Differential regulation of class IA phosphoinositide 3-kinase catalytic subunits p110 α and β by protease-activated receptor 2 and β -arrestins

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PAR-2 (protease-activated receptor 2) is a GPCR (G-protein-coupled receptor) that can elicit both G-protein-dependent and -independent signals. We have shown previously that PAR-2 simultaneously promotes $G\alpha_q/Ca^{2+}$ -dependent activation and β -arrestin-1-dependent inhibition of class IA PI3K (phosphoinositide 3-kinase), and we sought to characterize further the role of β -arrestins in the regulation of PI3K activity. Whereas the ability of β -arrestin-1 to inhibit p110 α (PI3K catalytic subunit α) has been demonstrated, the role of β -arrestin-2 in PI3K regulation and possible differences in the regulation of the two catalytic subunits (p110 α and p110 β) associated with p85 α (PI3K regulatory subunit) have not been examined. In the present study we have demonstrated that: (i) PAR-2 increases p110 α - and p110 β -associated lipid kinase activities, and both p110 α and p110 β are inhibited by over-expression of either β -arrestin-1 or -2; (ii) both β -arrestin-1 and -2 directly inhibit the p110 α catalytic subunit *in vitro*, whereas only β -arrestin-2 directly inhibited p110 β ;

(iii) examination of upstream pathways revealed that PAR-2-induced PI3K activity required the small GTPase Cdc (cell-division cycle)42, but not tyrosine phosphorylation of p85; and (iv) β -arrestins inhibit PAR-2-induced Cdc42 activation. Taken together, these results indicated that β -arrestins could inhibit PAR-2-stimulated PI3K activity, both directly and through interference with upstream pathways, and that the two β -arrestins differ in their ability to inhibit the p110 α and p110 β catalytic subunits. These results are particularly important in light of the growing interest in PAR-2 as a pharmacological target, as commonly used biochemical assays that monitor G-protein coupling would not screen for β -arrestin-dependent signalling events.

Key words: arrestin, phosphoinositide 3-kinase (PI3K), PI3K regulatory subunit (p85), PI3K regulatory subunit (p110), protease-activated receptor 2 (PAR-2).

INTRODUCTION

PAR-2 (protease-activated receptor 2) is a GPCR (G-protein-coupled receptor) that is capable of eliciting both $G\alpha_q$ -dependent and β -arrestin-dependent signals [1–6]. We have shown previously that PAR-2 simultaneously promotes $G\alpha_q/Ca^{2+}$ -dependent PI3K (phosphoinositide 3-kinase) activation and β -arrestin-1-dependent inhibition of PI3K activity. The $G\alpha_q/Ca^{2+}$ -dependent pathway involves PKC (protein kinase C) and SFK (Src-family kinase) activity, as well as the association of PYK2 (proline-rich tyrosine kinase 2) with p85 (PI3K regulatory subunit). The β -arrestin-dependent pathway involves the direct association of p85/p110 (PI3K catalytic subunit) α with β -arrestin-1, resulting in a decrease in p110 α catalytic activity [7]. In a cell line with high endogenous levels of β -arrestins (MDA MB-468, a breast cancer cell line), the inhibitory pathway dominates, resulting in a PAR-2-stimulated decrease in PI3K activity. In contrast, in a cell line with low endogenous levels of β -arrestins (NIH 3T3), an overall PAR-2-induced increase in PI3K activity is observed. These studies highlight the fact that PAR-2 can direct opposing signals in a cell-type-specific fashion, and that the prevalence of certain downstream signalling molecules, such as β -arrestins, can alter the outcome of PAR-2 activation. This inhibitory role for β -arrestins in PI3K activity is in contrast with studies on the receptor tyrosine kinase, IGF (insulin-like growth factor)-1, where β -arrestin was proposed to facilitate PI3K activation in the absence of IRS (insulin receptor

substrate)-1 [8]. In those studies, PI3K activity was measured after immunoprecipitation with the p110 α catalytic subunit, whereas our previous studies were performed by immunoprecipitation with the p85 subunit [7], which would co-immunoprecipitate both p110 α and p110 β [8a]. Also, there are two β -arrestin family members, both of which associate with p85 upon PAR-2 activation [7], but it is unknown whether both β -arrestins can inhibit PI3K activity and whether they display specificity for one catalytic subunit. Although they are redundant for some functions, the two β -arrestins have some specific binding partners and downstream effects, raising the possibility that they differentially regulate the PI3K pathway. We therefore investigated which catalytic subunit was preferentially targeted by the PAR-2 pathway and whether both β -arrestins inhibited p110 α and p110 β catalytic activity. We also examined what other upstream components are involved in PAR-2-induced PI3K signalling and their inhibition by β -arrestins.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma or Fisher Scientific except where otherwise indicated. FLAG-tagged β -arrestin-1 and β -arrestin-2 were gifts from Dr Robert Lefkowitz (Department of Medicine, Duke University Medical Center, Durham, NC, U.S.A.). LipofectamineTM was bought from

Abbreviations used: Cdc, cell-division cycle; CRIB, Cdc42/Rac-interacting binding; DKO, double knock-out; 2fAP, 2-furoyl-LIGRL-O-NH₂; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione transferase; HA, haemagglutinin; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; IPTG, isopropyl β -D-thiogalactoside; IRS, insulin receptor substrate; MEF, mouse embryonic fibroblast; PAK, p21-activated kinase; PAR-2, protease-activated receptor 2; PI3K, phosphoinositide 3-kinase; p85, PI3K regulatory subunit; p110, PI3K catalytic subunit; PKC, protein kinase C; PYK2, proline-rich tyrosine kinase 2; RT, reverse transcriptase; SFK, Src-family kinase; wt, wild-type.

