THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 11, Issue of March 12, pp. 10020–10031, 2004

Printed in U.S.A.

Termination of Protease-activated Receptor-1 Signaling by β-Arrestins Is Independent of Receptor Phosphorylation*

Received for publication, September 24, 2003, and in revised form, December 16, 2003
Published, JBC Papers in Press, December 29, 2003, DOI 10.1074/jbc.M310590200

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Protease-activated receptor 1 (PAR1), a G protein-coupled receptor (GPCR) for thrombin, is the prototypic member of a family of protease-activated receptors. PAR1 is irreversibly proteolytically activated; thus, the magnitude and duration of thrombin cellular responses are determined primarily by mechanisms responsible for termination of receptor signaling. Both phosphorylation and β-arrestins contribute to rapid desensitization of PAR1 signaling. However, the relative contribution of each of these pathways to the termination of PAR1 signaling is not known. Co-expression of PAR1 with β-arrestin 1 (βarr1) in COS-7 cells resulted in a marked inhibition of PAR1 signaling, whereas β-arrestin 2 (βarr2) was essentially inactive. Strikingly, signaling by a PAR1 cytoplasmic tail mutant defective in agonist-induced phosphorylation was also attenuated more effectively by βarr1 compared with βarr2. In contrast, both β-arrestin isoforms were equally effective at desensitizing the substance P receptor, a classic reversibly activated GPCR. PAR1 coimmunoprecipitated βarr1 in an agonist-dependent manner, whereas βarr2 association was virtually undetectable. Remarkably, βarr1 also interacted with phosphorylation defective PAR1 mutant, whereas βarr2 did not. Moreover, constitutively active β-arrestin mutants, βarr1 R169E and βarr2 R170E, that bind to activated receptor independent of phosphorylation failed to enhance either wild type or mutant PAR1 desensitization compared with normal versions of these proteins. In contrast, β-arrestin mutants displayed enhanced activity at desensitizing the serotonin 5-hydroxytryptamine$_{2A}$ receptor. Taken together, these results suggest that, in addition to PAR1 cytoplasmic tail phosphorylation itself, β-arrestin binding independent of phosphorylation promotes desensitization of PAR1 signaling. These findings reveal a new level of complexity in the regulation of protease-activated GPCR signaling.

Thrombin, a coagulant protease, is generated at sites of vascular injury and produces a variety of cellular effects critical for hemostasis, thrombosis, and inflammatory and proliferative responses triggered by vascular damage (1, 2). Thrombin activates cells through at least three proteolytically activated G protein-coupled receptors: PAR1,[1] -3, and -4 (3). The prototype of this family, PAR1, is activated by an unusual irreversible proteolytic mechanism in which thrombin binds to and cleaves the amino-terminal exodomain of the receptor. This cleavage generates a new amino terminus that functions as a tethered ligand by binding intramolecularly to the body of the receptor to cause transmembrane signaling (4–6). The synthetic peptide SFLLRN, which represents the newly formed amino terminus of the receptor, can activate PAR1 independent of thrombin and receptor cleavage. PAR1 is irreversibly activated; thus, the mechanisms that contribute to the termination of signaling are critical determinants of the magnitude and kinetics of the thrombin response in cells. Given the irreversible nature of PAR1 activation, we hypothesize that signal termination events are probably unique, since all other GPCRs are reversibly activated.

The molecular events responsible for GPCR desensitization and resensitization have been extensively studied using the β_2-adrenergic receptor (7, 8). In the classic paradigm, GPCRs are initially desensitized by rapid phosphorylation of activated receptors by G protein-coupled receptor kinases (GRKs) and other kinases. Receptor phosphorylation enhances the affinity of interaction with arrestins, and arrestin binding prevents receptor-G protein interaction, thereby uncoupling the receptor from signaling. Arrestins also interact with components of the endocytic machinery to facilitate recruitment of GPCRs to clathrin-coated pits and internalization from the plasma membrane (9, 10). Once internalized into endosomes, GPCRs dissociate from their ligands, become dephosphorylated, and then return to the cell surface in a state capable of responding to ligand. Thus, for most classic, reversibly activated GPCRs, signaling is terminated at the plasma membrane, and receptor trafficking is linked to resensitization of signaling.

Phosphorylation of activated PAR1 also appears to be important for rapid uncoupling from G protein signaling. Overexpression of either GRK3 or GRK5 enhances PAR1 phosphorylation and markedly inhibits inositol phosphate (IP) accumulation (11, 12). A PAR1 mutant in which all of the serines and threonines in the cytoplasmic tail (C-tail) are converted to alanines (S/T→A) is neither extensively phosphorylated nor inhibited by GRK3 overexpression in multiple cell types (11, 13, 14). In addition, we recently found that arrestins are also

* This work was supported in part by National Institutes of Health Grants HL67697 and HL073328 (to J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by an American Heart Association Predoctoral Fellowship.

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The abbreviations used are: PAR, protease-activated receptor; βarr, β-arrestin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; 5-HT$_{2A}$, 5-hydroxytryptamine$_{2A}$; IP, inositol phosphate; PI, phosphoinositide; SPR, substance P receptor; GRK, G protein-coupled receptor kinase; S/T→A, PAR1 mutant in which all of the serines and threonines in the cytoplasmic tail are converted to alanines; S^{297}SS^{299}, mutant in which serine residues Ser^{297}, Ser^{298}, and Ser^{299} are converted to alanine.

critical for the termination of PAR1 signaling. Desensitization of PAR1-promoted phosphoinositide (PI) hydrolysis is significantly impaired in mouse embryonic fibroblasts lacking both arrestin isoforms, arrestin 2 and arrestin 3 (also termed β-arrestin 1 and β-arrestin 2), whereas PAR1 internalization remained intact (15). However, in both wild-type and β-arrestin-deficient cells, phosphorylation of activated PAR1 is still necessary for internalization through clathrin-coated pits. Moreover, unlike classic GPCRs, proteolytically activated PAR1 is internalized and sorted rapidly to lysosomes, an event critical for termination of receptor signaling (16, 17). Thus, PAR1 defines a new class of GPCRs that utilize a phosphorylation-, clathrin-, and dynamin-dependent pathway for endocytosis that operates independent of β-arrestins and receptor trafficking is linked to termination of signaling.

The precise function of arrestins in signal regulation of a GPCR such as PAR1 that does not use these molecules for internalization through clathrin-coated pits has not been examined. Moreover, the relative contribution of phosphorylation *versus* β-arrestins to the termination of PAR1 signaling remains to be determined. In the present study, we used COS-7 cells to investigate the roles of phosphorylation and β-arrestins in uncoupling PAR1 from G protein signaling. Our findings strongly suggest that β-arrestins are able to bind and desensitize activated PAR1 independent of phosphorylation. Thus, these studies reveal a complex regulation of PAR1 signaling that involves both PAR1 C-tail phosphorylation and phosphorylation-independent binding of β-arrestins.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Human α-thrombin was purchased from Enzyme Research Laboratories. Agonist peptide SFLLRN was synthesized as the carboxyl amide and purified by reverse phase high pressure liquid chromatography (UNC Peptide Facility, Chapel Hill, NC). Substance P peptide was purchased from Phoenix Pharmaceuticals. 2,5-Dimethoxy-4-iodophenylisopropylamine was from Sigma.

