Protease-Activated Receptor-2 Simultaneously Directs β-Arrestin-1-Dependent Inhibition and Gαq-Dependent Activation of Phosphatidylinositol 3-Kinase†

Ping Wang‡ and Kathryn A. DeFea*,‡,§

Division of Biomedical Sciences and Cellular Molecular and Developmental Biology Program and Biochemistry and Molecular Biology Program, University of California, Riverside, California 92521

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ABSTRACT: Protease-activated receptor-2 (PAR-2) is a G-protein-coupled receptor (GPCR) activated upon proteolytic cleavage of its N-terminus by a number of serine proteases. We have previously reported that formation of a β-arrestin-dependent signaling scaffold is required for PAR-2-stimulated activation of extracellular signal regulated kinases 1 and 2 and chemotaxis. β-Arrestin-dependent pathways downstream of some GPCRs have been shown to function independently and sometimes in opposition to classic signaling through heterotrimeric G-proteins; however, this possibility has not been addressed with respect to PAR-2. Here we demonstrate that PAR-2 can increase PI3K activity through a Gαq/Ca^{2+}-dependent pathway involving PYK2 and a Src-family kinase, while inhibiting PI3K activity through a β-arrestin-dependent mechanism, and that β-arrestin-1 can directly associate with and inhibit the catalytic activity of p110α. Using size exclusion chromatography and co-immunoprecipitation, we demonstrate that the PI3K is recruited into a scaffolding complex containing PAR-2 and β-arrestins. Inhibition of PI3K activity blocks PAR-2-stimulated chemotaxis, and β-arrestin-1 colocalizes with p85 within the pseudopodia, suggesting that β-arrestin-1 association with PI3K may spatially restrict its enzymatic activity and that this localized inhibition may be crucial for PAR-2-stimulated chemotaxis.

Protease-activated receptors (PARs) are G-protein-coupled receptors (GPCR),[1] activated upon proteolytic cleavage of their N-termini, and are thought to play a major role in inflammation and cell migration (*1*−*5*). Cleavage of PAR-2 by serine proteases unveils the tethered ligand SLIGRL/GKV (mouse/human) which then binds to and activates the receptor. Synthetic peptides corresponding to the tethered ligand (SLIGRL, SLIGKV, and 2-furoyl-LIGRL-O) specifically activate PAR-2 in the absence of proteolytic cleavage (*6*−*9*). Recent studies from our laboratory have demonstrated that PAR-2 can promote chemotaxis in breast cancer cells and fibroblasts by a mechanism involving β-arrestin-dependent regulation of kinase activities (*8*, *10*); other studies suggest this mechanism may be shared by a number of receptors (*11*−*15*). β-Arrestins have been traditionally associated with GPCR desensitization and internalization; however, it is now widely accepted that they also serve as "endosomal scaffolds" that can determine the subcellular localization of signaling molecules and direct their downstream signaling properties (*16*). Our previous work revealed that the PAR-2-induced endosomal scaffold is retained at the leading edge of migrating fibroblasts and tumor cells, where it is associated with prolonged ERK1/2 activation (*8*). These data led to speculation that β-arrestins might sequester other signaling molecules at the leading edge in order to exert localized control over actin assembly machinery. Furthermore, recent studies on β_2-adrenergic receptor (β_2AR) and angiotensin II type 1a receptor (AT1aR) demonstrate that β-arrestin-dependent signals can oppose G-protein-dependent signals (*17*, *18*), providing a new paradigm for GPCR signaling. β-Arrestins have been demonstrated to regulate other signaling proteins, among them phosphatidylinositol 3-kinase (PI3K) (*19*, *20*); however, the role of this pathway in β-arrestin-dependent PAR-2 signaling has not been addressed.

Phosphatidylinositol 3-kinase (PI3K) has long been implicated in actin−cytoskeleton reorganization, chemotaxis, and tumor invasiveness (*21*−*23*) and is a likely candidate for regulation by β-arrestins in PAR-2-evoked chemotaxis. Involvement of β-arrestin-1 in PI3K activation has been reported previously downstream of insulin-like growth factor 1 receptor (IGF1R) (*19*) and endothelin A receptor (*24*). Furthermore, recruitment of PI3K to a β-arrestin/β_2AR kinase (β-ARK) complex is required for efficient endocytosis of β_2AR, suggesting reciprocal roles for PI3K in GPCR signaling and desensitization (*25*, *26*). Members of the most common class IA family of PI3Ks consist of an 85 kDa regulatory subunit (p85) and a catalytic subunit (110α or

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* Address correspondence to this author. Tel: 951-827-2871. Fax: 951-827-5504. E-mail: kathryn.defea@ucr.edu.

‡ Division of Biomedical Sciences, University of California.

§ Cellular Molecular and Developmental Biology Program and Biochemistry and Molecular Biology Program, University of California.

[1] Abbreviations: barr-1, β-arrestin-1; barr2, β-arrestin-2; CNT, control; DAG, diacylglycerol; GPCR, G-protein-coupled receptor; IF, immunofluorescence; IP, immunoprecipitation; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; p85, PI3K regulatory subunit; p110, PI3K catalytic subunit; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PAR-2, protease-activated receptor-2; PKC, protein kinase C; PBS, phosphate-buffered saline; PYK2, proline-rich tyrosine kinase 2; TBS, Tris-buffered saline; TF, transfection; WB, Western blot; WM, wortmannin.

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110β). p85 contains two SH2 domains that bind other phosphorylated SH2-domain-containing proteins, increasing p110 catalytic activity and localizing it to the membrane (*23*, *27*). The active enzyme phosphorylates the D3 ring of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI_4P), or phosphatidylinositol 4,5-bisphosphate (PIP2) to generate PI_3P, $PI_{3,4}P2$, and phosphatidylinositol 3,4,5-trisphosphate (PIP3), respectively. PIP3 can recruit and activate guanine exchange factors (GEFs) for RhoA GTPases and the Wiscott−Aldrich syndrome (WAS) protein, WAVE, which can then lead to formation of stress fibers, filipodia, and lamellipodia crucial for cell migration (*23*, *28*). $PI_{3,4}P2$ can activate the protein kinase Akt, which is implicated in tumor cell proliferation and metastasis (*15*, *23*). Here we investigate the role of PI3K in PAR-2 signaling and investigate (1) whether PAR-2 promotes PI3K activity, (2) whether PAR-2-stimulated PI3K activity is regulated by β-arrestins, and (3) the functional significance of β-arrestin-regulated PI3K activity in PAR-2 stimulated cell migration and cytoskeletal reorganization.

MATERIALS AND METHODS

Materials. All chemicals were from Sigma or Fisher Scientific except as otherwise indicated. GFP-tagged β-arrestin-1 and β-arrestin-2 were gifts from Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC). Lipofectamine 2000 was from Invitrogen Inc. 2-Furoyl-LIGRL-O-NH_2 (2fAP) was synthesized by Genemed Inc. [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences. GF109203X (GFX) was from CalBiochem, and LY294002 was from Echelon. Phosphatidylinositol (PI) was from Avanti Polar Lipids. Recombinant PI3K (p85α complexed to p110α) was from Upstate Technology, Inc. GST−β-arrestin was created by ligation of rat β-arrestin-1 (amplified by PCR) into pGEX KG (a modification of pGEX 4T3) at *Eco*RI sites. Recombinant β-arrestin−GST was expressed in BL21(DE3) cells by IPTG induction (1 mM) for 4 h, and the protein was purified with glutathione−agarose under standard nondenaturing conditions.

Antibodies. Mouse anti-β-arrestin-1 monoclonal antibody was from Transduction Laboratories. Rabbit β-arrestin-1 and -2 antibodies (A1CT and A2CT) were gifts from Dr. Robert Lefkowitz (Duke University Medical Center). Mouse anti-PAR-2 (SAM11), anti-PYK2, and anti-p110 were from Santa Cruz Biotechnology. Mouse anti-β-arrestin-1 was from BD-Pharmingen. Anti-GFP antibody was from Chemicon. Alexa-conjugated secondary antibodies and Alexa^{488}-phalloidin were from Molecular Probes. IRDye^{800}-conjugated secondary antibodies were from Rockland Immunochemicals. Anti-p85 antibodies and protein A−agarose were from Upstate Technology, Inc.

Transient Transfections. NIH3T3 and MDA MB-468 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% cosmic calf serum (Hyclone Laboratories) and maintained at 37 °C with 5% CO_2. NIH3T3 cells (5×10^5) grown on 10 cm dishes overnight (approximately 70% confluent) were transiently transfected with 10 μg of GFP-tagged β-arrestin-1 and -2 using Lipofectamine according to the manufacturer's instructions. Cells were harvested for experiments 24−36 h after transfection.