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Invitrogen. 2fAP (2-furoyl-LIGRL-O-NH₂) was synthesized by Genemed (San Francisco, CA, U.S.A.). [γ -³²P]ATP was purchased from PerkinElmer. PtdIns was bought from Avanti Polar Lipids and PtdIns(4,5)P₂ was purchased from Echelon (Salt Lake City, UT, U.S.A.). Recombinant PI3K (p85 α complexed to p110 α) was bought from Upstate. GST (glutathione transferase)-tagged β -arrestins were a gift from Dr Kerri Mowen (Department of Chemical Physiology, The Scripps Research Institute, San Diego, CA, U.S.A.). Dominant-negative RhoAGTPases: HA (haemagglutinin)-tagged RhoN19 (DNRHO), GFP-tagged CDC42N17 (DNCDC) (where GFP is green fluorescent protein and Cdc is cell-division cycle) and GFP-tagged RacN17 (DNRAC) were gifts from Dr Martin Schwartz (Department of Microbiology, University of Virginia, Richmond, VA, U.S.A.).

Antibodies

All anti-FLAG antibodies were purchased from Sigma. Rabbit anti-(PI3K p85), anti-p110 α and anti-p110 β antibodies and Protein A-agarose were bought from Upstate. Rabbit anti-GFP antibody was purchased from Chemicon and the mouse monoclonal anti-HA antibody was bought from Cell Signaling Technology. The IRDye[®] 800-conjugated mouse anti-phospho-tyrosine (RC20) was from Rockland Immunochemicals (Gilbertsville, PA, U.S.A.). Rabbit anti- β -arrestin-1 and anti- β -arrestin-2 antibodies (A1CT and A2CT) were gifts from Dr Robert Lefkowitz. Mouse anti- β -arrestin-1, anti-RhoA and anti-Cdc42 antibodies were purchased from BD Pharmingen. Rabbit anti-Rac antibody was bought from Cytoskeleton (Denver, CO, U.S.A.). Goat anti-actin was purchased from Santa Cruz Biotechnology. Alexa Fluor[®] 680-conjugated secondary antibodies were bought from Molecular Probes and IRDye[®] 800-conjugated secondary antibodies were purchased from Rockland Immunochemicals.

Cell culture and transient transfections

NIH 3T3, MEF (mouse embryonic fibroblast) wt (wild-type), MEF β -arrestin-1, β -arrestin-2-DKO (double knock-out), MEF β -arrestin-1, β -arrestin-2-DKO with β -arrestin-1 and MEF β -arrestin-1, β -arrestin-2-DKO with β -arrestin-2 cell lines were grown in DMEM (Dulbecco's modified Eagle's medium) (Mediatech) supplemented with 10% (v/v) cosmic calf serum (Hyclone) and maintained at 37°C with in an atmosphere of 5% CO₂. NIH 3T3 cells (5 × 10⁵ cells) were grown on 10 cm tissue-culture dishes overnight to approx. 70% confluency and then were transiently transfected with 10 µg of FLAG-tagged β -arrestin-1, β -arrestin-2, DNRHO, DNCDC or DNRAC using Lipofectamine[™] according to the manufacturer's instructions. Cells were harvested for experiments 36–48 h after transfection.

RT (reverse transcriptase)-PCR and electrophoresis of products

Oligonucleotide primers for PCR were synthesized (Sigma Genosys) as follows. Mouse p110 α : sense 5'-GTCACCCAA-GAAGCAGAAAG-3' and anti-sense 5'-TCTGCTTGTCGTTG-TTGG-3' (400 bp: size of amplified fragment); mouse p110 β : sense 5'-CAGATGTTATGGAAGCAAG-3' and anti-sense 5'-ATACTCCACTCTCCCACTG-3' (600 bp: size of amplified fragment); mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase): sense 5'-GCCATCACCATTCTCCAG-3' and anti-sense 5'-ACGCCACAGCTTTCCAGAG-3' (376 bp: size of amplified fragment). Total cellular RNA was isolated using TRIzol[®] (Invitrogen) according to the manufacturer's protocol. The concentration of total RNA was determined using spectrophotometric analysis in conjunction with the IBM program (Beckman Coulter), and 2 µg of total RNA was used as template for cDNA first-strand synthesis performed using a first-strand

cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. cDNA synthesized from reverse transcription was used as the DNA template for PCR amplifications using Taq polymerase and PCR was performed for 35 cycles. The amplified PCR products were resolved using a 1.5% (w/v) agarose gel containing 1 µg/ml of ethidium bromide, and the DNA bands were visualized by UV illumination. Stored images of the gels were analysed by densitometry using Gel Base/Gel Blot software (Ultraviolet Products, Upland, CA, U.S.A.). GAPDH levels were measured as an internal control.

Recombinant protein expression

GST-tagged β -arrestins, PAK (p21-activated kinase) 3 and Rhotekin were expressed and purified from *Escherichia coli* cells using glutathione-Sepharose (Pharmacia). Briefly, *E. coli* BL21DE3 cells were transformed with GST-tagged fusion proteins, grown to exponential phase [where D_{600} (attenuance) is 0.8 for Rhotekin and PAK3, and D_{600} is 0.6 for β -arrestin-1 and -2] and induced with IPTG (isopropyl β -D-thiogalactoside). Rhotekin and PAK3 were induced with 0.5 mM IPTG for 2 h, and β -arrestin-1 and -2 were induced for 8 h with 1 mM IPTG. Bacterial cells were subsequently lysed by sonication (6 bursts of 10 s at 30% power) in PBS supplemented with 10 µg/ml lysozyme, 10 µg/ml DNase 1 and 1% (v/v) Triton X-100. Cleared lysates were incubated with 300 µl of glutathione-Sepharose for 1 h, washed with 20 volumes of binding buffer supplemented with 1 mM ATP, and the bound protein was eluted using increasing concentrations of free reduced glutathione. Elution fractions containing the fusion protein were dialysed overnight against PBS and stored in 10% (v/v) glycerol at -80°C until needed. The protein concentration of eluates was determined using the Bradford assay, and the relative purity was determined by SDS/PAGE (10% gels), followed by staining with Coomassie Brilliant Blue R250. Some breakdown of both β -arrestin proteins was observed in each preparation, which was quantified by densitometric analysis of the Coomassie-stained gels. The protein concentrations shown in Figure 3 were calculated on the basis of total purified protein containing 60% full-length β -arrestin-1 and 50% full-length β -arrestin-2.