Monoclonal M1 and M2 anti-FLAG antibodies were from Sigma. Rabbit polyclonal anti-β-arrestin antibody A1CT was previously described (18) and generously provided by Robert J. Lefkowitz (Duke University). Anti-PAR1 rabbit polyclonal antibody was generated as previously described (19). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad.

cDNAs and Cell Lines—The cDNAs encoding FLAG-tagged PAR1 wild-type and C-tail phosphorylation site mutant (S/T→A) were previously described (11). The PAR1 third intracellular loop (IC_3) mutants in which serine residues Ser^{297}, Ser^{298}, and Ser^{299} were converted to alanine (IC_3 $S^{297}SS^{299}$ mutant) were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene), specific mutations were confirmed by dideoxy sequencing. A plasmid encoding wild type substance P receptor containing an amino-terminal FLAG epitope was generated as described (17). cDNAs encoding untagged and FLAG-tagged β-arrestins were gifts from Robert J. Lefkowitz (Duke University). Green fluorescent protein (GFP)-tagged β-arrestins were obtained from Marc Caron (Duke University). Mutant βarr1 R169E and βarr2 R170E were kindly provided by Vsevolod V. Gurevich (Vanderbilt University) and have been previously described (20). The FLAG-tagged human 5-hydroxytryptamine$_{2A}$ (5-HT_{2A}) serotonin receptor was generously provided by Bryan L. Roth (Case Western Reserve University). The plasmids encoding $G\alpha_q$ wild type and GTPase-deficient, constitutively active Q205L mutant were generously provided by T. Kendall Harden (University of North Carolina, Chapel Hill, NC).

COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% fetal bovine serum, 4.5 mg/ml glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Transient Transfection—COS-7 cells were plated at 4×10^4 cells/well in fibronectin-coated 24-well dishes (Falcon) and grown overnight. Cells were then transiently transfected with a total of 0.4 μg of either PAR1, PAR1 mutants, SPR, or 5-HT_{2A} receptor and either βarr1, βarr2, βarr mutants, or pcDNA using LipofectAMINE reagent according to the manufacturer's instructions (Invitrogen). Cells plated at 2×10^5 cells/well in 6-well dishes (Falcon) were grown overnight and transfected with a total of 2 μg of either PAR1 or PAR1 mutants and either βarr1, βarr2, or pcDNA using LipofectAMINE reagent.

Phosphoinositide Hydrolysis—COS-7 cells plated at 4×10^4 cells/well of 24-well dishes were grown overnight, transiently transfected, and then labeled with 2 μCi/ml *myo*-[^{3}H]inositol (American Radiolabeled Chemicals, Inc.) in serum-free DMEM containing 1 mg/ml bovine serum albumin for 18–24 h. Cells were washed with DMEM containing 1 mg/ml bovine serum albumin, 10 mM HEPES buffer, and 20 mM lithium chloride. Cells were then incubated in the absence or presence of either 10 nM α-thrombin, 100 nM substance P, or 10 μM 2,5-dimethoxy-4-iodophenylisopropylamine diluted in DMEM containing lithium chloride for various times at 37 °C. Cell incubation medium was removed, and [^{3}H]inositol phosphates ([^{3}H]IPs) were extracted with 50 mM formic acid. Cell extracts were neutralized with 150 mM NH_4OH, and IPs were isolated by column chromatography as described (15). Scintillation counting was then used to quantitate IPs eluted in this assay.

Data Analysis—Data were analyzed using Prism 3.0 software, and statistical significance was determined using InStat 3.0 (GraphPAD, San Diego, CA). The initial rate of PAR1 desensitization was determined by quantifying the decrease in thrombin response over time. The data were normalized to the amount of [^{3}H]IPs formed in untreated control cells for each time point.

Cell Surface ELISA—Transiently transfected COS-7 cells plated at 4×10^4 cells/well in 24-well dishes were either left untreated or treated with 50 μM SFLLRN or 100 nM substance P for 30 min at 37 °C. Cells were fixed with 4% paraformaldehyde for 5 min at 4 °C and then incubated with M1 anti-FLAG antibody for 1 h at 25 °C in DMEM containing 1 mg/ml bovine serum albumin and 10 mM HEPES, pH 7.4. Cells were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 1 h at 25 °C. Cells were washed again and incubated with one-step 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (Pierce) for 10–20 min at room temperature. An aliquot was removed, and the optical density was determined at 405 nm using a Molecular Devices SpectraMax Plus microplate reader.

Immunoblotting and Coimmunoprecipitation—Lysates were prepared as previously described (17) from cells plated at 4×10^4 cells/well of 24-well dishes (Falcon) transiently transfected with PAR1 and various amounts of FLAG-tagged β-arrestins. Protein concentrations were determined using BCA protein assay reagent (Pierce), and equivalent amounts of lysates were resolved by SDS-PAGE and transferred, and membranes were incubated with M1 anti-FLAG antibody (1:1000) overnight. To detect PAR1 and β-arrestin association, COS-7 cells were plated at 2×10^5 cells/well in a 6-well dish, transiently transfected with FLAG-tagged PAR1 and β-arrestin cDNAs, and then stimulated with 50 μM SFLLRN for 2.5 min at 37 °C. Cells were lysed in 1% Triton X-100 lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 200 μM sodium orthovanadate containing protease inhibitors, and equivalent amounts of lysates were used for immunoprecipitation with M2 anti-FLAG antibody. Immunoprecipitates were resolved by SDS-PAGE, transferred, and immunoblotted with rabbit polyclonal anti-β-arrestin A1CT antibody (1:5000) or anti-PAR1 antibody (1:1000). Membranes were washed, incubated with species-specific secondary antibodies conjugated to horseradish peroxidase (1:10,000), and washed again. Immunoblots were developed with ECL-Plus (Amersham Biosciences), imaged by autoradiography, and quantitated by a Bio-Rad Fluor-S MultiImager.

Immunofluorescence Confocal Microscopy—Transiently transfected COS-7 cells were grown on fibronectin-coated glass coverslips (22 × 22 mm) and incubated with M1 anti-FLAG antibody for 1 h at 4 °C, washed, and exposed to agonist at 37 °C. Cells were fixed and then processed for immunofluorescence microscopy as described (15). Images were collected using an Fluoview 300 laser-scanning confocal imaging system (Olympus) configured with an IX70 fluorescent microscope fitted with a PlanApo × 60 oil objective (Olympus). The final composite image was created using Adobe Photoshop 6.0 (Adobe Systems).

