for trypsin) was observed at 5 min; by 90 min, some of the cofilin remained dephosphorylated (53 ± 5% for 2fAP and 48 ± 16% for trypsin) (Fig. 1, A and C). No dephosphorylation was observed in response to reverse 2fAP (Fig. 1A). Dephosphorylation was observed even when agonist was removed at each time point (supplemental Fig. 1), suggesting that the prolonged response was due to maintenance of dephosphorylated cofilin by downstream mechanisms rather than to prolonged agonist exposure. Because peak dephosphorylation was observed at 5

**Microscopy**

Cells were seeded onto collagen-coated coverslips and allowed to attach overnight. After agonist treatment, cells were fixed in normal buffered formalin and prepared as described previously (9). Serial sections (1 μm; 100× and 63× objectives) were taken on a Zeiss LSM510 at 1× and 4× zoom. Overlays of four individual Z-sections and enlarged images were obtained using Adobe Photoshop 5.0.

**Data and Statistical Analysis**

All graphs and statistical analyses were performed using Kaleidagraph Version 4.0 or Microsoft Excel 2003. All experiments were performed a minimum of three times. Phosphoprotein levels were normalized to total protein levels before calculating fold change with respect to untreated controls. For LIMK assays, cpm values were normalized to total LIMK immunoprecipitated and total GST-cofilin levels before calculating fold change with respect to controls. Analysis of variance and Tukey t tests were used to determine statistical significance of and significant differences between values under different conditions.

**RESULTS**

PAR-2 Promotes Cofilin Dephosphorylation and Filament Severing Activity—Cofilin can be activated by dephosphorylation on Ser3; thus, we investigated whether cofilin phosphorylation was altered in response to the PAR-2 activation using the specific PAR-2 activating peptide 2fAP (49, 50), inactive reverse 2fAP, or trypsin and probing cell lysates with a phospho (Ser3)-specific cofilin antibody. In the human breast cancer cell line (MDA-MB-468), we observed a robust decrease in phosphorylation as early as 30 s after PAR-2 activation (Fig. 1, A–C). This effect was dose-dependent (Fig. 1B) and was observed in another breast cancer cell line, MDA-MB-231 (not shown). Maximum cofilin dephosphorylation (66% for 2fAP and 75% for trypsin) was observed at 5 min; by 90 min, some of the cofilin remained dephosphorylated (53 ± 5% for 2fAP and 48 ± 16% for trypsin) (Fig. 1, A and C). No dephosphorylation was observed in response to reverse 2fAP (Fig. 1A). Dephosphorylation was observed even when agonist was removed at each time point (supplemental Fig. 1), suggesting that the prolonged response was due to maintenance of dephosphorylated cofilin by downstream mechanisms rather than to prolonged agonist exposure. Because peak dephosphorylation was observed at 5

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\[ \text{**β-Arrestins and Cofilin Regulation**} \]

![Image](image_url)

**FIGURE 2.** siRNA knockdown of β-arrestin-1 and β-arrestin-2 inhibits PAR-2-stimulated cofilin dephosphorylation in MDA-MB-468 cells. A, Western blot with β-arrestin-1, β-arrestin-2 (recognizes β-arrestin-1 to a lesser extent), and tubulin (control) antibodies demonstrates knockdown efficiency and specificity. B, representative Western blot with phospho (Ser3)-cofilin (pC) and total cofilin (tC) antibodies after transfection of control siRNA (CNT) or siRNA to human β-arrestin-1, human β-arrestin-2, or both human β-arrestin-1 and -2 in MDA-MB-468 cells and treatment of cells with 2fAP for 0, 5, or 30 min. C, graph of normalized PAR-2-evoked changes in normalized phosphocofilin levels (±S.E.) after β-arrestin knockdown (n = 7). D and E, rescue experiments. D, upper panel, representative Western blot of PAR-2-evoked phospho- and total cofilin levels after transfection of control, human β-arrestin-1, or human β-arrestin-2 siRNA followed by transfection with the following DNA: empty vector (Mock) or FLAG-tagged rat β-arrestin-1 and 2. Inset, Western blot showing expression of FLAG-tagged β-arrestin-1 and -2 after siRNA knockdown. E, bar graph depicting PAR-2-evoked changes in normalized phosphocofilin levels (±S.E.) after rescue with each β-arrestin (n = 3). F, representative Western blot of PAR-2-evoked phospho- and total cofilin levels after knockdown of both β-arrestins simultaneously and subsequent rescue with the following DNA: empty vector (Mock) or FLAG-tagged rat β-arrestin-1 and 2. G, bar graph depicting PAR-2-evoked changes in normalized phosphocofilin levels (±S.E.) after double knockdown and rescue (n = 3). Significant differences between β-arrestin and control siRNA values in all bar graphs are indicated by * (p < 0.05) and ** (p < 0.005). Unt, control; Unt, untreated; β or βarr, β-arrestin.