Co-immunoprecipitations. After serum starvation for 16 h, cells were treated with 100 nM 2fAP for 0−30 min, washed with ice-cold PBS, and lysed with buffer containing TBS (pH 7.4), supplemented 1% NP-40 and phosphatase/protease inhibitors. Cleared lysates (500 μg of protein) were incubated with antibodies prebound to protein A−agarose, 0.5 μg of anti-GFP (NIH3T3 cells), 4 μg of anti-p85 (MDA MB-468 cells), or an equal amount of rabbit IgG at 4 °C overnight. GFP-transfected NIH3T3 cells were included as a negative control for β-arrestin−GFP immunoprecipitates (IPs). IPs were resolved by 10% SDS−PAGE and transferred to PVDF-FL membranes. Blots were probed with antibodies to p85 (1:1000), β-arrestin-1 (1:1000), β-arrestin-2 (A2CT, 1:500), or PAR-2 (1:1000), and bands were visualized with the LICOR Odyssey infrared imaging system using Alexa^{680}-conjugated rabbit and IR^{800}-conjugated mouse secondary antibodies (1:40000 each).

Cell Migration and Pseudopodia Assays. Serum-starved NIH3T3 cells (10^4 per well) and MDA MB-468 cells (10^5 per well) were seeded onto fibronectin-coated (10 μg/mL) 24-well Costar Transwell polycarbonate membranes (8 μm), and migration in response to 100 nM 2fAP was determined as described previously (*8*, *10*). To test the involvement of PI3K, wortmannin (WM, 100 nM) or LY294002 (20 μM) was added 30 min before 2fAP treatment. Pseudopodia purification has been described in detail previously (*8*, *29*). Briefly, serum-starved NIH3T3 cells (1.5×10^6 per well) were seeded into the upper chamber of six-well Transwell filters with 3 μm pores (too small to allow cell body translocation) and then treated with 100 nM 2fAP for 90 min to induce pseudopodia extension. Protein from either cell bodies on the upper membrane surface or pseudopodia on the underside was extracted, and 10 μg of protein from each was analyzed by SDS−PAGE, followed by Western blotting with antibodies to p85 (1:1000) and histone H1 (1:250, as a marker of cell body).

siRNA Transfections. Two separate targeting sequences were used to determine the requirement for β-arrestins in PAR-2-stimulated PI3K activity. Chemically synthesized double-stranded small interfering RNAs (siRNAs) with 19-nucleotide duplex RNA and 2-nucleotide 3′-dT overhangs were purchased from City of Hope Beckman Facility (Duarte, CA) in deprotected and desalted form. The siRNA sequences targeting β-arrestin-1 or -2 are 5′-AAAGCCUUCUGCGCG-GAGAAU-3′ and 5′-AAGGACCGCAAAGUGUUUGUG-3′, respectively, and have been described previously by our and other laboratories (*10*, *11*, *13*, *18*, *30*); the nontargeting sequence control is 5′-AAUUCUCCGAACGUGUCACGU-3′. A second set of siRNAs to β-arrestin-1 and -2 and siRNAs to Gαq were obtained from Santa Cruz Biotech (Santa Cruz, CA), the sequences of which are proprietary. Cells were transfected at 50% confluence with 20 μg of siRNA, using Genesilencer (Gene Therapy Systems. Inc.), according to manufacturer's instructions. Experiments were initiated at 72 h after siRNA transfection.

Immunofluorescence and Confocal Microscopy. NIH3T3 or MDA MB-468 cells (2×10^3), transiently transfected with GFP-tagged β-arrestin-1 or -2, were seeded onto collagen-coated (10 μg/mL) coverslips and incubated for 4 h in complete media followed by serum starvation for 16 h. Cells were treated with 100 nM 2fAP at 37 °C for 0−90 min and stained with AlexaFuor^{488}-conjugated phalloidin (finally

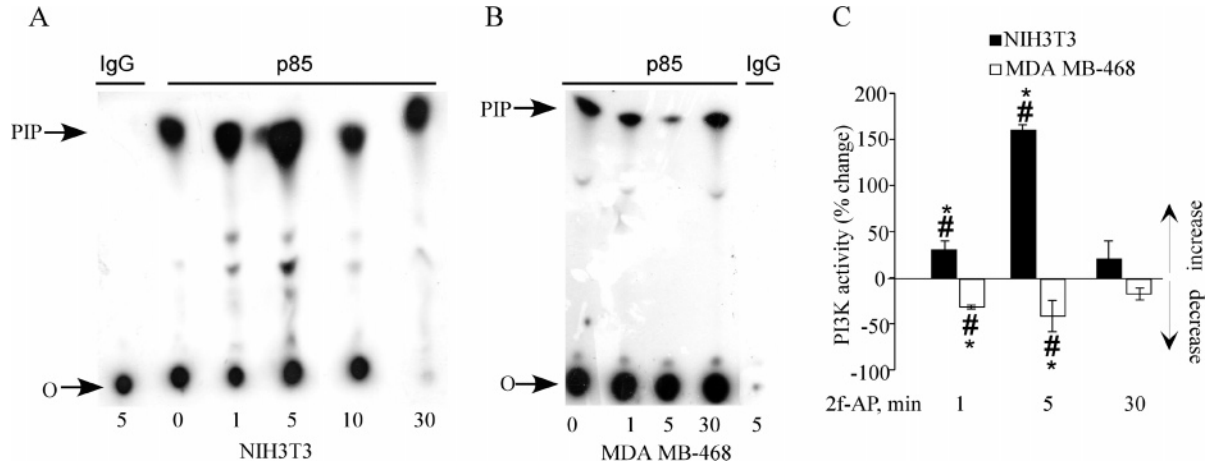

FIGURE 1: PAR-2 both promotes and inhibits PI3K activity. To determine PI3K activity, p85 was immunoprecipitated from NIH3T3 cells (A) or MDA MB-468 cells (B) after treatment with 100 nM PAR-2 agonist, 2fAP, for 0−30 min, and phosphorylation of exogenous PI was measured. (A, B) Representative autoradiograph of phospholipids separated by TLC. Migration rates of PI products were compared to those of stained standards; migration positions of PIP and the sample origin (O) are indicated by arrows. (C) Bar graph showing percent increase in the PI3K activity calculated from phosphoimage analysis of radioactive PIP (where 0% = value in untreated cells) after 1, 5, and 30 min of 2fAP treatment. Results represent the mean increase ± SEM; # indicates significant difference ($P < 0.05$) between treated and untreated; * indicates significant difference between NIH3T3 and MDA MB-468 cell PI3K activity; $n = 5$.

concentration 1:100; 1 h at room temperature) to visualize F-actin or with anti-mouse p85 (1:500). Z-sections were taken using a ZEISS LSM 510 with 100× oil objective (1 μm thick for each section). The final composite image was created using Adobe Photoshop 6.0 (Adobe) to overlay four Z-sections after analysis with LSM-Image software.

In Vitro Binding Assays. Recombinant p85α/^{6}His-p110α (100 ng, 0.5 pmol), immobilized on Ni^{+}-Sepharose, was incubated with 0−500 ng of β-arrestin-1−GST (0−10 pmol) or GST (0−18 pmol) for 30 min at 25 °C. His-bound proteins were collected by centrifugation, and beads were washed in PBS (containing 230 mM NaCl). Supernatants were incubated with glutathione−Sepharose to collect unbound GST fusion proteins, and beads were washed in PBS. Ni^{+} beads, glutathione beads, and supernatant fractions were analyzed by 10% SDS−PAGE and stained with Imperial protein stain (Pierce Biotech) to visualize proteins. p110α, β-arrestin−GST, and GST can be clearly seen, whereas p85α is approximately the same molecular weight as βarr-1−GST and is obscured. Replicate samples were analyzed by Western blot using anti-p85 to ensure equal recovery of p85α.

PI3K Assays. NIH3T3 cells (5×10^5) (80% confluence) were serum-starved for 16 h, stimulated with 100 nM 2fAP for 0−30 min at 37 °C, and lysed in ice-cold buffer A [137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1% NP-40, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM $NaVO_4$]. Cleared lysates (500 μg of protein) were incubated with polyclonal anti-p85 antibody (4 μg) at 4 °C for 2 h followed by 40 μL of protein A−agarose for 2 h at 4 °C. IPs were washed three times with each of following buffers: (1) buffer A; (2) 100 mM Tris-HCl (pH 7.4), 5 mM LiCl, and 0.1 mM $NaVO_4$; (3) TNE buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA, 0.1 mM $NaVO_4$]. PI3K reactions were performed in 50 μL of TNE buffer, supplemented with 10 μL (20 μg) of PI, 10 μL of 100 mM $MgCl_2$, and 5 μCi of [γ-^{32}P]ATP at 37 °C for 10 min and stopped by addition of 20 μL of 6 N HCl. For in vitro assays, 10 ng (∼50 pmol) of recombinant p85α/p110α was incubated with 20 μg of PI and 1 μCi of [γ-^{32}P]ATP in the presence of 0−400 ng of β-arrestin-1−GST (0−160 nM) or GST alone (0−500 nM) in a total volume of 30 μL. (Either 0, 2, 20, or 200 ng of recombinant GST fusion protein or 0, 20, 200, and 400 ng was added for each assay; assays were repeated a total of five times.) Lipids were extracted from the organic layer after addition of 160 μL of $CHCl_3$:MeOH (1:1), and ^{32}P-labeled phospholipids were resolved by thin-layer chromatography (TLC) on silica plates, pretreated with potassium oxalate, in chloroform−methanol−water−ammonium hydroxide (60:47:11.3:2). Migration rates of PI products were compared to those of molybdenum blue stained standards PI, PI_4P, $PI_{4,5}$-P2, and $PI_{3,4,5}$P3. Radioactive lipids were determined by phosphoimage analysis using a Bio-Rad Storm.