RESULTS

β-Arrestin-mediated Desensitization of PAR1 Signaling Is Independent of Receptor Phosphorylation—PAR1 couples to $G\alpha_q$ and stimulates PI hydrolysis through the activation of phospholipase C-β (21). Thus, we sought to determine the roles of phosphorylation and β-arrestins in PAR1 desensitization by measuring $G\alpha_q$ activation of PI hydrolysis in COS-7 cells. COS-7 cells are known to express low levels of endogenous

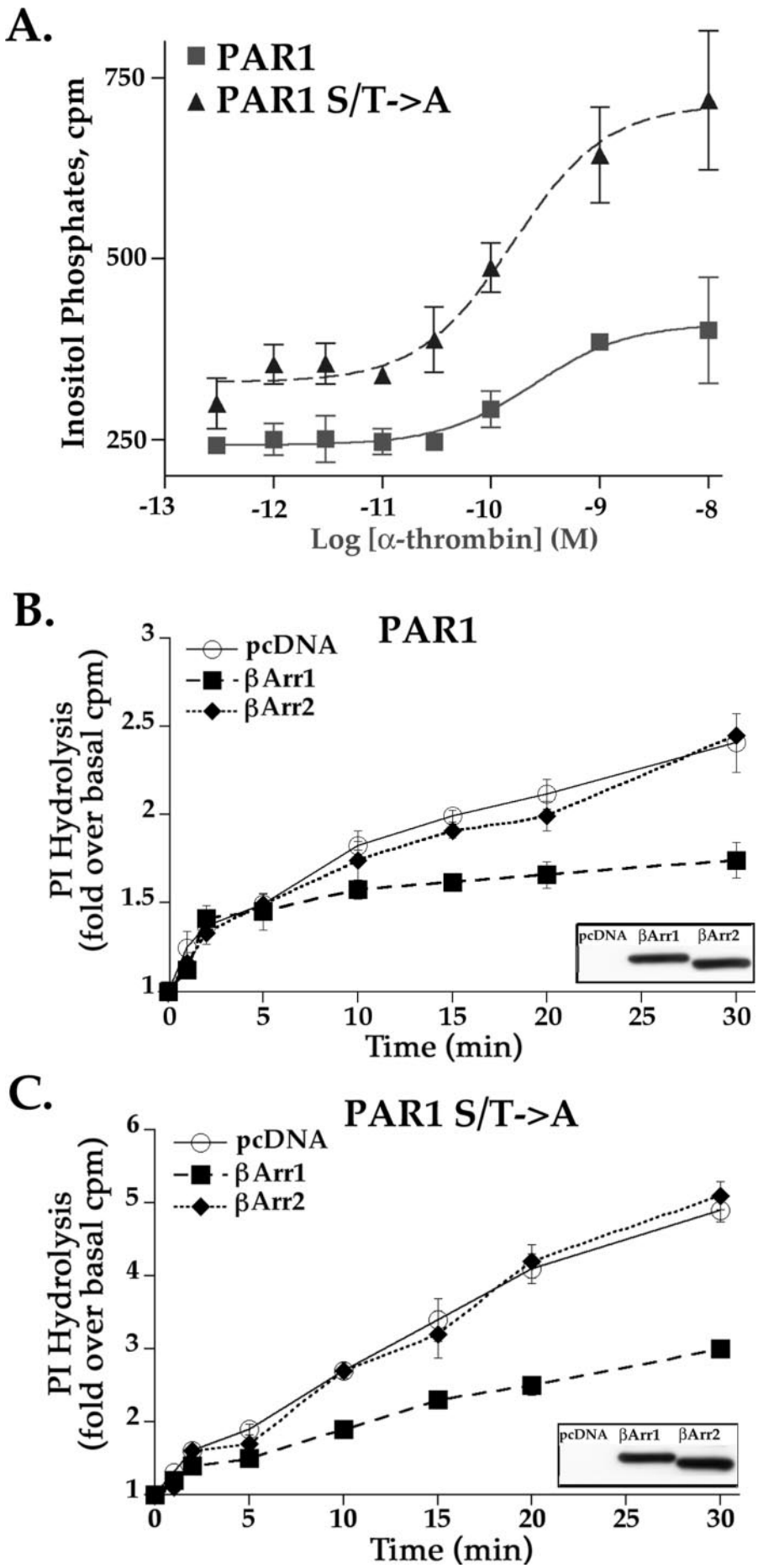

FIG. 1. **β-Arrestins promote termination of PAR1 signaling independent of receptor phosphorylation.** *A*, the concentration effect curves of α-thrombin at PAR1 wild type and S/T→A phosphorylation-defective mutant were determined in transiently transfected COS-7 cells labeled with *myo*-[^{3}H]inositol after 5 min of agonist incubation. The data shown are the mean ± S.D. for triplicates in one experiment and are representative of at least three separate experiments. *B* and *C*, COS-7 cells transiently transfected with PAR1 wild type or S/T→A mutant and either βarr1, βarr2, or pcDNA were labeled with *myo*-[^{3}H]inositol and incubated in the absence or presence of 10 nM α-thrombin for various times at 37 °C. The amounts of [^{3}H]IPs accumulated were then measured. The data shown (mean ± S.D.; n = 3) are expressed as -fold increase over basal [^{3}H]IPs of one experiment and are representative of six independent experiments. The initial level of receptor surface expression was similar for each transfection condition. The values (mean ± S.D.; n = 3) for PAR1 wild type and S/T→A mutant surface expression co-transfected with either βarr1, βarr2, or pcDNA were 0.112 ± 0.005, 0.123 ± 0.002, or 0.124 ± 0.008 and 0.173 ± 0.011, 0.171 ± 0.005, or 0.181 ± 0.003, respectively. The *insets* confirm a similar amount of βarr1 and βarr2 expression in total cell lysates of an equivalent well.