...min, many of the remaining studies examined events primarily after 5 min of PAR-2 activation. These data demonstrate that an early event in PAR-2 signaling is cofilin dephosphorylation and that after the initial cofilin activation some, but not all, is rephosphorylated over time. Although cofilin dephosphorylation results in its activation, there are still factors in the cell that can prevent its severing activity even when it is dephosphorylated (33, 51). We used a modified pyrene-actin assembly assay to confirm that PAR-2 actually promoted cofilin-dependent actin filament severing (see “Materials and Methods” for a detailed explanation of the assay). Briefly, addition of severing factors to filaments that are already at steady state results in a decrease in pyrene fluorescence, which provides a readout of filament severing activity (44, 47). Extracts were immunodepleted with either IgG, as a negative control (Fig. 1B, ■ and □), or cofilin antibody (Fig. 1B, ○ and ●) to determine whether cofilin was mediating the severing activity. Addition of IgG-depleted extract from 2fAP-treated MDA-MB-468 cells decreased pyrene fluorescence by 63%, whereas extract from untreated cells did not (Fig. 1, B and C). Addition of fresh pyrene-G-actin ~1200 s after 2fAP-treated lysate addition resulted in a sharp increase in fluorescence (Fig. 1D) that was not observed with untreated lysates, indicating that the observed fluorescence decrease was due to filament severing and the subsequent generation of free barbed ends for elongation and not to fluorescence quenching. Importantly the PAR-2-induced severing activity was lost by immunodepletion of cofilin (Fig. 1B, ○). Furthermore, severing activity in immunodepleted extracts could be restored by addition of recombinant cofilin (Fig. 1B, ●). These results clearly show that PAR-2 promotes cofilin dephosphorylation and activation, leading to increased actin filament severing activity.