Immunoblotting

All Western blots were performed by transfer on to PVDF-FI membranes and blocked in 50 mM Tris/HCl, pH 7.6, 150 mM NaCl and 1% (w/v) fish gelatin. Primary antibodies were added at the following concentrations: anti-(β -arrestin1/2) (A1CT), 1:500; anti-FLAG, 1:1000; anti-RhoA, 1:1000; anti-Rac, 1:250; anti-Cdc42, 1:1000; anti-p110 α , 1:1000; anti-p110 β , 1:1000; anti-p85, 1:1000; anti-HA-7, 1:750; anti-GFP, 1:1000 and anti-actin, 1:1000. Immunoreactive bands were visualized using the Odyssey infrared imaging system (LI-COR Biosciences) using Alexa Fluor[®] 680-conjugated and IRDye[®] 800-conjugated secondary antibodies at a concentration of 1:40000. Integrated band intensities were calculated using the LI-COR Odyssey software.

In vivo and in vitro PI3K assays

NIH 3T3 cells at 80% confluence were transfected with FLAG- β -arrestin-1 or FLAG- β -arrestin-2 and serum-starved for 16 h before stimulation with 100 nM 2fAP or IGF-1 for 0–5 min at 37°C. The cells were lysed in ice-cold buffer A [20 mM Tris/HCl, pH 7.4, 137 mM NaCl, 1% (v/v) NP40 (Nonidet P40), 1 mM CaCl₂, 1 mM MgCl₂ and 0.1 mM NaVO₄]. Cleared lysates (500 µg of protein) were incubated with 4 µg of either anti-p110 α or anti-p110 β antibodies at 4°C for 2 h, followed

by incubation with 40 μ l of Protein A-agarose for 2 h at 4°C. The immunoprecipitations were washed using various buffers, with three washes in each of: (i) buffer A, (ii) buffer B (100 mM Tris/HCl, pH 7.4, 5 mM LiCl and 0.1 mM NaVO₄) and (iii) TNE buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1 mM NaVO₄). PI3K reactions were performed in 50 μ l of TNE buffer supplemented with 20 μ g of PtdIns (approx. 400 μ M) or PtdIns(4,5)P₂ (approx. 320 μ M), 20 mM MgCl₂ and 5 μ Ci of [γ -³²P]ATP at 37°C for 10 min and the reactions were stopped by the addition of 20 μ l of 6 M HCl. For *in vitro* assays, 10 ng (approx. 50 pmol) of recombinant p85/p110 α or p85/p110 β were incubated with 20 μ g of PtdIns and 5 μ Ci of [γ -³²P]ATP in the presence of 20–40 μ l of purified β -arrestin-1 or β -arrestin-2. Purified β -arrestins were prepared from two sources: FLAG- β -arrestins were immunoprecipitated from transfected NIH 3T3 cells and compared with reactions containing anti-FLAG immunoprecipitates from untransfected NIH 3T3 cells as a negative control. Either 20 or 40 μ l of anti-FLAG-agarose (containing 25 ng/ μ l β -arrestin-1 or 2.5 ng/ μ l β -arrestin-2) was added to the PI3K reactions. The approximate protein concentrations of the purified β -arrestins were determined by SDS/PAGE (10% gels), followed by densitometric analysis of β -arrestin bands compared with serial dilutions of BSA. GST- β -arrestins expressed in *E. coli* were compared with reactions containing GST alone. Lipids were extracted from the organic layer which was formed after the addition of 160 μ l of chloroform/methanol (1:1 ratio), and ³²P-labelled phospholipids were resolved by TLC on potassium oxalate-treated silica plates, in chloroform/methanol/water/ammonium hydroxide (60:47:11.3:2 ratio). Migration rates of phospholipid products were compared with those of Molybdenum Blue-stained standards of PtdIns, PtdIns(4)P, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃. The density of the radioactive lipids was determined by phosphorimage analysis using a Storm PhosphorImager (Bio-Rad).

Rho GTPase assays

Activation of RhoA GTPases was measured using a CRIB (Cdc42/Rac-interacting binding) domain pull-down assay as described previously [9]. Briefly, GST fusion proteins containing the CRIB domain from PAK3 or Rhotekin were bound to glutathione-Sepharose and incubated for 30 min at 4°C with lysates from NIH 3T3 cells treated for 5 min with either 100 μ M PAR-2-activating peptide or 10% (v/v) serum as a positive control. The beads were washed three times and analysed by SDS/PAGE (12.5% gels), along with total lysates, then transferred on to PVDF membranes and blotted with rabbit anti-CDC42 and mouse anti-Rac-1 antibodies, or with rabbit anti-RhoA antibody.