Data Analyses. Percent changes in PI3K activity were calculated from phosphoimage band density in PAR-2-treated vs untreated cells for each experimental group. Band densities were normalized to total p85 immunoprecipitated, as determined by Western blotting. Baseline levels were those present in the absence of PAR-2 activation and were treated as 100%. Therefore, changes in baseline levels of PI3K activity were not apparent in the results. All experiments were repeated a minimum of three times and expressed as mean ± SEM as indicated in the Results. Differences between multiple groups were examined by two-way ANOVA and Bonferroni post-tests, with $P < 0.05$ considered to be significant. In this way, both the statistical significance of PAR-2 activation on PI3K activity at each time point and the difference between paired groups (i.e., PAR-2 activated compared with untreated, PAR-2 activated in the presence compared with the absence of inhibitor, or PAR-2 activated after Gαq/β-arrestin siRNA compared with control siRNA transfection) were determined. Apparent IC_{50} for in vitro PI3K assays was calculated using XLFit version 4. Data were fitted using a sigmoidal dose−response model with a 95% confidence interval.

RESULTS

PAR-2 Can Promote PI3K Activity through a $G\alpha_{q/11}$-Dependent Pathway Involving PYK2 and Src-Family Ki-

nases. While PI3K is thought to play a major role in cell migration downstream of a number of chemotactic receptors, its role in PAR-2 signaling has never been addressed. In these studies, we focused on the class IA PI3K family. We assayed PI3K activity in NIH3T3 and MDA MB-468 cells, after treatment with PAR-2 agonist (2fAP) for 0−30 min, by immunoprecipitation of the regulatory subunit of PI3K (p85α), followed by incubation of immune complexes with [γ-^{32}P]ATP and lipid substrate (PI). Radiolabeled phospholipids were then analyzed by TLC, and the migration rates of PI products were compared to those of stained standards. As shown in Figure 1, PAR-2 activation increased PI3K activity in NIH3T3 cells (160 ± 6% after 5 min) but decreased PI3K activity in MDA MB-468 cells (40 ± 9%). PAR-2 has been demonstrated to signal through Gαq coupling, which leads to mobilization of Ca^{2+} and activation of PKC. However, some PAR-2-dependent effects, such as activation of ERK1/2, require both β-arrestin-1 and -2. Given the recent emergence of a new paradigm in GPCR signaling from studies demonstrating β-arrestin-dependent, G-protein-independent signaling, we next investigated whether the increase in PI3K activity observed in NIH3T3 cells was mediated by the classic $G\alpha_{q/11}$ signaling axis. We addressed the role of Gαq signaling at three levels, by knocking down $G\alpha_{q/11}$ expression with siRNA, blocking downstream Ca^{2+} mobilization with BAPTA-AM, or blocking PKC activation with GFX. The specific PKC isoform used by PAR-2 is unknown; thus we chose a broad spectrum inhibitor. Mouse $G\alpha_{q/11}$-specific siRNA sequences are proprietary (purchased from Santa Cruz Inc.), but their specificity was confirmed by quantitative Western blot analysis using tubulin as an internal protein loading/expression control (Figure 2C). Knockdown of $G\alpha_{q/11}$ expression (by ∼50%), treatment with BAPTA-AM, and treatment with GFX abolished the PAR-2-stimulated PI3K activity in NIH3T3 cells (Figure 2). In fact, after $G\alpha_{q/11}$ knockdown or GFX treatment, PAR-2 activation promoted a 53 ± 9.7% and 30 ± 10% decrease in PI3K activity, respectively, similar to what we had observed in MDA MB-468 cells, suggesting that PAR-2 sends opposing signals to PI3K through Gαq-dependent and independent pathways. $G\alpha_{q/11}$ knockdown also increased baseline PI3K activity, suggesting it may play multiple roles in regulating this pathway.

The proline-rich tyrosine kinase 2 (PYK2) can be activated by Ca^{2+} and PKC, leading to binding and activation of Src, followed by binding and activation of PI3K (*31*−*34*). Since PAR-2 has been shown to activate PYK-2 and promote its association with Src (*31*), we wished to examine whether PAR-2 also promotes its association with PI3K and whether Src-family kinases mediate the PAR-2-induced PI3K activity. Co-immunopreciptiations with p85 revealed that, upon PAR-2 activation, PYK2 associated with p85 (Figure 3A). Furthermore, inhibitor SU6656, which is specific for members of the Src family of tyrosine kinases (*35*), inhibited PAR-2-stimulated PI3K activity in NIH3T3 cells by 48 ± 6% (Figure 3B).

PAR-2 Can Inhibit PI3K Activity through a β-Arrestin-Dependent Pathway. Opposing G-protein and β-arrestin-dependent pathways have been demonstrated downstream of other GPCRs (*17*, *18*). Furthermore, we had previously demonstrated that PYK2 activation and Src association, which are involved in PAR-2-stimulated PI3K activity, were

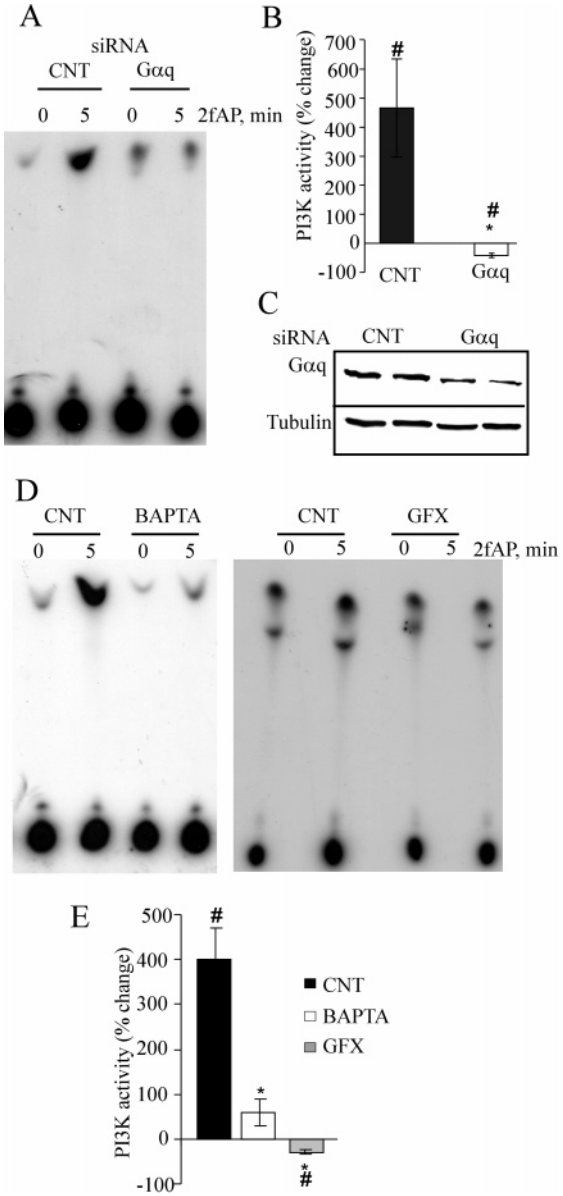

FIGURE 2: PAR-2-stimulated PI3K activity in NIH3T3 cells requires $G\alpha_{q/11}$ and Ca^{2+}. (A−C) NIH3T3 cells were either transfected with nontargeting control (CNT) and $G\alpha_{q/11}$-specific siRNA or pretreated with or without BAPTA-AM followed by activation of PAR-2 with 100 nM 2fAP. PI3K activity was assayed as described in Figure 1. (A) Representative TLC autoradiograph of PI3K activity after $G\alpha_{q/11}$ knockdown. (B) Bar graph depicting percent increase or decrease in PI3K activity in control and Gαq siRNA transfected cells. (C) Quantitative Western analysis of Gαq and tubulin (internal control) protein levels after siRNA transfection. (D, E) NIH3T3 cells were treated with BAPTA-AM or GFX, and PI3K activity was measured. (D) Representative TLC autoradiograph of PI3K activity after 30 μM BAPTA-AM (left) or 10 μM GFX (right) treatment. (E) Bar graph depicting percent increase or decrease in PI3K activity with BAPTA-AM or GFX treatment. Results represent the mean increase ± SEM; * indicates significant difference between pretreated or Gαq siRNA transfected and control groups; # indicates significant difference in PI3K activity between untreated and PAR-2 activated groups ($P < 0.05$, $n = 3$).

profoundly increased in response to a mutant PAR-2, deficient in β-arrestin-1 binding and desensitization (*36*), suggesting that β-arrestins might oppose this pathway. We next investigated whether the PAR-2-mediated inhibition of PI3K observed in MDA MB-468 cells and after blockade of the Gαq signaling axis might be dependent on β-arrestins.