**PAR-2-stimulated Cofilin Dephosphorylation and Filament Severing Require β-Arrestins**—To determine whether β-arrestins were required for cofilin activation, we assessed cofilin dephosphorylation in MDA-MB-468 cells after siRNA knockdown of either β-arrestin-1, β-arrestin-2, or both (Fig. 2 and supplemental Fig. 2) or in embryonic fibroblasts from wild type (MEFwt) and β-arrestin-1/2 knock-out mice (MEFβarrDKO) (Fig. 3). PAR-2 signaling in MDA-MB-468 cells has been well characterized in our laboratory; thus, they serve as an excellent model system for studying PAR-2-induced cofilin activation (9, 10, 27). MEFs from β-arrestin knock-out mice provide the best model to address the role of β-arrestins in signaling events, but they express less PAR-2; thus PAR-2 responses are less robust (40). Two sets of siRNA oligos that have been described previously (10, 16, 17, 40–43) were used to knock down each human β-arrestin separately or both simultaneously; data from one set is shown. Specificity of siRNA oligos in these studies was confirmed by quantitative Western analysis, which showed a 70–80% knockdown of each β-arrestin with no discernible effect on expression of an unrelated protein, tubulin (Fig. 2A), and by rescue of knockdown...
effects with expression of FLAG-tagged rat β-arrestin-1 and -2 (Fig. 2, D and E). A full time course revealed that in MDA-MB-468 cells knockdown of either β-arrestin inhibited PAR-2-evoked cofilin dephosphorylation between 30 and 90 min (supplementary Fig. 2). The maximum difference between control siRNA-transfected and either β-arrestin-1 or -2 siRNA-transfected cells was observed at 30 min; thus, subsequent knockdown experiments were performed at 0, 5, and 30 min. Expression of β-arrestin-1 siRNA alone had no significant effect on cofilin dephosphorylation at 5 min but inhibited it by 75% at 30 min (Fig. 2, B and C). Expression of β-arrestin-2 siRNA alone inhibited cofilin dephosphorylation by 43% at 5 min and by 90% at 30 min (Fig. 2, B and C). siRNA oligos that target both β-arrestin-1 and -2 inhibited cofilin dephosphorylation by greater than 85% at both 5 and 30 min (Fig. 2, B and C). Expression of FLAG-tagged rat β-arrestin-1 and -2 rescued the effect of individual siRNA knockdown (Fig. 2, D and E). The inhibition of cofilin dephosphorylation observed with double knockdown at 5 min could be rescued by expression of either β-arrestin-1 or -2 (Fig. 2, F and G), suggesting that the two β-arrestins may be redundant with respect to initial cofilin dephosphorylation. In MEFs, cofilin dephosphorylation was abolished by genetic deletion of both β-arrestins (Fig. 3). These data demonstrate that PAR-2 promotes cofilin dephosphorylation by a β-arrestin-dependent mechanism and that initial cofilin dephosphorylation can be mediated by either β-arrestin. To confirm that the requirement for β-arrestins in cofilin dephosphorylation was reflected in filament severing activity, we performed pyrene-actin severing assays in the presence of extracts from cells transfected with β-arrestin-1 or -2 siRNA. β-Arrestin-2 siRNA eliminated and β-arrestin-1 siRNA reduced PAR-2-induced severing (by 60%) compared with control siRNA-transfected cells (supplemental Fig. 3). These data clearly demonstrate that PAR-2-induced cofilin dephosphorylation and filament severing activity are mediated by β-arrestins.

PAR-2 Promotes Association of β-Arrestins with Cofilin and Redistribution of Both to Membrane Protrusions—To address whether PAR-2 promotes interaction of cofilin with β-arrestins, cofilin was immunoprecipitated from MDA-MB-468 cells after PAR-2 activation with rabbit anti-cofilin, and immune complexes were analyzed by SDS-PAGE followed by Western blotting with mouse anti-β-arrestin. Both β-arrestin-1 and -2 (Fig. 4A) associated with cofilin at 5 and 90 min of PAR-2 activation. Furthermore when β-arrestin-2 was overexpressed, association with cofilin was observed even in the absence of receptor activation (Fig. 4B). PAR-2 promoted redistribution of both β-arrestins (Fig. 5A) and cofilin (Fig. 5B) to membrane protrusions where they were colocalized (Fig. 5C). Interestingly although β-arrestin-2 was found predominantly...
in the back of the F-actin-rich protrusions, β-arrestin-1 was found predominantly at the tips (Fig. 5C). These data are consistent with a role for β-arrestins in the spatial restriction of cofilin activity.

**PAR-2 Promotes Cofilin Dephosphorylation Independently of G-protein Coupling**—To determine whether β-arrestin-dependent cofilin activation represented a G-protein-independent pathway, we analyzed cofilin dephosphorylation after 1) treatment with BAPTA-AM to block intracellular Ca^{2+} mobilization (Fig. 6, A and C), 2) treatment with U73122 to block PLC activity (Fig. 6, B and C), 3) treatment with a Go_{q} antagonist (GP2A peptide (52, 53)) (Fig. 6, B and C), or 4) siRNA knockdown of Go_{q/11} (Fig. 6, E and F). Cofilin dephosphorylation was potentiated by BAPTA-AM pretreatment and Go_{q/11} knockdown, suggesting that it does not require Go_{q/11} activation and subsequent Ca^{2+} mobilization and may be negatively regulated by the Go_{q}/Ca^{2+} signaling axis (Fig. 6, A–E). Further confirming independence of Go_{q} signaling, neither the GP2A peptide nor U73122 significantly inhibited PAR-2-stimulated cofilin dephosphorylation (Fig. 6, B and C). The ability of BAPTA-AM, U73122, and GP2A to block PAR-2/Go_{q} signaling was confirmed by their ability to inhibit PAR-2-stimulated Ca^{2+} mobilization (Fig. 6D). Go_{q/11} knockdown was confirmed by quantitative Western analysis; note that levels of another heterotrimeric G-protein (Go_{i}) were unaltered, whereas levels were reduced by ~70% (Fig. 6E, lower panel). Although the pharmacological agents used in these studies may affect pathways other than Go_{q}, the fact that they do not decrease