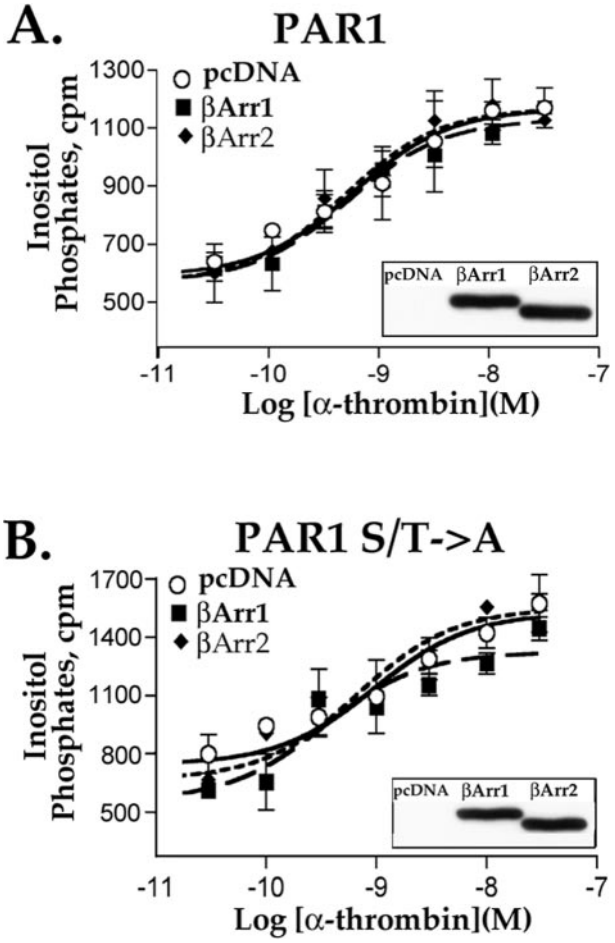

FIG. 2. **Activated PAR1 initial coupling to G protein-mediated PI hydrolysis is not affected by β-arrestins.** *A* and *B*, the concentration effect curves of α-thrombin at PAR1 wild-type and S/T→A mutant were determined in COS-7 cells transiently co-transfected with either βarr1, βarr2, or pcDNA. Cells labeled with *myo*-[^{3}H]inositol were incubated with varying concentrations of α-thrombin for 5 min at 37 °C, and [^{3}H]IPs were then measured. The data shown are the mean ± S.D. for triplicates in one experiment and are representative of at least three separate experiments. The initial surface levels of PAR1 wild type and S/T→A mutant in cells co-transfected with either βarr1, βarr2, or pcDNA were 0.336 ± 0.003, 0.335 ± 0.010, 0.345 ± 0.009 and 0.472 ± 0.008, 0.449 ± 0.005, or 0.459 ± 0.014, respectively. The amount of antibody binding to untransfected cells was less than 5% of that observed in transfected cells. The *insets* reveal a similar amount of βarr1 and βarr2 expression in total cell lysates from an equivalent well.

β-arrestins (22). We initially compared the signaling properties of PAR1 wild type and a phosphorylation-defective mutant that lacks all potential C-tail phosphorylation sites (S/T→A) and is insensitive to GRK-mediated desensitization in multiple cell types including COS-7 (11, 14). The concentration effect curves for thrombin at wild type and mutant PAR1 were determined by incubating cells labeled with *myo*-[^{3}H]inositol and varying concentrations of thrombin for 5 min at 37 °C. The accumulation of [^{3}H]IPs was then measured. The effective concentration of thrombin to stimulate a half-maximal response after 5 min was similar for both PAR1 wild type and S/T→A mutant in these studies (Fig. 1*A*). However, activated PAR1 S/T→A mutant caused an enhanced maximal signaling response compared with wild type receptor (Fig. 1*A*). These findings suggest that each activated PAR1 S/T→A mutant coupled longer to PI hydrolysis before signaling was shut off.

Both phosphorylation and β-arrestins contribute to PAR1 desensitization (11, 15). However, the relative contribution of each of these pathways to termination of PAR1 signaling remains to be determined. We initially compared the rates of agonist-induced PI hydrolysis in COS-7 cells transiently transfected with PAR1 and either βarr1 or βarr2 to establish that β-arrestins are capable of regulating PAR1 signaling in these cells. Cells were incubated in the absence or presence of a saturating concentration of thrombin for various times at 37 °C, and [^{3}H]IPs were then measured. The initial rate of thrombin-induced PI hydrolysis was similar in all transfection conditions (Fig. 1*B*). After 30 min of agonist exposure, a marked ~2.5-fold increase in PI hydrolysis was detected in cells expressing PAR1 only (Fig. 1*B*). Interestingly, agonist caused a similar ~2.5-fold increase in IP accumulation in cells expressing PAR1 and βarr2 (Fig. 1*B*), suggesting that βarr2 does not play a significant role in PAR1 uncoupling from G protein signaling. In contrast, agonist-stimulated signaling was markedly impaired in cells expressing PAR1 and βarr1; an ~1.5-fold increase in PI hydrolysis was detected after 30 min of agonist treatment (Fig. 1*B*), indicating that βarr1 is more effective than βarr2 at terminating PAR1 signaling.

TABLE I
Effect of β-arrestins on relative efficacy and potency of thrombin at PAR1 wild type and S/T→A mutant

COS-7 cells transiently expressing PAR1 and S/T→A mutant together with βarr1, βarr2, or vector were incubated with varying concentrations of α-thrombin for 5 min at 37 °C, and [^{3}H]inositol phosphates were then measured. The data are representative of three independent experiments (mean ± S.E.) performed in triplicate.

Receptor	Relative efficacy	EC_{50}
		nM
PAR1		
+ pcDNA	1.000 ± 0.000	0.476 ± 0.066
+ βarr1	0.918 ± 0.018	0.391 ± 0.135
+ βarr2	0.953 ± 0.040	0.428 ± 0.027
PAR1 S/T→A		
+ pcDNA	1.000 ± 0.000	1.090 ± 0.119
+ βarr1	0.938 ± 0.029	1.451 ± 0.790
+ βarr2	1.049 ± 0.078	1.002 ± 0.275

To examine the contribution of phosphorylation *versus* β-arrestin binding to PAR1 desensitization, we assessed signaling by the PAR1 S/T→A phosphorylation-defective mutant in cells co-expressing either βarr1 or βarr2. In COS-7 cells expressing the PAR1 S/T→A mutant, thrombin stimulated an ~5-fold increase in PI hydrolysis (Fig. 1*C*), a response substantially greater than that observed with comparable amounts of wild type receptor in these same cells (Fig. 1*B*). Expression of βarr2 failed to significantly decrease signaling by PAR1 S/T→A mutant (Fig. 1*C*), similar to that observed with wild type receptor. In contrast, however, βarr1 caused a marked ~50% inhibition of PAR1 S/T→A signaling (Fig. 1*C*), suggesting that βarr1-mediated PAR1 uncoupling from G protein signaling is independent of phosphorylation.

We next examined whether the initial coupling of activated PAR1 to $G\alpha_q$-promoted PI hydrolysis was affected by either βarr1 or βarr2. PAR1 wild type or S/T→A mutant was transiently co-expressed with either βarr1 or βarr2, and the capacity of receptor to promote IP accumulation was compared. The concentration effect curves for thrombin at wild type and mutant PAR1 co-expressed with either βarr1, βarr2, or vector was shown in Fig. 2. The EC_{50} values for stimulation (5-min assay) of IP accumulation by thrombin were comparable in each transfection condition (Fig. 2, Table I). The maximal effect of 30 nM thrombin for stimulation of IP accumulation by PAR1 wild type and S/T→A mutant co-expressed with either βarr1, βarr2, or vector was also similar (Fig. 2, Table I). Together, these findings imply that the initial coupling of activated PAR1 wild type and S/T→A mutant to G protein-induced signaling response is not affected by βarrestins.