Data analysis

Percentage changes in PI3K activity were calculated from the phosphorimage band density in PAR-2-treated compared with untreated cells for each experimental group. Band densities were normalized to the total p85 immunoprecipitated as determined by Western blotting. The baseline PI3K levels were those present in the absence of PAR-2 activation and were treated as 100%. Therefore changes to the baseline levels of PI3K activity were not apparent in the results. All experiments were repeated a minimum of three times and results are means $\pm$ S.E.M. Differences between multiple groups were examined by two-way ANOVA and Tukey *t*-tests, with *P* < 0.05 considered 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 of inhibitor compared with the absence

Figure 1 Expression of p110 α and p110 β subunits in NIH 3T3 cells

(A) Specificity of antibodies in NIH 3T3 cell lysates. Lysates were immunoprecipitated (IP) with anti-IgG and anti-p110 β antibodies (left-hand side panel) or anti-IgG and anti-p110 α antibodies (right-hand side panel), followed by Western blotting with antibodies against p110 α , p110 β and p85. No p110 α was co-immunoprecipitated with anti-p110 β antibody and vice versa. (B) Protein expression of p110 α and p110 β in NIH 3T3 whole-cell lysates. Representative Western blots probed with anti-p110 α and anti-actin antibodies (top panel) or p110 β and actin (bottom panel). (C) mRNA expression of p110 α and p110 β in NIH 3T3 cells. cDNA was synthesized from total RNA isolated from NIH 3T3 cells, and used as a template for PCR. A representative agarose gel of PCR products using specific primers directed against p110 α , p110 β and GAPDH (internal control) is shown.

of inhibitor) was determined. Apparent IC₅₀ values for *in vitro* PI3K assays were calculated using Kaleidagraph 4.0 (Synergy Software, Reading, PA, U.S.A.). Data were fitted using a sigmoidal dose-response model with a 95% confidence interval.

RESULTS

PAR-2 can activate both p110 α and p110 β

We have shown that PAR-2 promotes β -arrestin-dependent inhibition and G α q/Ca²⁺/SFK-dependent stimulation of p85-associated PI3K activity. This was in contrast to previous studies on the IGF-1R (IGF-1 receptor), which suggested that β -arrestin-1 facilitated PI3K activity. Since p85 can associate with both p110 α and p110 β [8a], we examined the possibility that PAR-2 and IGF-1R can differentially modulate the activity of these two subunits. We first established that both subunits were expressed in NIH 3T3 cells, and that the antibodies used were specific for each catalytic subunit. The specificity of each p110 antibody was confirmed by immunoprecipitation with either anti-p110 α or anti-p110 β antibodies, followed by Western blotting with antibodies against p110 α , p110 β and the p85 regulatory subunit (which binds both p110 subunits). There is no visible p110 β immunoreactivity in p110 α immunoprecipitates and vice versa, but both can co-immunoprecipitate p85 (Figure 1A), confirming their specificity and the existence of separate p85/p110 α and p85/p110 β complexes. To compare the expression of both the

Figure 2 PAR-2 activates both p110 α and p110 β subunits of the class IA PI3K kinase

NIH 3T3 cells were treated with or without 100 nM 2fAP (PAR-2 activating peptide) or 100 nM IGF-1 for 5 min, lysed and immunoprecipitated (IP) with antibodies against p110 α or p110 β . Immune complexes were incubated with PtdIns in the presence of [γ - 32 P]ATP and phospholipids were separated by TLC to determine PI3K activity. (A) Western blot with anti-IgG and anti-p85 antibodies to demonstrate equal immunoprecipitation levels. (B) Representative autoradiograph of phospholipids separated by TLC. Migration rates of PtdIns product (PIP) were compared with rates of stained standards, and the migration position is indicated by an arrow. A full TLC plate with both origin and phospholipids is shown. (C) Histogram depicting baseline PI3K activity in p110 α and p110 β immunoprecipitates as normalized phosphate incorporation into PtdIns (left-hand side panel), and fold increase in PI3K activity after treatment with 2fAP or IGF-1 as a fraction baseline (right-hand side panel). (D) PI3K assay was performed as described above using PtdIns(4,5) P_2 as a substrate. Incorporation of phosphate in all PI3K assays was determined by phosphorimage analysis. Fold change was determined by the ratio of band density in treated samples compared with untreated samples. Arbitrary units refer to raw band density, as determined by phosphorimage analysis. All densities were normalized to levels of p85 in p110 α or p110 β immunoprecipitates.

catalytic subunits in NIH 3T3 cells, we examined protein levels from whole-cell lysates by Western blotting and mRNA levels by RT-PCR from total cellular RNA (Figures 1B and 1C). In NIH 3T3 cells, the expression of mRNA and protein for both p110 α and p110 β catalytic subunits was detected.

We then compared the activation of either p110 α or p110 β by PAR-2 and IGF-1 using a standard PI3K assay [10]. NIH 3T3 cells were treated with IGF-1 or 2fAP to activate PAR-2, then the cells were lysed and immunoprecipitated with antibodies against either p110 α or p110 β . PI3K activity was determined by phosphorylation of purified PtdIns. PAR-2 significantly increased p110 α activity 4.2-fold and increased p110 β activity 2-fold (Figures 2A–2C). The baseline activity of p110 β appeared to be higher than that for p110 α (Figure 2C, left-hand side panel), indicating that the induction in response to PAR-2 was lower. In contrast, IGF-1 treatment increased the activity of p110 α only 2-fold and had no measurable effect on p110 β (Figures 2A–2C). Similar results were observed when the assays were repeated using PtdIns(4,5) P_2 as a substrate, confirming that the observed activity was not a result of co-immunoprecipitation of phosphoinositide 4-kinases with any of the PI3K subunits (Figure 2D). These data demonstrate that PAR-2 can activate both of the class IA p110s; thus the p85-associated PI3K activity reported previously [7] is most likely to represent a combination of p110 α and p110 β catalytic activity.