PAR-2-evoked cofilin dephosphorylation strongly supports a model for Go_{q/PLC\(\beta1\)/Ca^{2+}-independent activation of cofilin. Involvement of Go_{i} and Go_{q/12} is unlikely as PAR-2-evoked cofilin dephosphorylation was also insensitive to pretreatment with pertussis toxin or transfection of dominant negative RhoA (not shown). The data thus far demonstrate that PAR-2 can promote cofilin activation via a Go_{q} protein/Ca^{2+-independent, β-arrestin-dependent pathway (Fig. 6G).

**PAR-2-stimulated Cofilin Dephosphorylation Requires CIN**—The next two sets of studies were directed at elucidating the molecular mechanisms by which PAR-2 promotes cofilin dephosphorylation by examining the role of the positive (phosphatase) and negative (LIMK) regulators of cofilin activity. We first addressed the possibility that PAR-2 promotes activation of a phosphatase leading to cofilin dephosphorylation. GST-cofilin was phosphorylated with recombinant LIMK in vitro and then incubated with lysis buffer alone or with extracts from cells treated with 2fAP for 0–45 min. Addition of PAR-2-activated extracts decreased phosphocofilin levels by 50%, suggesting that PAR-2 activates a cofilin phosphatase (Fig. 7A). This activity was insensitive to nonspecific phosphatase inhibitors NaVO_{4} and NaF. We next examined whether the recently identified cofilin phosphatase, CIN, was required for PAR-2-stimulated cofilin dephosphorylation. Expression of dominant negative CIN (DN-CIN-GFP) inhibited cofilin dephosphorylation by 50% at 5 min and abolished cofilin dephosphorylation at 90 min, demonstrating that PAR-2-stimulated cofilin dephosphorylation requires the activity of CIN (Fig. 7B). CIN also was redistributed to the membrane protrusions upon PAR-2 activation similar to what was observed with cofilin and β-arrestins (Fig. 8A). Co-immunoprecipitations revealed that β-arrestin-1 associated with CIN upon 5 and 90 min of PAR-2 activation (Fig. 8, B and C), whereas β-arrestin-2 could not be co-precipitated with CIN (not shown). Thus, β-arrestin-1 may spatially restrict cofilin dephosphorylation through scaffolding of its upstream phosphatase in membrane protrusions.

**PAR-2 Activation Antagonizes LIMK Activity by a β-Arrestin-independent Mechanism**—Another mechanism by which PAR-2 signaling might lead to dephosphorylation of cofilin is through inhibition of LIMK activity. To address this possibility, MDA-MB-468 cells were treated with 2fAP for 0–45 min, and phosphorylation of recombinant cofilin by immunoprecipitated

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5 L. Ge and K. DeFea, unpublished observations.
LIMK was determined. Consistent with its ability to trigger rapid cofilin dephosphorylation, PAR-2 promoted a rapid decrease in LIMK activity (Fig. 9, A and C). Interestingly knockdown of β-arrin-1 abolished and knockdown of β-arrin-2 attenuated LIMK inhibition; in fact inhibition of β-arrin-2 unmasked a PAR-2-stimulated increase in LIMK activity (Fig. 9, B and C). These findings demonstrate that the PAR-2-evoked inhibition of LIMK activity requires β-arrins and once again suggest that, in their absence, PAR-2 has the opposite effect, i.e. promoting events leading to cofilin phosphorylation. Confocal microscopy revealed a redistribution of LIMK to membrane protrusions and colocalization with β-arrins upon PAR-2 activation similar to what was observed with cofilin and CIN (Fig. 10A). Furthermore we were able to detect interaction between β-arrins and the C terminus of LIMK in vitro. Recombinant GST-tagged β-arrin-1 expressed in E. coli was pulled down by cobalt-Sepharose-bound His-tagged LIMK (amino acids 285–639), whereas GST alone and GST-β-arrin-2 were not. No β-arrin-1 was bound to cobalt in the absence of LIMK (Fig. 10B). When the concentration of the LIMK was increased by 5-fold, we observed a corresponding increase in β-arrin-1 binding (Fig. 10B, compare upper and lower panels). Surprisingly when FLAG-tagged β-arrins were expressed in and immunoprecipitated from mammalian cells, both proteins pulled down the recombinant LIMK (Fig. 10C), suggesting that β-arrin-2 might be modified or might interact with another protein, either of which confers the ability to interact with LIMK. Although we could not detect PAR-2-evoked interaction of β-arrins with LIMK in intact cells by co-immunoprecipitation (perhaps due to a transient interaction), these data suggest that β-arrins may also contribute to the spatial regulation of cofilin phosphorylation through localized interaction with LIMK.