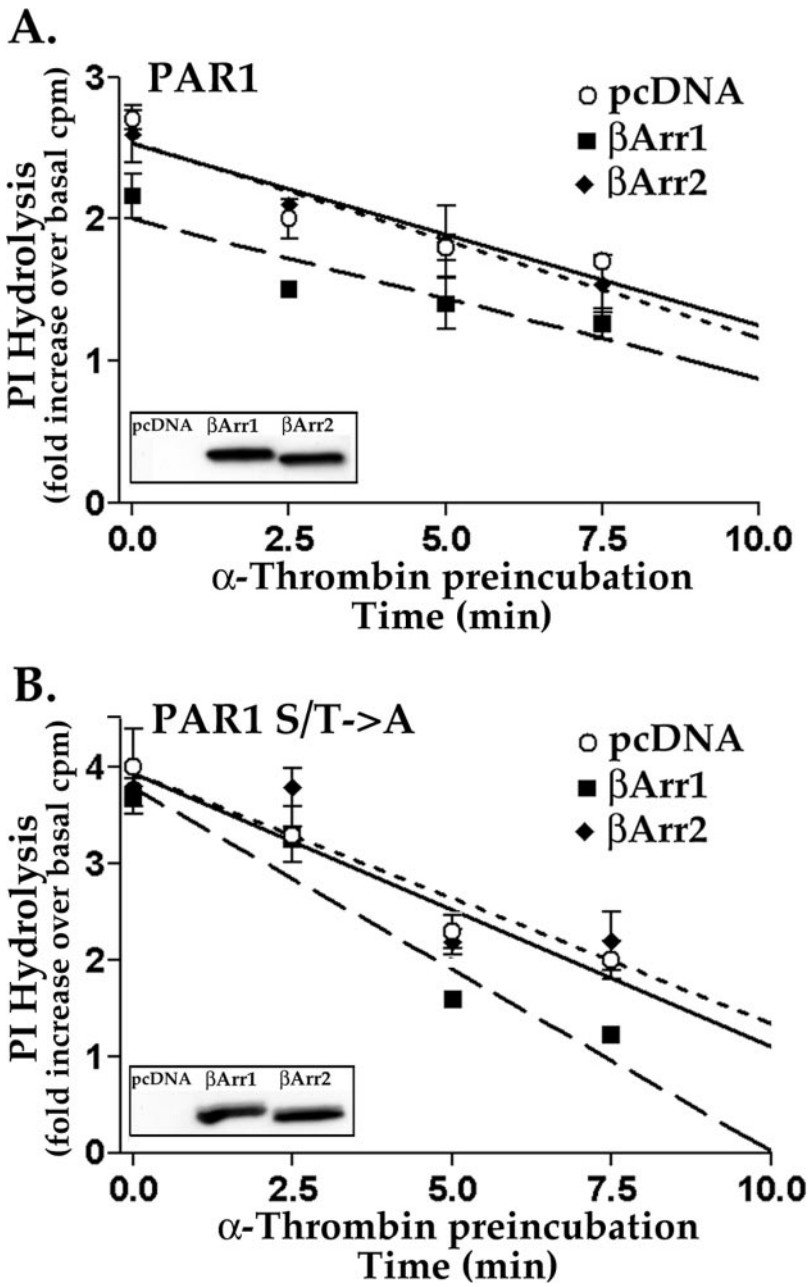

FIG. 3. **Effect of β-arrestins on initial rate of PAR1 wild type and S/T→A mutant desensitization.** *A* and *B*, COS-7 cells transiently expressing PAR1 wild type or S/T→A mutant together with either βarr1, βarr2, or pcDNA labeled with *myo*-[^{3}H]inositol were incubated with 10 nM α-thrombin for 10 min at 37 °C. Lithium chloride was added after various times of agonist exposure, and the amounts of [^{3}H]IPs formed were then measured. The data shown (mean ± S.D.; $n = 3$) are expressed as -fold increase over basal [^{3}H]IPs determined at each time point of one experiment and are representative of three independent experiments. Each time point on the graph corresponds to the amount of PAR1 signaling activity remaining after various times of thrombin exposure. The *insets* indicate a comparable amount of βarr1 and βarr2 expression in total cell lysates from an equivalent well.

To assess desensitization rates, COS-7 cells transiently expressing PAR1 wild type or S/T→A mutant together with either βarr1, βarr2, or vector were exposed to a saturating concentration of thrombin for 10 min at 37 °C. The extent of PAR1 signaling activity remaining after various times of thrombin incubation was then determined by the addition of lithium chloride and quantification of the amounts of IPs formed. In the absence of lithium chloride, thrombin-induced IP formation was not detectable in these cells (data not shown). In cells expressing PAR1 wild type only and either βarr1 or βarr2, the apparent rates of desensitization were not significantly different (Fig. 3*A*). These findings suggest that the major initiating event of PAR1 wild type desensitization is independent of β-arrestin binding. Interestingly, PAR1 S/T→A phosphorylation-defective mutant also showed a similar rate of desensitization in cells co-transfected with either βarr2 or vector only (Fig. 3*B*). In contrast, in cells co-expressing βarr1, the PAR1 S/T→A mutant desensitization appeared to occur more rapidly (Fig. 3*B*). At face value, these findings suggest that βarr1 enhances the rate of PAR1 desensitization independent of receptor phosphorylation.

To determine whether other $G\alpha_q$-linked GPCRs are similarly regulated by β-arrestins in COS-7 cells, we examined the effects of β-arrestins on signaling by the substance P receptor (SPR), also known as the neurokinin-1 receptor. In COS-7 cells expressing SPR only, a ~3.3-fold increase in IP formation was measured after 30 min of agonist exposure (Fig. 4*A*). In contrast to responses observed with PAR1, agonist-stimulated SPR signaling is substantially diminished in cells expressing either βarr1 or βarr2 (Fig. 4*A*), suggesting that both βarr1 and βarr2 are equally effective at uncoupling activated SPR from G protein signaling in these cells. These findings are consistent with previous studies demonstrating that activated SPR is rapidly desensitized via a GRK-mediated redistribution, and presumably binding, of β-arrestins to the receptor in other cell types (23, 24). In addition, these results establish that heterologous expression of βarr2 is able to desensitize GPCR signaling in COS-7 cells.

We also examined the ability of βarrestins to directly modulate signaling by $G\alpha_q$ to ensure that ectopic expression of β-arrestins does not globally disrupt signaling by this G protein in COS-7