β -Arrestins inhibit PAR-2-induced p110 α and p110 β activation

In previous studies, over-expression of β -arrestin-1 inhibited PAR-2-induced PI3K activity associated with p85. However, the ability of β -arrestin-2 to inhibit PI3K was not examined. To

determine whether the two β -arrestins differ in their ability to inhibit PAR-2-induced activation of either p110 α or p110 β , the assay described above was repeated in the presence and absence of over-expressed FLAG- β -arrestin-1 and FLAG- β -arrestin-2. Similar to what was reported previously for p85 immunoprecipitates [7], over-expression of either β -arrestin-1 or β -arrestin-2 eliminated 2fAP-induced p110 α and p110 β activity (Figure 3). β -Arrestin-2 was a more potent inhibitor of p110 β : when β -arrestin-2 was over-expressed, PAR-2 inhibited p110 β activity by 50 % (Figure 3B), comparable with what was observed previously in MDA MB-468 cells, which express high levels of both β -arrestins [7]. Together with the previous results comparing PI3K signalling in NIH 3T3 and MDA MB-468 cells, these data demonstrate that both β -arrestin-1 and β -arrestin-2 inhibit PAR-2-induced PI3K activity.

Inhibition of p110 α and p110 β catalytic activity by both β -arrestin-1 and β -arrestin-2

To determine whether the β -arrestin-mediated inhibition of PI3K was direct, we investigated the ability of recombinant GST-tagged β -arrestin-1 and β -arrestin-2 to inhibit the activity of recombinant p85/p110 α or p85/p110 β complexes *in vitro* (Figures 4A–4D). Expanding on our previous findings that β -arrestin-1 directly binds to and inhibits p85/p110 α complexes, we incubated purified GST-tagged β -arrestin-1, GST-tagged β -arrestin-2 and GST alone with either recombinant p85/p110 α or p85/p110 β complexes and assayed the phosphorylation of PtdIns *in vitro* (Figures 4A and 4B). β -Arrestin-1 and β -arrestin-2 were equally potent inhibitors of p110 α activity, as the apparent IC₅₀ was

Figure 3 PAR-2 inhibits p110 α and p110 β activity through both β -arrestin-1 and β -arrestin-2

NIH 3T3 cells transfected with FLAG-tagged β -arrestin-1 (Flag- β arr1), β -arrestin-2 (Flag- β arr2) or empty vector as a control (Cnt) were treated with 100 nM 2fAP for 0 or 5 min, and PI3K activity was measured from samples immunoprecipitated (IP) using anti-p110 α (A) and anti-p110 β (B) antibodies as performed in Figure 2. A representative autoradiograph of phospholipids separated by TLC is shown, where PIP is the PtdIns product and a histogram showing PI3K activity in response to 2fAP as fraction baseline. (C) Representative Western blot showing anti-FLAG antibody immunoreactivity in cells transfected with empty vector as a control (cnt) or either FLAG- β -arrestin-1 (Flag- β arr1) or FLAG- β -arrestin-2 (Flag- β arr2).

8.6 nM for β -arrestin-1 and 6.2 nM for β -arrestin-2, with the Pearson correlation coefficient for sigmoidal dose curve fit (R) > 0.99, but β -arrestin-2 was a more efficacious inhibitor, providing a maximum inhibition of $94 \pm 7\%$ compared with $64 \pm 8\%$ for β -arrestin-1. Surprisingly, whereas β -arrestin-2 strongly inhibited p110 β activity with an apparent IC_{50} of 10.6 nM (R > 0.99) and maximum inhibition of $97 \pm 2\%$, β -arrestin-1 stimulated p110 β activity 2.4 ± 0.2 -fold. In contrast, when β -arrestin-1 or β -arrestin-2 was purified from NIH 3T3 cells and added to cell-free kinase reactions, they each inhibited the catalytic activity of both p110 α and p110 β (Figures 4C and 4D). SDS/PAGE analysis of GST-tagged and FLAG-tagged proteins is displayed in Figures 4(E) and 4(F). These data suggest that β -arrestin-2 may be a more efficacious inhibitor of PI3K activity than

β -arrestin-1. Furthermore, p110 β may be differentially affected by β -arrestin-1 depending on the presence of post-translational modifications or accessory proteins, and these factors in turn may vary between cell types and the activation state of the cell.

Mechanism of PAR-2-stimulated PI3K activity

Previous studies have demonstrated that PAR-2 promotes Ca^{2+} -dependent activation of PYK2 and Src, and association of PYK2 with p85. Furthermore, inhibition of SFKs, Ca^{2+} chelation or $G\alpha_q$ knock-down blocked PAR-2-induced PI3K activity [7,11]. As multiple signalling pathways can converge on PI3K in order to increase its activity, the stimulatory effect of PAR-2 may reflect a contribution from multiple signalling arms. The most common means of PI3K activation by receptor tyrosine kinases such as IGF-1R involves the phosphorylation of tyrosine residues in the SH2 domain of p85 and its association with other phosphotyrosine proteins. In order to determine whether PAR-2 induced the tyrosine phosphorylation of p85, NIH 3T3 cells were treated with or without 2fAP for 5 min (when maximal PI3K activity is observed), and lysates were immunoprecipitated with anti-p85 antibody, followed by Western blot analysis with anti-phosphotyrosine antibody (Figure 5A). No significant phosphorylation of p85 was observed. However, two tyrosine phosphorylated bands (at approx. 55 kDa and approx. 100–120 kDa) were associated with p85 after PAR-2 stimulation, consistent with what has been reported for $G\alpha_q$ -coupled chemokine receptors. The 100–120 kDa phospho-tyrosine band is consistent with the migration of p110 (Figure 5A, bottom panel), but could also be consistent with the size of PYK2, which has been demonstrated previously to associate with p85 on PAR-2 activation [7].