**DISCUSSION**

Identification of a Major Gαq/Ca2+-Independent PAR-2 Signaling Arm—We and others have shown previously that β-arrins are required for activation of a number of signaling proteins and that they can scaffold these molecules to regulate their localization and activity (4, 9, 27). Furthermore a recent paradigm shift suggesting that some receptors can signal through β-arrins (16, 38, 42), independently of G-protein engagement, has led to the speculation that β-arrin-dependent events may represent an independent signaling arm for a subset of receptors. The studies described here are the first to demonstrate regulation of the cofilin pathway by either PAR-2 or β-arrins and point to the importance of evaluating both β-arrin- and Ca2+-dependent signaling events when assessing PAR-2 action. We show that PAR-2, a Gαq-coupled receptor, pro-
motes β-arrestin-dependent cofilin dephosphorylation and activation by a mechanism that is independent of the classic Gαq/Ca²⁺ signaling axis. β-Arrestin dependence is demonstrated by the fact that siRNA-mediated silencing of either β-arrestin-1 or -2 partially inhibited cofilin dephosphorylation and knockdown or genetic ablation of both β-arrestins abolished it. Independence from the classic G-protein pathway is demonstrated on multiple levels: siRNA-mediated silencing of Gαq/11 itself, inhibition of Gαq/receptor coupling by the GP2A, and inhibition of signaling intermediates PLCβ (by U73122) and Ca²⁺ (by BAPTA-AM). The effects of U73122 were less pronounced than those of the other inhibitors possibly because it can also block PLCγ activation, which is not activated by Gαq; a possible β-arrestin-dependent role for PLCγ in PAR-2 signaling has never been investigated. In the case of Gαq/11 siRNA and BAPTA-AM treatment, cofilin dephosphorylation was actually potentiated. Conversely in the absence of both β-arrestins, PAR-2 was able to promote a mild increase in cofilin phosphorylation. The prevalence of one pathway over the other in a given cell type may depend not only on the relative abundance of β-arrestins and Gαq/11 but on the expression of other components of the cofilin pathway. Together these results suggest that β-arrestin-dependent signaling can operate not just independently of, but in opposition to, G-protein-dependent signaling. At first glance, this model may appear counterintuitive; i.e. why would a cell want a single receptor to send opposing signals simultaneously? However, it is consistent with the concept that during a process in which a cell must polarize the same molecular events occurring in one cellular microdomain need to be suppressed in another. The physiological relevance of this β-arrestin-dependent pathway is highlighted by the fact cofilin activation can lead to increased cell motility and tumor metastasis (31, 54–57), and we have demonstrated previously that constitutive activation of β-arrestin-dependent PAR-2 signaling is associated with the basal migration of metastatic tumor cells (10).