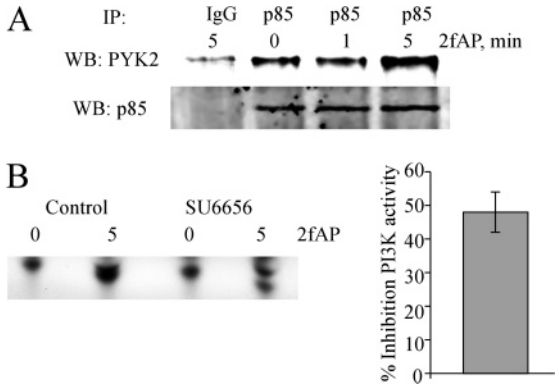

FIGURE 3: PYK-2 and Src-family kinase involvement in PAR-2 stimulated PI3K activation. (A) Lysates from NIH3T3 cells treated with 2fAP for 0, 1, or 5 min were immunoprecipitated (IP) with p85 or IgG (negative control), and Western blots (WB) were probed with PYK2 (upper panel) or p85 (lower panel). (B) NIH3T3 cells were treated with 2fAP for 0−5 min with or without inhibition of Src-family kinases with 20 μM SU6656, and PI3K assay was performed as described previously. The bar graph represents percent inhibition of PAR-2 stimulated PI3K activity by SU6656 compared with control cells.

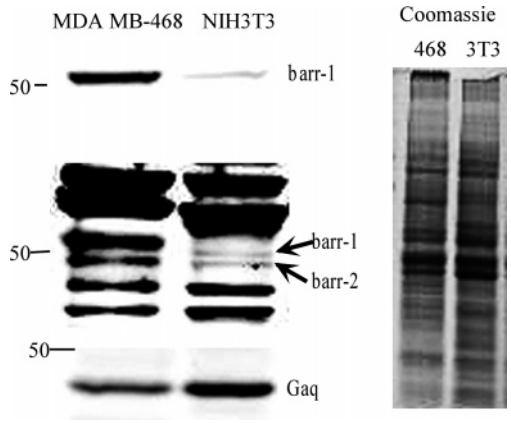

FIGURE 4: Differential expression of β-arrestins and Gαq in NIH3T3 and MDA MB-468 cells. Equal amounts (25 μg) of protein extract from NIH3T3 and MDA MB-468 cells were run in duplicate and analyzed for β-arrestin-1 (upper panel), β-arrestin-1 and -2 (middle panel), and Gαq (lower panel) expression by Western blot. The left panel is a Coomassie-stained gel to demonstrate equal loading of samples (right panel).

First, we compared levels of β-arrestin and Gαq expression in the two cell lines by quantitative Western blot analysis and found that β-arrestin-1 and -2 are more highly expressed in MDA MB-468 cells, while Gαq was more highly expressed in NIH3T3 (Figure 4). Thus, it is possible that β-arrestins might play a role in mediating the PAR-2-stimulated decrease in PI3K activity. To address this possibility, we examined whether siRNA knockdown of β-arrestins in MDA MB-468 cells, or overexpression of β-arrestins in NIH3T3 cells, would alter PI3K activity in response to PAR-2 activation. SiRNA oligos specific for human β-arrestin-1 and -2 have been described previously by our and other laboratories (*10*, *13*, *18*, *30*, *37*); specificity of knockdown in these studies was confirmed by quantitative Western analysis compared to an internal protein loading control (Figure 5A). Knockdown of either β-arrestin obliterated the PAR-2-stimulated inhibition of PI3K activity (Figure 5B,C). Surprisingly, when β-arrestin-1 was knocked down, PAR-2 activation resulted in a 260 ± 60% increase in PI3K activity, similar to what was previously observed in NIH3T3 cells (see Figure 1). Treatment of MDA MB-468 cells with BAPTA-AM potentiated the PAR-2-stimulated decrease in PI3K activity (not shown), consistent with the hypothesis that β-arrestin-dependent signaling opposes Gαq/Ca^{2+}-dependent signaling downstream of PAR-2. Conversely, in NIH3T3 cells, overexpression of β-arrestin-1−GFP inhibited PAR-2-stimulated PI3K activity by 77 ± 16% (Figure 5D−F). Sufficient overexpression of β-arrestin-2−GFP could not be achieved so we could not determine whether β-arrestin-2 would have the same inhibitory effect on PI3K activity. These data demonstrate that β-arrestin-1 is capable of directing PAR-2-mediated inhibition of PI3K activity, which opposes a Gαq-mediated stimulation of PI3K. Furthermore, the difference in PI3K response to PAR-2 activation may be partially determined by the relative levels of β-arrestin and Gαq.

PI3K Incorporates into a Macromolecular Complex Containing β-Arrestins and PAR-2. If β-arrestin-1 is capable of regulating PI3K activity, we might expect it to directly associate with the enzyme. We next examined whether either or both β-arrestins physically associated with PI3K by co-immunoprecipitation with p85. In resting MDA MB-468 cells a low level of β-arrestin-1 coprecipitated with p85; association of both β-arrestin-1 and -2 with p85 was increased for 5−60 min after PAR-2 activation (Figure 6A). Consistent with our previous studies on β-arrestin-dependent scaffolding (*36*), PAR-2 also associated with p85 within 5 min of receptor activation but did not appear to remain associated at 60 min (Figure 6A, bottom panel). Interactions of β-arrestins and p85 were confirmed in NIH3T3 cells; where due to the low endogenous levels of β-arrestins, we transfected epitope-tagged β-arrestin-1 and -2. Even in untreated NIH3T3 cells, both p85 and the catalytic subunit (p110α) strongly associated with transfected β-arrestin-1, perhaps as a result of overexpression (Figure 6B, left panel). Association of p85 with β-arrestin-2 increased upon PAR-2 activation (Figure 6B, right panel). Importantly, β-arrestins associated with p85 in both cell types. To confirm that β-arrestin-1 directly interacts with PI3K, GST−β-arrestin-1 or GST alone was added to Ni^{+}-Sepharose-bound ^{6}His-p110α complexed to p85α; unbound GST fusion proteins were then bound to glutathione−Sepharose (Figure 7A). GST−β-arrestin-1 strongly associated with p85 α/p110α, while GST alone did not (Figure 7B,C). Thus, it appears that β-arrestin can directly bind the PI3K complex, suggesting that it might directly regulate PI3K activity.

These data suggest that PAR-2 might promote formation of an endosomal scaffold, similar to that described for ERK1/2 (*36*), to regulate PI3K activity. However, in this case, β-arrestin-1 exerts a negative rather than a positive regulatory effect. We further investigated whether p85, β-arrestin-1 and -2, and PAR-2 were present in a macromolecular complex by size exclusion chromatography. By examining coelution of all four proteins on an S300 Sephacryl column, we could ascertain whether they were present in one complex or whether co-immunoprecipitations reflected separate interactions. By concentrating eluted proteins prior to Western blot analysis, we were able to visualize endogenous β-arrestin-1 and -2 in NIH3T3 cell fractions containing PAR-2 and p85 (Figure 8). Furthermore, a higher molecular weight species of both p85 and β-arrestin