RhoA GTPases have also been identified as upstream regulators, as well as downstream effectors, of PI3K [12–14]. To investigate whether RhoA GTPases were involved in PAR-2-stimulated PI3K activation, p85-associated PI3K activity was assayed after treatment of NIH 3T3 cells transfected with dominant negative mutants of RhoA (DNRHO), Cdc42 (DNDCDC) and Rac-1 (DNRAC) with 2fAP. Expression of DNDCDC, but not DNRHO or DNRAC, abolished PAR-2-stimulated PI3K activation (Figure 5B). p85 was used in these studies, as it co-immunoprecipitates both p110 α and p110 β . Although p85 has a GTPase-binding domain, we could not detect association of Cdc42 with p85 by co-immunoprecipitation (results not shown). Expression of the dominant-negative GTPases was assessed by Western blotting, and this is shown in Figure 5(D). PAR-2-induced regulation of RhoA, Rac-1 and Cdc42 was confirmed using a GST-CRIB-domain pull-down assay (Rhotekin CRIB domain for RhoA and PAK3 CRIB domain for Cdc42 and Rac-1) [9]. Treatment of NIH 3T3 cells with 2fAP increased the binding of Rac-1 and Cdc42 to PAK3-GST, but did not increase RhoA binding to Rhotekin-GST (Figures 6A and 6B), suggesting that PAR-2 activates both Rac-1 and Cdc42.

To examine the role of β -arrestins in this pathway, we compared Cdc42 activation in mouse embryonic fibroblast (MEF) cell lines from wild-type mice (MEFwt), β -arrestin-1/2 $^{-/-}$ mice (MEF β arr DKO) or MEF β arr DKO cells transfected with either β -arrestin-1 (DKO + β arr1) or β -arrestin-2 alone (DKO + β arr2). In MEFwt, PAR-2 promoted a decrease in Cdc42 activity, whereas in the absence of both β -arrestins, PAR-2-promoted a 2-fold increase in Cdc42 activation, similar to that observed in NIH 3T3 cells. Transfection of either β -arrestin-1 or β -arrestin-2 inhibited Cdc42 activation (Figures 6C and 6D). These data suggests that the mechanism by which PAR-2 activates PI3K involves input from multiple upstream pathways and differs from that used by the classical receptor-tyrosine kinases.

Figure 4 Effect of β -arrestins on the catalytic activity of p110 α and p110 β *in vitro*

(A,B) Increasing concentrations (nM) of GST- β -arrestin-1 (β 1GST), GST- β -arrestin-2 (β 2GST) or GST alone (negative control) were added to reaction mixtures containing [γ - 32 P]ATP, purified PtdIns and either recombinant p85/p110 α (A) or recombinant p85/p110 β (B). Upper panels show representative autoradiographs of phospholipids separated by TLC. Lower panels show semi-log plots of PI3K activity (defined as the fraction of control) as a function of the GST fusion protein concentration (in M). The control activity is that observed in the absence of added GST fusion protein. (C,D) Increasing amounts of anti-FLAG antibody immunoprecipitates from NIH 3T3 cells transfected with FLAG- β -arrestin-1 (β 1-Flag), FLAG- β -arrestin-2 (β 2-Flag) or empty vector as a control (CT) were added to *in vitro* recombinant p85/p110 α (C) and recombinant p85/p110 β (D) and kinase activity was measured as described above. The amount of anti-FLAG-agarose added (20 and 40 μ l) corresponded to approx. 500 and 1000 ng of β -arrestin-1 and approx. 50 and 300 ng of β -arrestin-2. Upper panels show representative autoradiographs of phospholipids separated by TLC. Lower panels show plots of PI3K activity as a function of β -arrestin added (in ng). PI3K activity is defined as the fraction of control (activity observed in the presence of an equal volume of anti-FLAG immunoprecipitates from untransfected cells). (E) Representative Coomassie-stained gels of increasing amounts of β -arrestin-2-GST (β -arr-2GST) and GST (left-hand side panel) and β -arrestin-1-GST (β 1GST) (right-hand side panel). (F) Representative Coomassie-stained gel of increasing amounts of FLAG- β -arrestin-1 (β 1-Flag) and FLAG- β -arrestin-2 (β 2-Flag).

DISCUSSION

These results are the first to demonstrate the regulation of the individual catalytic subunits of class IA PI3K by PAR-2 and β -

arrestins. We have shown that PAR-2 can activate both p110 α and p110 β in NIH 3T3 cells, and that β -arrestin-1 and β -arrestin-2 can differentially regulate the activity of each subunit.

Figure 5 PAR-2-stimulated PI3K activity requires Cdc42 but not p85 tyrosine phosphorylation

(A) NIH 3T3 cells were treated with or without 100 nM 2fAP for 5 min and immunoprecipitated (IP) with anti-IgG or anti-p85 antibodies, followed by Western blotting with anti-phospho-tyrosine (upper left-hand side panel). Blots were then reprobed with anti-p85 antibody (upper right-hand side panel), then stripped and the region corresponding to 100–200 kDa was reprobed with anti-p110 antibody, which recognizes both p110 α and p110 β (lower panel). (B–D) NIH 3T3 cells were transfected with EGFP (enhanced GFP) N1 control vector (CNT), DNRHO, DNCDC or DNRAC, treated with or without 2fAP, and PI3K activity in anti-p85 antibody immunoprecipitates was determined as described above. (B) Representative autoradiograph of phospholipids separated by TLC. (C) Histogram depicting PAR-2-induced PI3K activity (as fraction of baseline) under each transfected condition. (D) Representative Western blot of GTPase over-expression (upper panel: anti-GFP antibody; lower panel: anti-HA-7 antibody).