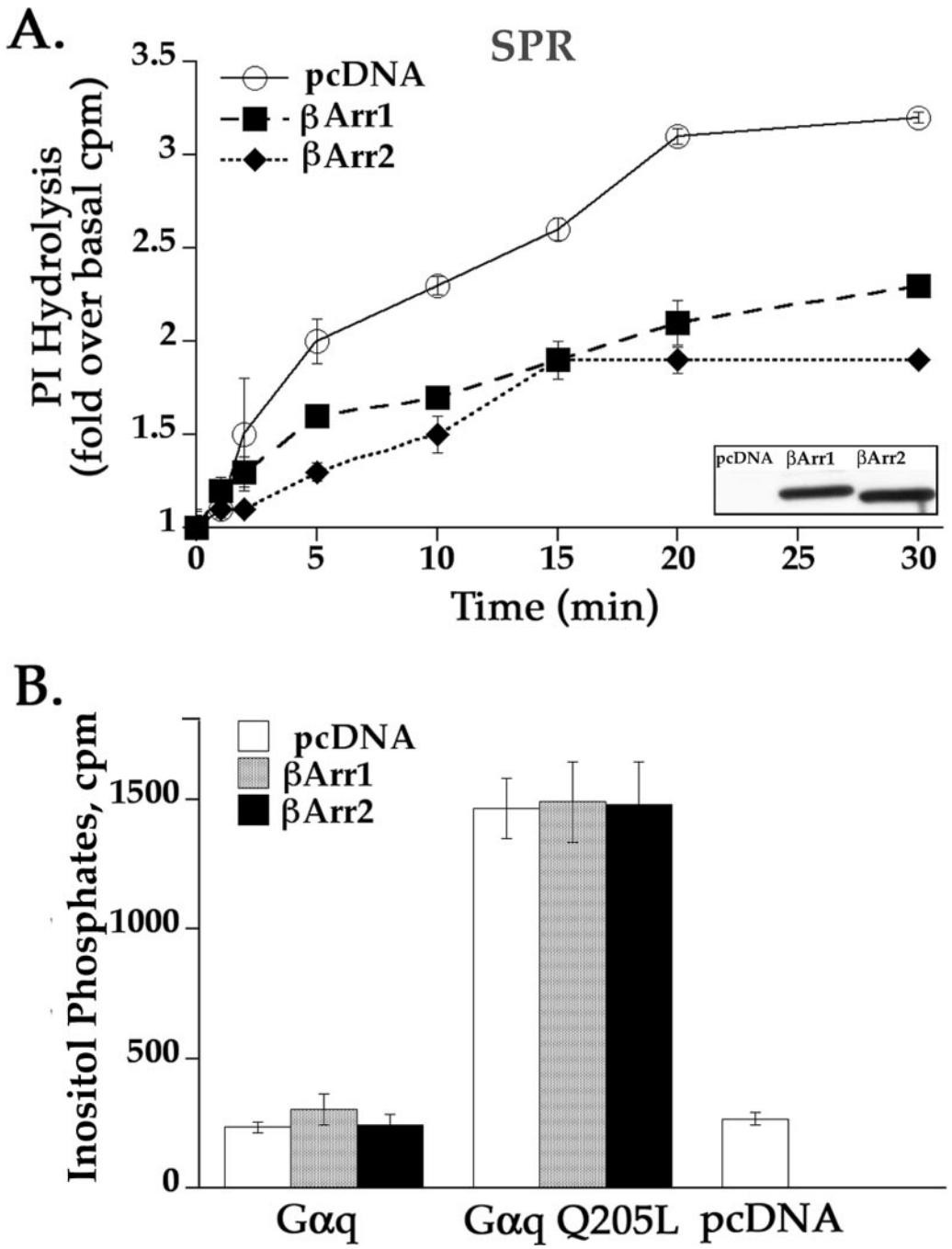

FIG. 4. **Both of the β-arrestin isoforms are equally effective at desensitizing SPR signaling.** *A*, COS-7 cells transiently expressing SPR and either βarr1, βarr2, or pcDNA were labeled with *myo*-[^{3}H]inositol and treated with or without 100 nM substance P for various times at 37 °C and processed as described above. The data (mean ± S.D.; $n = 3$) shown are expressed as -fold increase in [^{3}H]IPs over basal and are representative of three or more experiments. The initial levels of surface SPR expression in cells co-transfected with βarr1, βarr2, or pcDNA were 0.148 ± 0.006, 0.160 ± 0.002, or 0.155 ± 0.002, respectively. The *inset* shows a comparable amount of βarr1 and βarr2 expression in total cell lysates from an equivalent well. *B*, COS-7 cells were co-transfected with $G\alpha_q$ or $G\alpha_q$ Q205L mutant plasmids and either βarr1, βarr2, or pcDNA vector, and [^{3}H]IP accumulation was measured after a 30-min agonist incubation. The data are the mean ± S.D. for triplicate samples in one experiment and are representative of three separate experiments.

cells. In cells overexpressing wild type $G\alpha_q$, the basal IP accumulation measured after 30 min of incubation in medium containing lithium chloride was comparable with that measured in vector control cells (Fig. 4*B*). Compared with $G\alpha_q$ wild type or vector control cells, the GTPase deficient, constitutively active mutant of $G\alpha_q$ Q205L caused a ~5.5-fold increase in PI hydrolysis (Fig. 4*B*). Interestingly, however, expression of either βarr1 or βarr2 failed to diminish the $G\alpha_q$ Q205L signaling response (Fig. 4*B*), suggesting that neither βarr1 nor βarr2 globally disrupts signaling by $G\alpha_q$ in COS-7 cells.

We next assessed thrombin-stimulated PI hydrolysis in cells expressing wild type and mutant PAR1 and varying amounts of the individual β-arrestin isoforms to exclude the possibility that the differential effects of β-arrestins on PAR1 signaling are due to differences in the levels of β-arrestin expression. COS-7 cells transiently transfected with either PAR1 wild type or S/T→A mutant and varying amounts of FLAG-tagged βarr1 or FLAG-tagged βarr2 were incubated in the absence or presence of agonist for 30 min at 37 °C. The generation of IPs was then measured, or cell lysates were prepared, and β-arrestin