Molecular Mechanisms of β-Arrestin-dependent Cofilin Dephosphorylation—Although β-arrestins have been demonstrated to facilitate cell migration in a number of systems (9–13;15), few studies have revealed a molecular link between β-arrestins and proteins that are known to directly affect actin assembly. The cofilin pathway is of particular interest in this regard because it is widely accepted that its activation by upstream kinases, or both. Further characterization of the opposing signals from PAR-2 to LIMK is necessary to fully elucidate this mechanism.
characterization of CIN activity and its regulation is an uncharted territory, and future studies will be necessary to determine whether β-arrestin-1 is required for PAR-2-stimulated CIN activation or whether it merely exerts spatial control over cofilin dephosphorylation by scaffolding the phosphatase. Association of cofilin with β-arrestins may affect both its localization and its phosphorylation by LIMK. In support of the former, overexpression of β-arrestin-2 appeared to restrict cofilin to the back of membrane protrusions (compare cofilin localization in Fig. 5, B and C). Another possibility is that β-arrestin-2 binding might stabilize dephosphorylated cofilin similar to the way in which 14-3-3 was shown to bind and stabilize phosphorylated cofilin (59).

Our previous studies have strongly suggested that both β-arrestins are required for ERK1/2 activation, but identifying a selective role for each has remained elusive (10). More recently, we have evidence that β-arrestin-1 mediates early ERK activation, receptor internalization, and lysosomal targeting, whereas β-arrestin-2 mediates prolonged ERK1/2 activation and internalization (60). When investigating the effects of silencing individual β-arrestins on PAR-2-induced cofilin activation here, there were apparent differences in the temporal regulation of cofilin activation by each β-arrestin with β-arrestin-2 having a greater effect at earlier time points and β-arrestin-1 having a greater effect at later time points. Initial dephosphorylation (at 30 s or less) was abolished only when both β-arrestins were deleted, suggesting that for the initial increase in cofilin activity the two β-arrestins are functionally redundant. However, the two proteins may differ in the mechanisms by which they regulate cofilin. We detected interactions between CIN and β-arrestin-1 but not β-arrestin-2, suggesting that regulation of cofilin phosphatase activity might be mediated primarily by β-arrestin-1. Conversely cofilin constitutively associated with overexpressed β-arrestin-2 but not β-arrestin-1. Teasing out the subtle differences between β-arrestin-1 and -2 in PAR-2 signaling may further clarify the molecular mechanisms demonstrated here.

β-Arrestin-dependent Actin Filament Severing—Why might β-arrestins be necessary for cofilin activation? Other studies have demonstrated that cofilin localizes to the lamellipodium tip immediately upon epidermal growth factor stimulation where it facilitates actin assembly by creating free barbed ends (55, 57), and site-specific activation of cofilin in live cells leads to membrane protrusion at that site (56). These observations are consistent with the idea that localized filament-severing promotes actin reorganization and assembly through treadmilling of actin filaments at the leading edge and point to the importance of spatially restricting cofilin activity. We observed a PAR-2-induced increase in actin filament severing activity that was abolished by immunodepletion of cofilin and restored by addition of 10 nm recombinant cofilin. Furthermore PAR-2-evoked filament severing activity was severely impaired after β-arrestin knockdown. Thus, it is likely that β-arrestins play a key role in the spatial restriction of cofilin-induced filament severing during PAR-2-evoked cell migration. However, recent studies have raised some controversy as to whether cofilin is merely an actin filament-severing protein because at high concentrations cofilin can promote actin filament assembly instead of severing (61). A model where β-arrestins facilitate nucleation through localized cofilin accumulation is also plausible and should be addressed in future studies.

To summarize, PAR-2 activates the actin filament-severing protein cofilin through two molecular mechanisms, both apparently dependent on β-arrestins: inhibition of LIMK activity and activation of the cofilin phosphatase. Cofilin activation is independent of PAR-2-mediated Goq coupling and mobilization of intracellular Ca2+. These observations are important from both a biomedical and a basic signaling perspective. First, PAR-2 activation has become an increasingly popular thera-
peutic target in inflammatory diseases, and its activation is associated with increased migration of tumor cells (10, 62–65). Because Ca\(^{2+}\)/H\(^{1001}\)mobilization and G\(^{\alpha}/H^{9251}\)q coupling are the most commonly used readouts of PAR-2 activation, studies using PAR-2 agonists and antagonists in disease models might need to be revisited in the context of \(\beta\)-arrestin-dependent signaling. Also tissue-specific differences in molecular components of