A model for PAR-2 regulation of the PI3K pathway

Previous research has demonstrated that PAR-2 sends opposing signals to PI3K by affecting both $G\alpha_q$ /Ca²⁺-dependent activation and β -arrestin-1-dependent inhibition. PAR-2-dependent PI3K activity was inhibited by broad spectrum SFK and PKC inhibitors, and involved the association of p85 with PYK2 [7]. PAR-2 has also been shown to activate both PYK2 and Src, via a Ca²⁺-dependent mechanism, and activation of both was enhanced by the inhibition of β -arrestins [1,11]. Together with the findings described here, these data point to a model where PAR-2 regulates PI3K through multiple G-protein-dependent pathways (Figure 7A). We propose that PAR-2-induced PI3K activity involves Ca²⁺/PKC-dependent

activation of tyrosine kinases (PYK2 and Src), incorporation of PI3K into a complex containing PYK2 and possibly other tyrosine phosphorylated proteins, and activation of Cdc42. The identity of the approx. 110–120 kDa phospho-protein that was observed to associate with p85 on PAR-2 activation has not been confirmed. The size is consistent with p110, but other candidates include PYK2 [7] or ACK1, a tyrosine kinase whose activity is increased by association with Cdc42 and clathrin, and decreased by β -arrestin-1 [15]. The ability of β -arrestins to inhibit PAR-2-induced PI3K activity (Figure 7B) probably results from a combination of direct inhibition of the p110 catalytic subunits and their more traditional roles as terminators of G-protein signalling. Both β -arrestin-1 and -2 were shown previously to associate with p85 and with PAR-2 on receptor activation [1,6,7,16,17], and both β -arrestins can inhibit PI3K activity at multiple levels through classic uncoupling of $G\alpha_q$ [1,16,17], through inhibition of Cdc42, and through direct inhibition of the catalytic subunits. Thus there are multiple mechanisms by which PAR-2 can both promote and inhibit PI3K activity. Whether the overall result of PAR-2 activation is stimulation or inhibition of PI3K activity in a given cell depends on the combined effects of each signalling arm, which in turn is partially dependent on the level of β -arrestin expression [7]. β -Arrestins can be viewed as molecular switches, capable of turning off one signal and turning on another.

The β -arrestin paradox: a facilitator or inhibitor of PI3K activity?

In contrast with the findings described in this present paper, previous reports on IGF-1R indicated that β -arrestin-1 facilitated PI3K activity in HEK-293 cells (human embryonic kidney cells) [8]. There are several possibilities which may account for this difference. First, the mechanism of PI3K activation differs between the two receptors. IGF-1 is predicted to utilize two pathways, one involving tyrosine phosphorylation of IRS and p85 and the docking of p85/p110 on to IRS-1 or IGF-1R [18–20], and the other pathway involving β -arrestin-dependent PI3K activation [8] (Figure 7B). Whereas PAR-2-induced PI3K activity is dependent on SFKs and Cdc42, both proteins lie downstream of PI3K in IGF-1 signalling [7,8,21]. Second, no direct interaction between β -arrestin-1 and PI3K has been demonstrated downstream of IGF-1R, whereas PAR-2 promotes association of both β -arrestin-1 and β -arrestin-2 with the PI3K complex [7]. Other work has suggested that β -arrestins can bind IGF-1R and other phospho-tyrosine proteins implicated in PI3K activity, such as p62yes and Src [22–25]. Thus the facilitative effects of β -arrestin-1 on IGF-induced PI3K activity may reflect positive effects on an upstream PI3K regulator. In previous studies, both β -arrestin-1 and β -arrestin-2 appeared to exist in a macromolecular complex with p85/p110 and PAR-2; thus incorporation of β -arrestin-1 into distinct signalling complexes may be able to switch it from an activator to an inhibitor of PI3K [7]. One possibility that should be examined in the future is whether PAR-2 activation can inhibit IGF-1-induced PI3K activity through β -arrestins. Finally, the studies shown here suggest that the two receptors activate different catalytic subunits. Although activation of either IGF-1R or PAR-2 increased p110 α activity, only PAR-2 also promoted p110 β activity. These data are consistent with other reports in cultured cells and whole animals. For example, insulin receptor and IGF-1R preferentially activate p110 α in adipocytes, myotubes and several cancer cell lines [26–28]. Furthermore, transgenic mice expressing kinase-inactive p110 α had severe defects in insulin and IGF-1 signalling, whereas specific inhibition of p110 β had little effect on IRS-1-associated PI3K activity [29]. We demonstrate that both β -arrestins are capable of inhibiting

Figure 6 β -arrestin-1 and β -arrestin-2 inhibit PAR-2-stimulated Cdc42 activity

(A–B) NIH 3T3 cells were treated with or without 2fAP or serum (ser) as a positive control, and lysates were incubated with either PAK3 CRIB–GST or Rhotekin–GST. Total lysates and bound proteins were analysed by SDS/PAGE followed by Western blotting with anti-Cdc42, anti-Rac-1 or anti-RhoA antibodies. (A) Representative Western blots, where the left-hand side lanes contain total lysates and right-hand side lanes contain bound proteins. (B) Histogram depicting PAR-2 and serum-induced changes in CRIB domain binding (as a fraction of baseline). (C–D) MEFwt, MEF β -arrestin-1, β -arrestin-2-DKO, MEF β -arrestin-1, β -arrestin-2-DKO with β -arrestin-1 (DKO+ β arr1) and MEF β -arrestin-1, β -arrestin-2-DKO with β -arrestin-2 (DKO+ β arr2) cells were treated with 100 nM 2fAP for 0 or 5 min and activation of Cdc42 was determined by association with GST–PAK3 CRIB domain. (C) Representative Western blot of bound proteins (B) and total lysates (TL) blotted with anti-Cdc42 antibody. (D) Histogram depicting PAR-2-stimulated changes in Cdc42/CRIB domain binding (as a fraction of baseline) in each cell line. Unt, untreated.