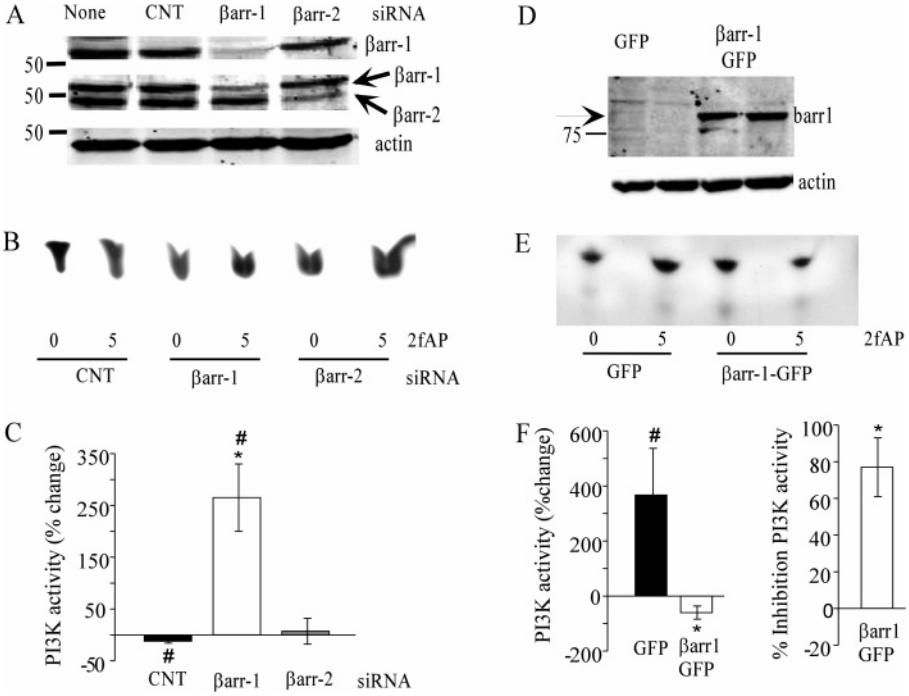

FIGURE 5: Effect of β-arrestin knockdown and overexpression on PI3K activity. (A−C) PI3K activity in MDA MB-468 cells after transfection of control versus β-arrestin-1 and -2 siRNA. (A) Quantitative Western analysis of β-arrestin-1 and -2 levels (upper two panels) or actin as an internal control (lower panel) after transfection with control (CNT), β-arrestin-1 (β-arr1), or β-arrestin-2 (β-arr2) siRNA. Typical knockdown was 80−90% for β-arrestin-1 and 70−80% for β-arrestin-2. (B) Representative TLC autoradiograph of PI3K activity after β-arrestin knockdown. (C) Quantification of percentage increase in PI3K activity after β-arrestin knockdown. (D−F) PI3K activity after overexpression of GFP alone or β-arrestin-1−GFP in NIH3T3 cells. (D) Representative Western blot of GFP, β-arrestin-1−GFP expression in NIH3T3 cells using anti-β-arrestin-1 (upper panel) and anti-actin as a loading control (lower panel). (E) Representative TLC autoradiograph of PI3K activity after β-arrestin-1 overexpression. (F) Quantification of percentage increase in PI3K activity after β-arrestin-1 overexpression. The effect of β-arrestin-1−GFP overexpression is also represented as percentage inhibition of PI3K activity compared to GFP transfected cells. * indicates significant difference in PI3K activity between β-arrestin siRNA or β-arrestin−GFP transfected and control groups; # indicates significant PAR-2 stimulated change in PI3K activity ($P < 0.05$, $n = 5$).

appears upon PAR-2 activation (compare panels A and B of Figure 8), which may reflect a posttranslational modification such as phosphorylation, as has been reported for both proteins (*23*, *38*, *39*). The two PAR-2 bands observed correspond to glycosylated and unglycosylated species (*40*, *41*). Upon PAR-2 activation, all four proteins redistribute (Figure 8, compare protein profile in panel A vs panel B), and both β-arrestin-1 and p85 coelute in two separate protein peaks. Coelution of β-arrestin and p85 is also observed in untreated cells, which may reflect some baseline level of interaction between the two proteins; however, the expected Stokes radius of proteins eluting in those fractions would be about 4.5 nm. Fractions 50−52 contain p85, β-arrestin-1, and PAR-2, suggesting they are present in a macromolecular complex with a Stokes radius of approximately 5.0 nm (Figure 8B). Phosphorylated ERK1/2, which was previously found to coelute with β-arrestin-1 in KNRK cells and colonic enterocytes (*36*), coeluted with β-arrestin-1 and p85 in fractions 40−42 (not shown), suggesting that two separate complexes exist.

β-Arrestin-1 Inhibits PI3K Activity. There are several ways in which β-arrestin might inhibit PI3K activity: by directly inhibiting its kinase activity, by activating an inhibitory factor, or by inducing dissociation of p85 and p110, such that less catalytic activity is present in p85 immunoprecipitates. Because β-arrestin-1 directly associates with PI3K and its overexpression inhibits PAR-2-stimulated PI3K activity, it might directly inhibit the enzymatic activity of the catalytic p110α subunit. To address this hypothesis, increasing concentrations of either β-arrestin-1−GST or GST alone were added to recombinant PI3K (p85 complexed with p110). β-Arrestin-1−GST inhibited PI3K activity by a maximum of 63 ± 8%, with an apparent IC_{50} of 13 nM, while GST alone had a nominal effect (Figure 9A−C). To address whether the PAR-2-induced association of β-arrestin-1 with p85 decreased its association with p110, the presence of p110 in p85 and β-arrestin immune complexes was examined. β-Arrestin binding to the regulatory p85 subunit did not disrupt its interaction of the 100 kDa catalytic subunit as similar levels of p110 could be coprecipitated with p85 in the presence and absence of PAR-2 activation (Figure 9D). Thus, it appears that β-arrestin-1 can associate with PI3K and directly inhibit the catalytic activity of p110α. PAR-2 activation increases this association in whole cells leading to inhibition of PI3K activity, although overexpressed β-arrestin-1 can associate with PI3K in the absence of receptor activation (see Figure 6).

PAR-2 Promotes Colocalization of p85 and β-Arrestin-1 at the Leading Edge. To examine the possible functional significance of these opposing signals to PI3K, we turned to PAR-2-stimulated cytoskeletal reorganization and cell migration. A requirement for β-arrestins in cell migration has been demonstrated by a number of groups, including ours (*11*, *12*, *14*, *16*, *42*−*44*). Since spatial regulation of signaling molecules, including the products of PI3K activity, during cell migration is thought to be essential for directed

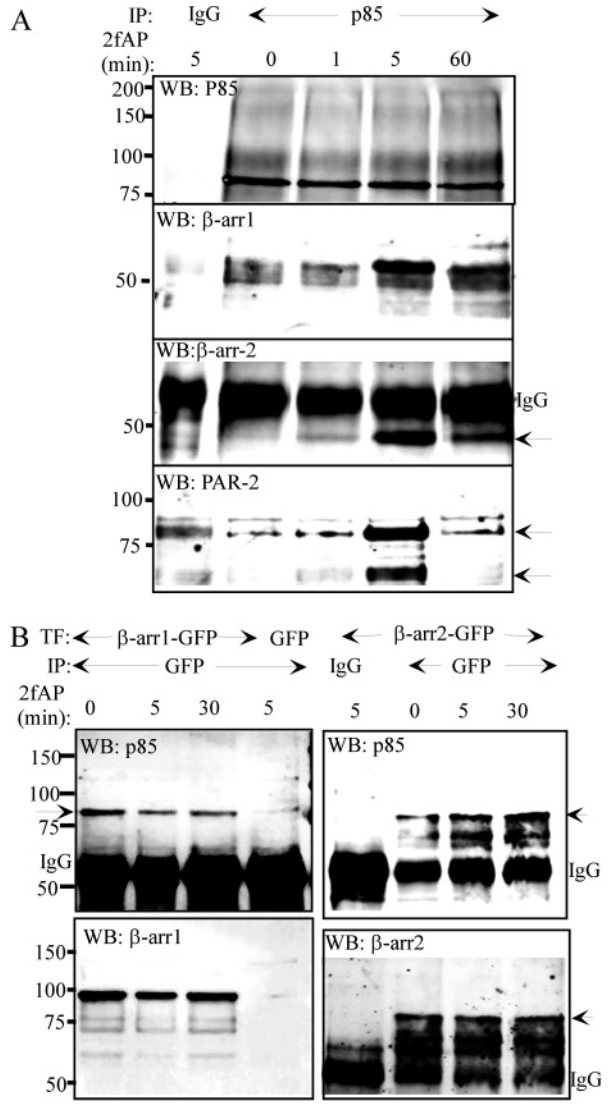

FIGURE 6: Association of p85 with β-arrestins-1 and -2 in response to PAR-2 activation. (A) Coprecipitation of endogenous β-arrestin-1, PAR-2, and p85 in MDA MB-468 cells. Cell extracts after PAR-2 activation with 2fAP for 0−60 min were immunoprecipitated with rabbit anti-p85 or rabbit IgG (first lane) and Western blotted with anti-p85 to demonstrate equal protein levels (upper panel), mouse β-arrestin-1 (second panel), rabbit β-arrestin-2 (third panel), and mouse PAR-2 (bottom panel). IgG is visible in the third panel; β-arrestin-2 is indicated by the arrow. PAR-2 migrates as glycosylated and unglycosylated species in the bottom panel. (B) NIH3T3 cells were transfected (TF) with β-arrestin-1−GFP (left panels), β-arrestin-2−GFP (right panels), or GFP alone (left panel, last lane) and treated with 2fAP for 0−60 min, and cell extracts were subjected to immunoprecipitation with anti-GFP or IgG (right panels, first lane). Western blots representative of four independent experiments are shown. (Upper panel) WB: p85; (lower panel), WB: β-arrestin-1 (left) or β-arrestin-2 (right).