**FIGURE 9.** \(\beta\)-Arrestins inhibit LIMK activation downstream of PAR-2. A, LIMK assay: phosphorylation of recombinant cofilin by LIMK immunoprecipitated from MDA-MB-468 cells after treatment with 2fAP for 0, 1, 5, and 45 min. Upper panel, representative autoradiograph showing radiolabeled cofilin. Lower panel, Coomassie-stained gel showing total cofilin levels. B, LIMK assay after siRNA knockdown of \(\beta\)-arrestin: representative autoradiographs and Coomassie-stained gels of LIMK reactions after transfection with control siRNA (upper panels), \(\beta\)-arrestin-1 siRNA (middle panels), or \(\beta\)-arrestin-2 siRNA (lower panels). C, bar graph depicting PAR-2-stimulated LIMK activity (mean fold base line ± S.E.) with and without \(\beta\)-arrestin knockdown. Significant difference between \(\beta\)-arrestin and control siRNA values are indicated by * (\(p < 0.01\)) and **(\(p < 0.001\)), and significant decreases in LIMK activity at each time point with respect to untreated cells are indicated by # (\(p < 0.01\)).

**FIGURE 10.** LIMK binds to and colocalizes with \(\beta\)-arrestins. A, MDA-MB-468 cells were treated with 2fAP for 0–45 min and costained with anti-\(\beta\)-arrestin-1/2 (red) and anti-LIMK (green). Arrows indicate regions of colocalization after 5 min of PAR-2 activation. Scale bar = 10 μm. B, 5 μg of recombinant GST-\(\beta\)-arrestin-1 or GST-\(\beta\)-arrestin-2 was incubated with 10 μg (upper panel) or 2 μg (lower panel) of His\(_{6}\)-LIMK (amino acids 285–638), and His\(_{6}\)-LIMK was precipitated with cobalt resin. Cobalt resin alone was included as a control for non-specific protein precipitation. Both bound and unbound proteins were analyzed by SDS-PAGE followed by Coomassie staining. C, FLAG-\(\beta\)-arrestin-1 and FLAG-\(\beta\)-arrestin-2 were purified with anti-FLAG-agarose from crude cell lysates and incubated with 20 ng of His\(_{6}\)-tagged recombinant LIMK (amino acids 285–638). Anti-FLAG-agarose pulldowns from untransfected cells were used as a negative control. Protein was eluted from beads (25 μl), and Western blots were probed with anti-FLAG (upper panel) or anti-His\(_{6}\) (lower panel) to demonstrate interaction of both \(\beta\)-arrestins. CNT, control; \(\beta\) or \(\beta\)-arr, \(\beta\)-arrestin.
each PAR-2 signaling arm could explain the somewhat disparate set of cellular responses reported for PAR-2. Second, because β-arrestins have been implicated in G-protein-independent signaling downstream of other receptors, the molecular mechanisms elucidated here might be utilized by other G-protein-coupled receptors.

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FIGURE 11. Model for regulation of cofilin by PAR-2. Upon PAR-2 activation, two pathways are evoked: (a) classic Gq, activation leading to PLCβ, inositol 1,4,5-trisphosphate (IP3) generation, and mobilization of intracellular Ca2+. (b) β-arrestin-dependent cofilin dephosphorylation/activation. β-Arr-2 can scaffold both negative and positive regulators of cofilin: the inhibitory kinase (LIMK) and the stimulatory phosphatase (CIN). β-Arestins inhibit LIMK activity and may facilitate CIN activity, thus maintaining a pool of cofilin in its dephosphorylated/activated state. Genetic deletion or siRNA-mediated knockdown of β-arrestins increases LIMK activity and decreases cofilin activation downstream of PAR-2. In contrast Gqα,11 knockout, chelation of intracellular Ca2+ with BAPTA-AM, and pharmacological inhibition of G-protein coupling increase cofilin dephosphorylation possibly through stimulation of LIMK, suggesting that this pathway opposes the β-arrestin-dependent pathway. Spatially restricted, β-arrestin-dependent cofilin activation may promote filament treadmilling at the leading edge, whereas in other domains Gqα,2/Ca2+-dependent cofilin phosphorylation may protect stability of actin filaments. β-arr, β-arrestin.
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