PI3K activity. In particular, PAR-2-stimulated p110 β activity was highly sensitive to inhibition by β -arrestin-2. Thus the difference in β -arrestin requirements in total IGF-1R- and PAR-2-induced PI3K activity may partially reflect the added contribution of β -arrestin-2-dependent inhibition of p110 β , induced by PAR-2. This contribution may differ between cell types depending on the relative expression of each subunit.

Differential regulation of p110 α and p110 β by β -arrestins

We have demonstrated that β -arrestins can directly inhibit the catalytic subunits of PI3K, although the mechanism remains undetermined. Although both β -arrestins inhibit both p110 subunits *in vitro*, in the absence of PAR-2 activation, they do not inhibit PI3K activity in intact cells, even when constitutively associated with p85 [7]. This suggests that although direct association with PI3K is a key factor in the ability of β -arrestins to inhibit its activity, additional factors contribute to this inhibitory activity. Furthermore, the two β -arrestins are not interchangeable in their regulation of the two catalytic PI3K subunits. β -Arrestin-2 directly inhibits both p110 α and p110 β , and does so with a higher efficacy, whereas β -arrestin-1 directly inhibits only p110 α . The fact that β -arrestin-1 only inhibited p110 β when purified from mammalian cells suggests that it may either associate with other proteins in intact cells or undergo a post-translational modification that switches it from a facilitator to an inhibitor of the p110 β subunit. One such post-translational modification might be phosphorylation, as β -arrestin-1 is reported to be phosphorylated by MAPK (mitogen-activated protein kinase), which in turn affects its ability to associate with β_2 -adrenergic receptors [30].

The ability of β -arrestin-1 to act as both a facilitator and inhibitor of p110 β activity *in vitro* may partially explain its positive role in the activation of PI3K downstream of some receptors and in some cell types, whereas it is inhibitory downstream of PAR-2 in NIH 3T3 cells. Future studies are required to address the possibility that PAR-2 promotes association of phosphorylated β -arrestin-1 with p85/p110 β and to address whether phosphorylation affects the ability of β -arrestin-1 to activate or inhibit p110 β .

The ability of β -arrestins to differentially regulate p110 α and p110 β has implications beyond explaining their divergent roles in the regulation of PI3K downstream of various receptors. Although the two catalytic subunits are sometimes treated as redundant mediators of PI3K activity, numerous reports suggest distinct and, in some cases, opposing roles in signal transduction. Recent studies on EGFR (epidermal growth factor receptor) have suggested that p110 α is the primary mediator of PI3K-induced actin assembly and cell migration in a breast cancer cell line, whereas p110 β inhibits these same events [27,28]. Furthermore, several studies suggested that insulin/IGF-1-dependent signalling relies more on p110 α than on p110 β activity [26,29,31] and that p110 β is oncogenic, whereas p110 α is not [31a]. Another study found that p110 α activity was higher than that of p110 β in areas of high phospholipid concentration, whereas in areas of low phospholipid concentration p110 β had a higher activity, suggesting that the two subunits might exhibit spatially distinct patterns of activation [32]. PAR-2 promotes β -arrestin-dependent cell migration by a process involving spatial regulation of opposing pathways involved in actin assembly [1,3,18]. Thus regulation of specific p110 catalytic subunits by β -arrestins may

Figure 7 Model of PI3K regulation by PAR-2.

(A) $G\alpha_q$ compared with β -arrestin-dependent pathways. On activation, PAR-2 promotes $G\alpha_q$ coupling and subsequent mobilization of intracellular Ca^{2+} and activation of PKC (i). Ca^{2+} and PKC can promote activation of PYK2 and Src [1,11], association of PYK2 with p85 [7] and possibly phosphorylation of p110 (shown in Figure 5) (ii). PAR-2 also promotes activation of Cdc42 by an unknown mechanism (shown in Figures 5 and 6), perhaps through $G\alpha_q/Ca^{2+}$ -dependent mechanisms or through coupling to a different G-protein (iii). Activation of Cdc42 and Src leads to activation of p110 α and p110 β (p110*) (iv). β -Arrestin signalling through PAR-2 opposes the $G\alpha_q$ pathway through multiple mechanisms. First, PAR-2 promotes recruitment of both β -arrestins and p85/p110 to the receptor [1,7,17] (v), which can lead to inhibition of p110 catalytic activity (vi). β -Arrestins also inhibit PAR-2-stimulated Cdc42 activation (shown in Figure 6) (vii) and promote receptor uncoupling from $G\alpha_q$ [17] (viii), both of which would inhibit PAR-2-induced PI3K activity. (B) Model of IGF-1-stimulated PI3K activity (for comparison with PAR-2). IGF-1 has been shown to promote tyrosine phosphorylation of p85 SH2 domains, and its recruitment to phospho-tyrosines on IRS-1 or the IRS-1 receptor itself [18,19] (i). β -Arrestins were shown to facilitate IRS-1-induced PI3K activity, possibly through its ability to bind both the IGF-1R and SH3 domain-containing proteins [8,24] (ii). This may indirectly recruit the p85/p110 complex to the receptor. (C) PAR-2 can promote β -arrestin-dependent inhibition of both p110 subunits. Both β -arrestin-1 and β -arrestin-2 can directly inhibit p110 α *in vitro* (i), but can only do so *in vivo* on PAR-2 activation (ii). Only β -arrestin-2 can directly inhibit p110 β *in vitro* (iii), however, on activation of PAR-2 *in vivo* β -arrestin-1 becomes inhibitory. This may reflect an association with a third factor ("factor X") in cells (iv) or post-translational modification of the receptor, e.g. phosphorylation (v).

represent another mechanism for the spatial control of actin assembly during PAR-2-induced cell migration.

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