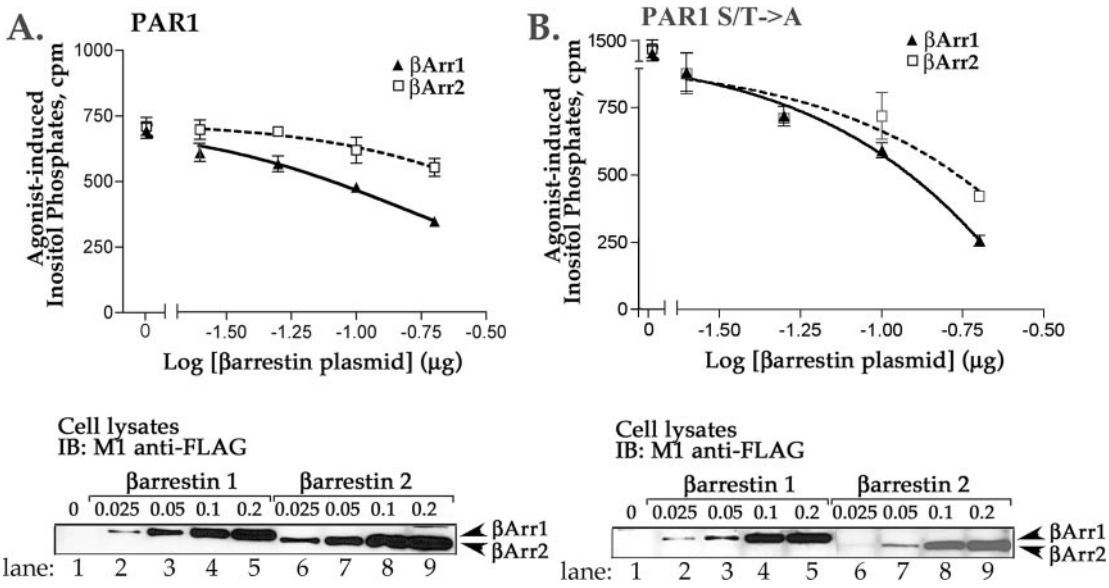

FIG. 5. **β-Arrestin isoforms differentially regulate PAR1 signaling in an expression-dependent manner.** *A* and *B*, COS-7 cells were transiently co-transfected with a constant 0.2 μg of PAR1 wild type or S/T→A mutant and varying amounts of either FLAG-βarr1, FLAG-βarr2, or pcDNA vector equaling 0.2 μg such that the total plasmid amount equaled 0.4 μg. Cells were then labeled with *myo*-[^{3}H]inositol and incubated in the absence or presence of 10 nM α-thrombin for 30 min at 37 °C and processed as described above. The data are the mean ± S.D. of triplicates determined in one experiment and are representative of three or more individual experiments. The basal [^{3}H]IPs determined from cells co-transfected with wild type PAR1 and either pcDNA, βarr1, or βarr2 were on average 230 ± 59, 226 ± 43, and 214 ± 44 cpm per well, respectively. PAR1 S/T→A mutant co-transfected with either pcDNA, βarr1, or βarr2 yielded basal [^{3}H]IPs of 354 ± 76, 227 ± 35, or 256 ± 33 cpm/well, respectively. Lysates were prepared from cells transfected exactly as described above and immunoblotted (*IB*) to detect β-arrestin expression.

expression was detected by immunoblotting. In the absence of β-arrestin expression, an ~2-fold and ~4-fold increase in IP accumulation was detected in PAR1 wild type- and S/T→A mutant-expressing cells following 30 min of agonist exposure, respectively (Fig. 5, *A* and *B*, *lane 1*). In cells expressing wild type PAR1 and maximum amounts of βarr2, activated PAR1 signaling was modestly diminished by ~20% (Fig. 5*A*), whereas βarr1 caused a significantly greater 50% inhibition of agonist-stimulated signaling (Fig. 5*A*). In PAR1 S/T→A mutant-expressing cells, agonist-stimulated PI hydrolysis was decreased more effectively by βarr1 compared with βarr2 (Fig. 5*B*), similar to the results observed with wild type receptor. However, both β-arrestin isoforms were quite efficacious at attenuating thrombin-induced PI hydrolyis, suggesting that the PAR1 S/T→A mutant is more sensitive than wild type receptor to β-arrestins. Regardless, in cells expressing comparable amounts of βarr1 and βarr2, the βarr1 isoform appears more effective than βarr2 at terminating activated PAR1 signaling even in the absence of receptor phosphorylation.

β-Arrestins Fail to Enhance PAR1 Internalization in COS-7 Cells—To determine whether the differential effects of β-arrestins on PAR1 signaling result from differences in receptor trafficking, we examined agonist-induced receptor internalization. COS-7 cells transiently expressing FLAG-tagged PAR1 and either βarr1 or βarr2 were incubated in the absence or presence of saturating concentrations of SFLLRN for 30 min at 37 °C. Since thrombin removes the amino terminus of PAR1 containing the FLAG epitope, the peptide agonist SFLLRN was used instead. After agonist treatment, the amount of PAR1 remaining on the cell surface was measured by cell surface ELISA. In PAR1-expressing cells, agonist induced an ~30% loss of receptor from the cell surface (Fig. 6*A*), consistent with PAR1 internalization observed in other cell types (15, 25). A similar extent of PAR1 internalization was induced by agonist in cells expressing either βarr1 or βarr2 (Fig. 6*A*). The failure of β-arrestins to enhance PAR1 internalization suggests that receptor trafficking occurs independent of β-arrestins in COS-7 cells. These data are consistent with β-arrestin-independent internalization of activated PAR1 observed in mouse embryonic fibroblasts deficient in β-arrestin expression (15).

We next examined the effects of β-arrestins on agonist-induced internalization of PAR1 S/T→A phosphorylation-defective mutant. Consistent with phosphorylation-dependent internalization of activated PAR1 reported previously (15, 25), agonist fails to promote PAR1 S/T→A internalization (Fig. 6*B*), whereas wild type PAR1 is robustly internalized (Fig. 6*A*). Moreover, neither βarr1 nor βarr2 significantly enhance agonist-induced PAR1 S/T→A mutant internalization (Fig. 6*B*), suggesting that the differential regulation of PAR1 S/T→A signaling by the individual isoforms of β-arrestins is not due to effects on receptor trafficking. We also determined whether SPR internalization is similarly regulated by β-arrestins in COS-7 cells. In contrast to wild type and mutant PAR1, both βarr1 and βarr2 significantly enhance agonist-induced internalization of SPR (Fig. 6*C*), consistent with a β-arrestin-dependent internalization of SPR reported previously (26). Together, these results further suggest that the differential regulation of PAR1 signaling by the individual isoforms of β-arrestin is not due to differences in their ability to affect receptor trafficking.

Immunofluorescence confocal microscopy studies are consistent with a failure of β-arrestins to enhance internalization of PAR1. COS-7 cells were transiently co-transfected with PAR1 wild type or S/T→A mutant together with either GFP-tagged βarr1 or GFP-βarr2, and internalization of PAR1 was assessed by confocal microscopy. In the absence of agonist, both wild type and mutant PAR1 are localized predominantly to the cell surface (Fig. 7, *A* and *B*, *top panels*). However, a small fraction of unactivated receptor was found in an intracellular pool in both wild type- and mutant PAR1-expressing cells, consistent with tonic cycling of these receptors as previously reported (25). In cells expressing wild type PAR1, exposure to SFLLRN for 10 min at 37 °C caused substantial internalization of receptor into endocytic vesicles (Fig. 7*A*). A similar extent of agonist-induced PAR1 internalization was observed in both βarr1- and βarr2-expressing cells (Fig. 7*A*). In contrast, agonist failed to promote