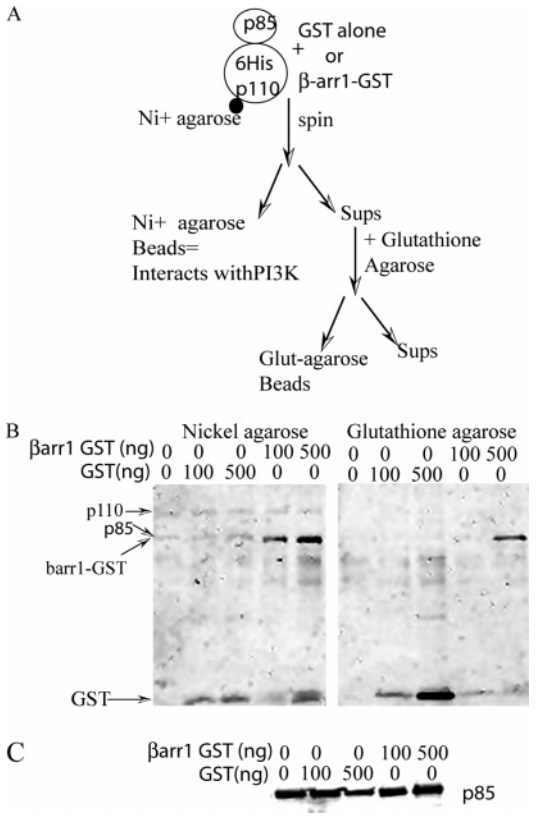

FIGURE 7: Direct association of β-arrestin-1 with PI3K. (A) Schematic of in vitro PI3K binding to β-arrestin-1. 100 ng (0.5 pmol) of ^{6}His-tagged p110α complexed with p85α was immobilized on Ni^+-Sepharose and incubated with 0−500 ng of recombinant GST (0−18 pmol) or GST−β-arrestin-1 (0−10 pmol) for 30 min. p110α/p85α-bound protein was pelleted, and supernatants were incubated with glutathione−agarose to collect GST-bound protein. (B) Representative gel of Ni^+-Sepharose and glutathione−Sepharose bound proteins. Supernatants had no visible protein (not shown). (C) Equal pull-down of p85α was confirmed by Western blotting with p85α antibody. (GST−β-arrestin-1 is the same size as p85α, so p85α is obscured when β-arrestin-1 is added.)

migration (*21*, *23*, *45*−*47*), one possible explanation for the observations thus far is that β-arrestins play a role in localized quenching of PI3K activity. In fact, a role for β-arrestins in the sequestration of signaling molecules to specific cellular microdomains has been reported for a wide variety of receptors (*36*, *48*−*54*). We previously demonstrated that, upon PAR-2 activation, both β-arrestins are found in isolated pseudopodia (*8*, *10*), where they appear to prolong ERK1/2 activation during PAR-2-stimulated cell migration. To determine whether p85 was also enriched in pseudopodia in response to PAR-2 activation, we grew NIH3T3 cells on Transwell filters with 3 μm pores (large enough for pseudopodial extension but too small to allow translocation of cell bodies) and exposed them to 2fAP added to the bottom well only. Equal amounts of protein extracted from either the pseudopodia (underside of filter) or cell body (top of filter) were analyzed by SDS−PAGE followed by Western blotting with antibodies to p85 or histone H1 (as a control for a cell body-specific protein). Approximately 70% of p85 was found in the pseudopodia, while histone H1 was restricted to the cell body (Figure 10A). To investigate where p85 and β-arrestin-1 interact within the cell, we examined their colocalization by confocal microscopy. In resting cells, both β-arrestin-1 and p85 were diffusely localized throughout the cytosol. After PAR-2 activation, some β-arrestin-1 and p85 colocalized both at the tips of and to the base of membrane protrusions (Figure 10B). A considerable portion of β-arrestin-1, but not p85, also redistributes to a perinuclear region, consistent with observations by our and other laboratories. Since β-arrestins have a variety of functions, including receptor endocytosis, this pool of β-arrestin is probably engaged in other cellular processes (*16*, *55*). Notably, p85 is also found, without β-arrestin-1, along the length of membrane protrusions after 5 min of PAR-2 activation,

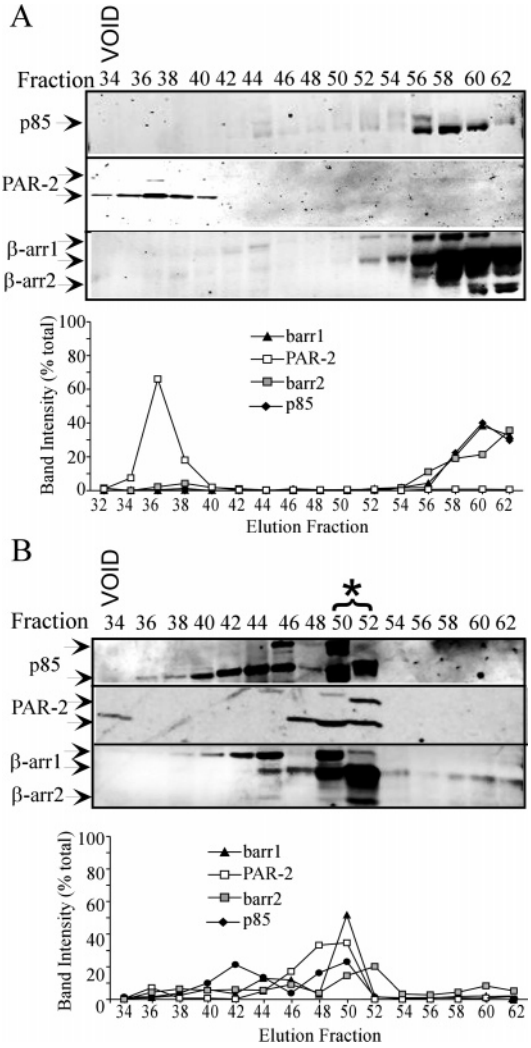

FIGURE 8: Identification of a β-arrestin/p85/PAR-2 complex by gel filtration. Extracts from approximately 10^8 NIH3T3 cells, either untreated (A) or treated with 2fAP for 5 min (B), were separated by size exclusion on a 400 mL S300 Sephacryl column. Protein from every other sample in the included volume (34−62) was concentrated and analyzed by SDS−PAGE followed by Western blotting for p85 (top panels), PAR-2 (middle panels), and β-arrestin-1/2 (bottom panels). Graphs below each set of Western blots depict the relative amounts of protein in each fraction. (Total band density was calculated, and values were represented as a percentage of the total for each protein.) Void volume fractions are indicated; higher fraction numbers correspond to proteins or protein complexes with smaller Stokes radii. An increase in modified species, indicated by arrows, of β-arrestin and p85 appears upon PAR-2 activation (compare panels A and B). The braced fractions (50−52) in panel B represent the region where all three proteins coelute upon PAR-2 activation.

consistent with a model where β-arrestin might exert spatial control over PI3K by inhibiting its activity in distinct cellular microdomains. This raises the question of whether PAR-2 can promote PI3K-independent cell migration. To test this possibility, we quantified cell migration in the presence of two inhibitors of PI3K activity, WM or LY294002. Baseline levels of cell migration were greatly reduced by pretreatment with either 100 nM LY294002 or WM; PAR-2-induced migration was abolished in NIH3T3 cells (Figure 11A) and reduced by 70% in MDA MB-468 cells (Figure 11B). Consistent with its effect on cell migration, formation of actin-rich membrane protrusions in response to 2fAP treatment was also disrupted by pretreatment of cells with WM (Figure 11C).

DISCUSSION

The studies described here suggest that PAR-2 promotes both positive and negative signals to the PI3K pathway through opposing Gαq and β-arrestin-dependent pathways, findings with numerous important implications. Most cells appear to have some baseline level of PI3K activity, which may be essential for cell survival. Interestingly, upon activation of PAR-2 in NIH3T3 cells, PI3K activity is increased, whereas in another cell line (MDA MB-468), PAR-2 inhibits baseline PI3K activity. Inhibition of the Gαq signaling axis in NIH3T3 cells not only inhibits PAR-2-stimulated PI3K activity but unmasks a PAR-2-stimulated decrease from baseline. Conversely, knockdown of β-arrestins in MDA MB-468 cells does not simply abolish the PAR-2-stimulated decrease in PI3K activity but unmasks a PAR-2-stimulated increase. Furthermore, overexpression of β-arrestin-1 in NIH3T3 cells decreases PAR-2-stimulated PI3K activity and addition of recombinant β-arrestin-1 to the purified p110α/p85α complex directly inhibits PI phosphorylation. β-Arrestin appears to bind tightly to PI3K (resistant to 230 mM salt wash) and inhibits with an apparent IC_{50} of 13 nM, suggesting a high-affinity interaction and raising the question of whether it negatively regulates PI3K in response to other signals as well. Taken together, these data suggest that PAR-2 is exerting both positive and negative signals on PI3K activity, the former through classic Gαq signaling and the latter through β-arrestin-dependent signaling.

β-Arrestin-dependent signaling and spatial regulation of kinases have been demonstrated for a number of receptors; in some cases the β-arrestin-dependent signal occurs independent of G-protein engagement and opposes the G-protein-dependent signal (*8*, *11*, *12*, *16*−*19*, *30*, *36*, *48*, *56*, *57*). However, in most of those studies, the β-arrestin-dependent activity was stimulation rather than inhibition of a kinase. We have previously demonstrated that, in some cell types, PAR-2 activates ERK1/2 independent of Ras and tyrosine kinases, and the active ERK1/2 is prevented from translocating to the nucleus to promote proliferation through interaction with β-arrestin-1 (*8*, *10*, *36*). In those studies, a mutant PAR-2, deficient in β-arrestin binding but able to signal through Gαq, activated PYK2 and Src, resulting in Ras-dependent activation of ERK1/2, nuclear translocation, and proliferation (*36*). Consistent with those results, we now find that PAR-2/Gαq coupling promotes binding of PYK2 to p85 and Src-dependent activation of PI3K, while PAR-2/β-arrestin coupling results in recruitment of p85 into a complex and inhibition of PI3K activity. While these studies clearly show that β-arrestin-1 can directly inhibit the catalytic activity of p110α, there are several other ways in which β-arrestins might inhibit PI3K activity in intact cells. First, their ability to uncouple $Gαq/Ca^{2+}$ signaling from PAR-2 activation almost certainly contributes to the inhibition of PI3K activity that appears to be mediated by β-arrestin-1 in these studies. Second, β-arrestin binding to p85 may disrupt its interaction with PYK2, which appears to be involved in PAR-2-stimulated PI3K activation, perhaps acting synergistically with its direct inhibitory effect on p110α. A precedent for β-arrestin-dependent inhibition of the PI3K signaling axis

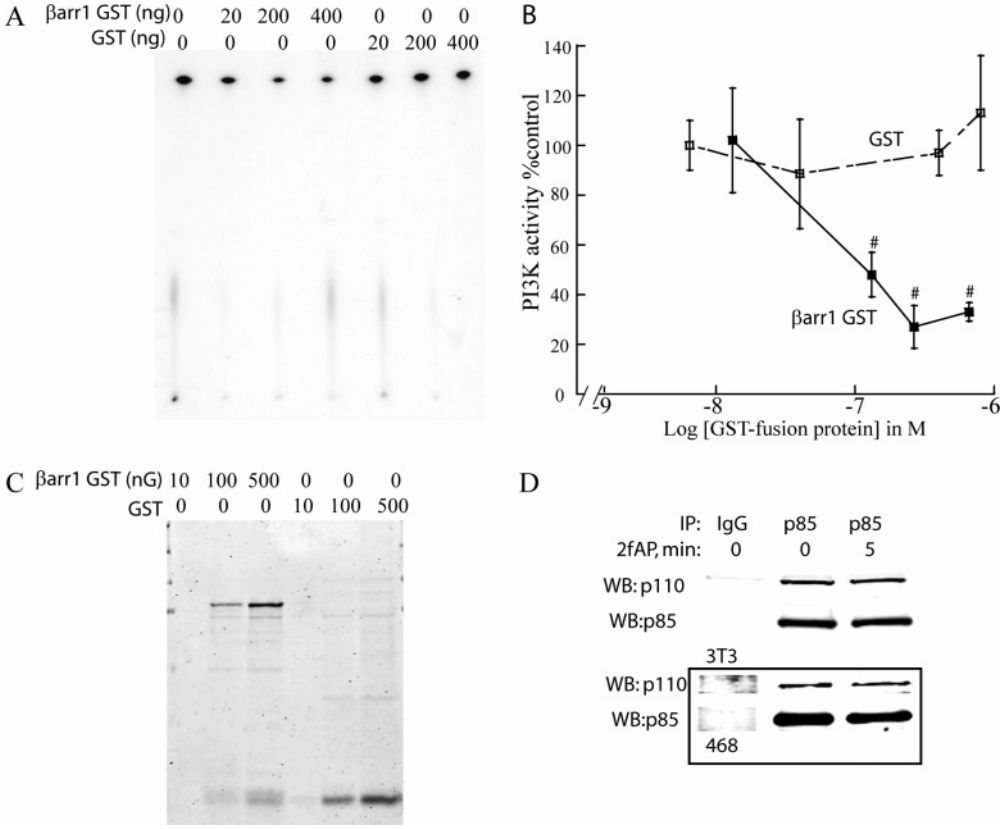

FIGURE 9: β-Arrestin-1 directly inhibits PI3K activity in a dose-dependent fashion. (A−C) Increasing concentrations of purified GST−β-arrestin-1 or GST alone (0−8 nM) were added to purified p110α precomplexed with p85α, and phosphorylation of the PI substrate was determined as described in Figure 1. (A) Representative TLC autoradiograph. (B) Graph depicting inhibition of PI3K activity by GST−β-arrestin-1 or GST alone, where 100% is activity of p110α/p85α alone. (C) Representative gel demonstrating protein levels of β-arrestin-1 and GST added to PI3K reactions. # represents significant difference in PI3K activity with respect to control (no added GST fusion protein). (D) NIH3T3 (upper) or MDA MB-468 cells (lower panel) were treated with or without 2fAP for 5 min and immunoprecipitated with either IgG or anti-p85 followed by Western blotting with p110.

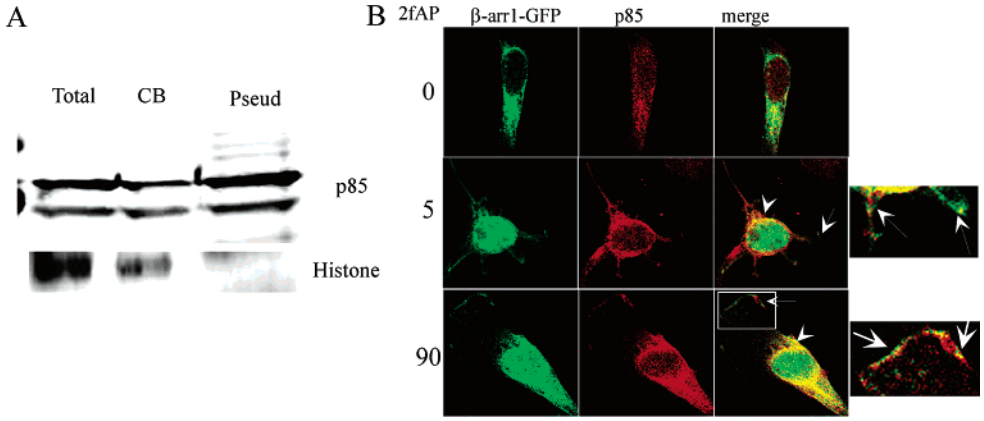

FIGURE 10: p85 colocalizes with β-arrestin-1 in both cell bodies and tips of extending pseudopodia. (A) NIH3T3 cells grown on Transwell filters were treated with 2fAP (added to bottom chamber) for 90 min, and total cell extract (total) total protein was extracted from either pseudopodia (pseud) on the filter underside or cell bodies (CB) on the upper side. The relative amount of p85 found in pseudopodia compared with CB fractions was determined by Western blotting with anti-p85. Total cellular protein is shown in the left lane, and blots were probed with histone H1 to demonstrate the absence of cell body protein in the pseudopodial preparation. (B) NIH3T3 cells, transfected with β-arrestin-1−GFP grown on fibronectin-coated coverslips, were treated with or without 2fAP for 0−60 min, fixed, and stained with anti-p85 (red). Arrows indicate regions of colocalization in the pseudopodia top, and arrowheads point to cell body and at the base of the extending pseudopodia, as demonstrated by yellow in merged images.

was reported for the dopamine receptor, D2A; in those studies, β-arrestin-2 was shown to promote inactivation of Akt downstream of PI3K through direct interaction with its inhibitory phosphatase, PP2A (*58*). Thus, another possible explanation for the results presented here is that β-arrestins counteract PI3K activity by scaffolding a tyrosine phosphatase (tyrosine dephosphorylation would disrupt interaction of p85 with SH2 domain containing proteins) or PTEN (which would dephosphorylate the lipid products of PI3K activity).

There are also reports of β-arrestin-1 facilitating rather than inhibiting PI3K activity. Studies on endothelin A have

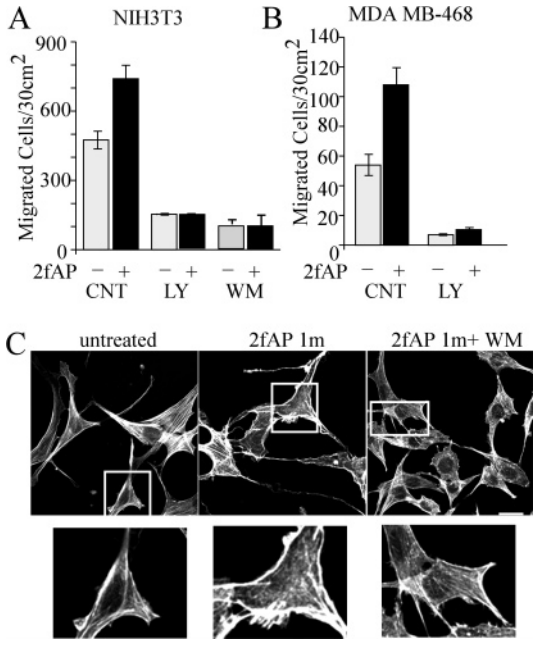

FIGURE 11: PAR-2-induced chemotaxis and cytoskeletal reorganization require PI3K. NIH3T3 (A) and MDA MB-468 (B) cell migration in response to 100 nM 2fAP, with or without inhibition of PI3K with 100 nM wortmannin (WM) or 20 μM LY294002, was determined using a Transwell filter migration assay. Data are presented graphically in terms of cells migrated. NIH3T3 cells are more motile than MDA MB-468 cells; thus, the total numbers differ between the cell lines. (C) Phalloidin staining of NIH3T3 cells after treatment with vehicle, 2fAP (100 nM), or 2fAP plus WM (100 nM, pretreated for 30 min). Note decrease in stress fibers accompanied by increased membrane ruffling observed with 2fAP treatment is inhibited by WM. Insets represent 4× magnification of boxed regions. Scale bar = 10 μm.

demonstrated that β-arrestin-1 leads to recruitment and activation of a Src-family kinase (Yes), which in turn phosphorylates Gαq, leading to activation of PI3K and glucose transport (*24*). We did not observe any significant tyrosine phosphorylation of Gαq in response to PAR-2 activation (P. Wang and K. DeFea, unpublished observations), suggesting that this mechanism may not be involved downstream of PAR-2. Studies on the IGF1R showed that β-arrestin-1 facilitates IGF-stimulated PI3K and Akt activity, perhaps through its ability to bind and recruit Src into a macromolecular complex (*8*, *19*, *49*). On the other hand, previous studies revealed that Src was not present in PAR-2-induced β-arrestin-1 scaffolds and β-arrestin actually inhibited PAR-2-stimulated Src activation (*8*). The main p110 isoform in the cell types examined is p110α; however, it is possible that β-arrestin might differentially affect specific p110 subunits and might even have a potentiating effect on p110β or p110γ, perhaps explaining why β-arrestin is facilitative in some systems but inhibitory in these studies. Another anomaly in these studies was the fact that, in the absence of Gαq, baseline levels were elevated, while PAR-2-stimulated PI3K activity was abolished in the absence of Gαq. There is some precedent for this result, as Gαq can directly bind and inhibit p110α downstream of some receptors (*59*), while upon activation of other receptors, it activates PI3K through β-arrestin-dependent recruitment of Src-family kinases. Furthermore, these intrinsic variations in baseline PI3K activity, which are most likely due to other environmental factors, are likely to contribute to the PAR-2 response that is observed.

We have previously hypothesized that the two β-arrestins may have nonredundant functions, but we have not yet been able to elucidate separate roles (*10*). In these studies, β-arrestin-1 knockdown appeared to have a more potent stimulatory effect on PI3K activity than β-arrestin-2, but both proteins associated with p85 and appeared to be together in a macromolecular complex. Because knockdown efficiency of β-arrestin-2 was lower and we could not achieve high enough levels to examine the effect of overexpression, we cannot rule out a role for β-arrestin-2 at this point. It is possible that β-arrestin-2 is acting in a redundant or a synergistic manner with β-arrestin-1 or that the inhibitory effect of β-arrestin-1 is due to an alteration in β-arrestin-2 interaction with p85 and PAR-2.

Why would one receptor simultaneously activate and inhibit the same enzyme? A logical answer to this question is that one or more cellular responses to PAR-2 activation require spatial regulation of PI3K activity. Because cell migration requires tight spatial regulation of a number of signaling molecules, including the production of acidic phospholipids, it is possible that β-arrestin serves to spatially restrict PI3K activity to regulate localized pools of acidic phospholipids or promote localized activation of signaling molecules, such as RhoA GTPases or Akt, that lie downstream of PI3K. In favor of this hypothesis, p85 can be found in extending membrane protrusions after PAR-2 activation, both with and without β-arrestin-1. There are numerous scenarios where spatial regulation of PI3K activity at the leading edge would be conducive to directed cell migration. PIP3, a product of PI3K activity, can promote activation of RhoA GTPases through interactions with PH-domain-containing guanine exchange factors. RhoA GTPases are, in turn, essential for the formation of lamellipodia, filipodia, and stress fibers as well as for the regulation of actin filament severing through control of LIMK activity (*47*). Furthermore, PIP2 is an important mediator of actin filament capping activity, which controls filament length and preserves filament stability (*47*). Localized uncapping is essential during cell motility, and PI3K-mediated conversion of PIP2 to PIP3 could destabilize filaments by inhibition of capping activity. The data presented here support a model where, upon activation, PAR-2 engages $G\alpha_{q/11}$ and mobilizes Ca^{2+} to PI3K activity, possibly leading to activation of downstream effectors such as Akt and RhoA GTPases, whose activation by PAR-2 has been reported (*60*, *61*) (Figure 12). Simultaneously, β-arrestin-1-bound receptor directly binds to PI3K in specific locations to inhibit its activity; integration of both pathways is essential for directed cell migration.

Gαq signaling is generally considered the predominant mechanism by which PAR-2 signals, and many studies use generation of IP_3 or mobilization of intracellular Ca^{2+} as a readout for PAR-2 activation. Although it has been accepted for some time that β-arrestins are involved in PAR-2 signaling, independent of their role as signal desensitization molecules, the possibility that β-arrestin-dependent signals can occur independent of Gαq engagement and directly oppose Ca^{2+}-dependent signaling has never been proposed. As PAR-2 is becoming an increasingly popular therapeutic target, and studies in whole animals have revealed both pro- and antiinflammatory actions in response to PAR-2 agonists

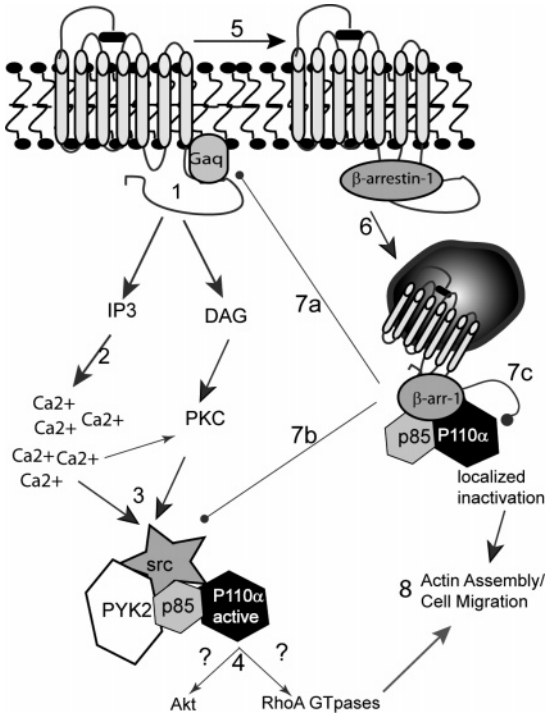

FIGURE 12: Model for opposing PAR-2 signals to PI3K. Upon activation (1) PAR-2 couples to Gαq to produce IP3 and DAG, leading to (2) mobilization of Ca^{2+} and activation of PKC. (3) PYK2 and Src are activated downstream of PAR-2 and bind PI3K to increase its activity. (4) Increased PI3K activity leads to activation of downstream effectors, which may include RhoA GTPases and Akt. (5) Simultaneously, activated PAR-2 binds β-arrestins, (6) recruiting PI3K into a complex, leading to inhibition of PI3K activity, through (7a) uncoupling Gαq from PAR-2, (7b) preventing association of PYK2 and Src with PI3K, and/or (7c) directly inhibiting PI3K activity. (8) The end result is localized activation and inhibition of PI3K activity. Both pathways are essential for actin reorganization and cell migration and are likely spatially separated within the cell to create microdomains of PI3K products.

(*5*), the elucidation of these opposing pathways has great biomedical relevance. Extrapolating from the difference in PI3K response observed here between NIH3T3 cells, which express low levels of β-arrestin-1, and MDA MB-468 cells, which express higher levels, we would predict that one might observe a completely different set of PAR-2-induced responses depending on the level of β-arrestin, and possibly Gαq, expression in a given tissue.

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