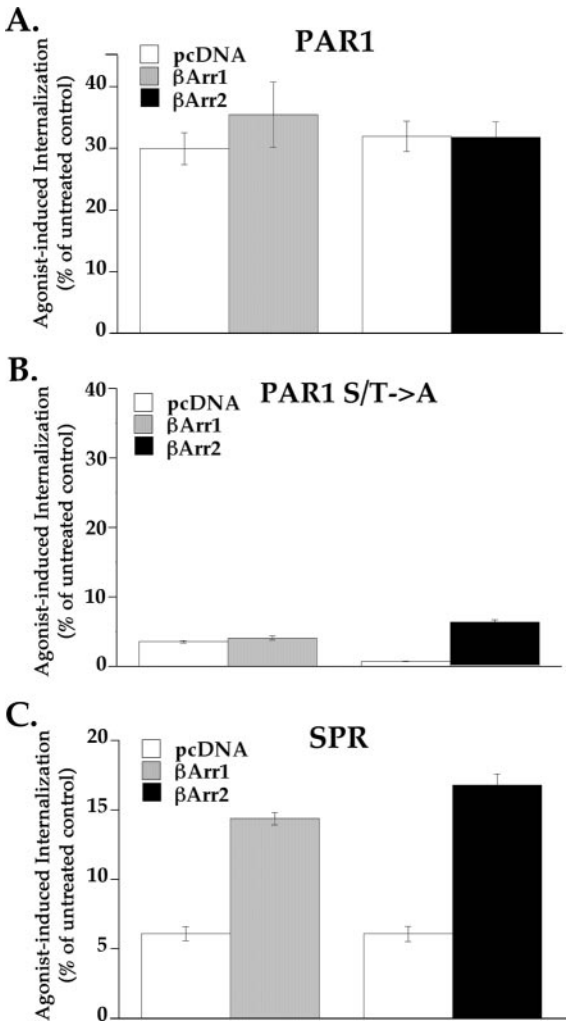

FIG. 6. **β-Arrestins fail to enhance agonist-induced PAR1 internalization.** *A* and *B*, COS-7 cells transiently co-transfected with PAR1 wild type or S/T→A mutant and either βarr1, βarr2, or pcDNA were incubated with or without 50 μM SFLLRN for 30 min at 37 °C. Cells were then fixed, and the amount of PAR1 remaining on the cell surface was measured by ELISA and used as an index for receptor internalization. The data are expressed as a percentage of the total amount of antibody bound to the cell surface of a transfected untreated control for each transfection condition. The data (mean ± S.D.) of triplicate samples of one experiment shown are representative of five experiments. *C*, transiently transfected COS-7 cells expressing SPR and either βarr1, βarr2, or pcDNA were incubated in the absence or presence of 100 nM substance P for 30 min at 37 °C. Cells were fixed, and the amount of receptor remaining on the cell surface was determined as described above. Data (mean ± S.D.; $n = 3$) are expressed as a percentage of total receptor measured in transfected untreated control cells and are representative of three separate experiments performed in triplicate.

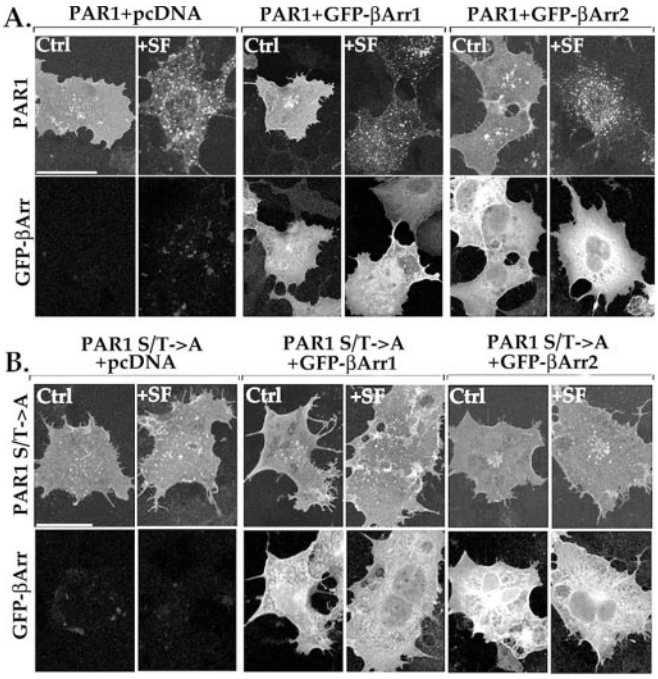

FIG. 7. **Effect of β-arrestins on agonist-induced internalization of PAR1 wild type and S/T→A mutant examined by immunofluorescence microscopy.** *A* and *B*, COS-7 cells transiently expressing PAR1 wild type or S/T→A mutant together with pcDNA, GFP-βarr1, or GFP-βarr2 were incubated in the absence (*Ctrl*) or presence of 50 μM SFLLRN for 10 min at 37 °C. Cells were fixed, immunostained for PAR1 (*top panels*) and GFP-β-arrestin expression (*bottom panels*), and imaged by confocal microscopy. These images are representative of many cells examined in three different experiments. The *scale bar* represents 10 μm.

PAR1 S/T→A mutant internalization, even in cells overexpressing βarr1 and βarr2 (Fig. 7*B*). These findings provide further support for an arrestin-independent internalization of PAR1 in COS-7 cells.

β-Arrestins Interact with Activated PAR1 Independent of Receptor Phosphorylation—We next determined whether activated PAR1 and β-arrestins directly associate by coimmunoprecipitation. COS-7 cells transiently co-transfected with FLAG-PAR1 and either βarr1 or βarr2 were incubated with or without SFLLRN for 2.5 min at 37 °C. Cells were lysed, and PAR1 was immunoprecipitated with M2 anti-FLAG antibody, and the presence of β-arrestins was detected by immunoblotting. In untreated control cells expressing βarr1, PAR1 was immunoprecipitated, and a small amount of βarr1 coimmunoprecipitated with the receptor, suggesting that unactivated receptor weakly associates with βarr1 (Fig. 8*A*). In contrast, immunoprecipitates from agonist-treated cells revealed a significant more than ~2-fold increase in βarr1 associated with activated PAR1, whereas βarr2 was at most weakly associated with PAR1 (Fig. 8*A*). Strikingly, however, a substantial amount of βarr1 associated with PAR1 S/T→A phosphorylation-defective mutant in both agonist-treated and untreated control cells (Fig. 8*B*); this may result from partial constitutive activity observed with this mutant (Fig. 9*C*). Consistent with a lack of robust interaction between wild type PAR1 and βarr2, a weak association between βarr2 and PAR1 S/T→A mutant was observed even in cells where a substantial amount of receptor was immunoprecipitated (Fig. 8*B*, *middle panel*). The apparent differences in the amount of βarr1 *versus* βarr2 expression detected in COS-7 cell lysates is due to the greater affinity of A1CT anti-arrestin antibody for βarr1 protein (Fig. 8, *bottom panels*) (18). This differential affinity is not responsible for the lack of association observed between PAR1 and βarr2, since similar results were found in cells expressing PAR1 and FLAG-β-arrestins, where the presence of β-arrestins in immunoprecipitates was detected using anti-FLAG antibody (data not shown). Together, these findings suggest that agonist enhances binding of βarr1 to wild type PAR1, and the phosphorylation-defective PAR1 S/T→A mutant binds βarr1 even in the absence of receptor phosphorylation.

Constitutively Active β-Arrestin Mutants Fail to Enhance PAR1 Desensitization—To further investigate whether β-arrestins are capable of binding to activated PAR1 independent of phosphorylation, we utilized the "constitutively active" β-arrestin mutants, βarr1 R169E and βarr2 R170E, that bind with high affinity to agonist-activated receptors independent of phosphorylation (20, 27). We first evaluated the ability of wild

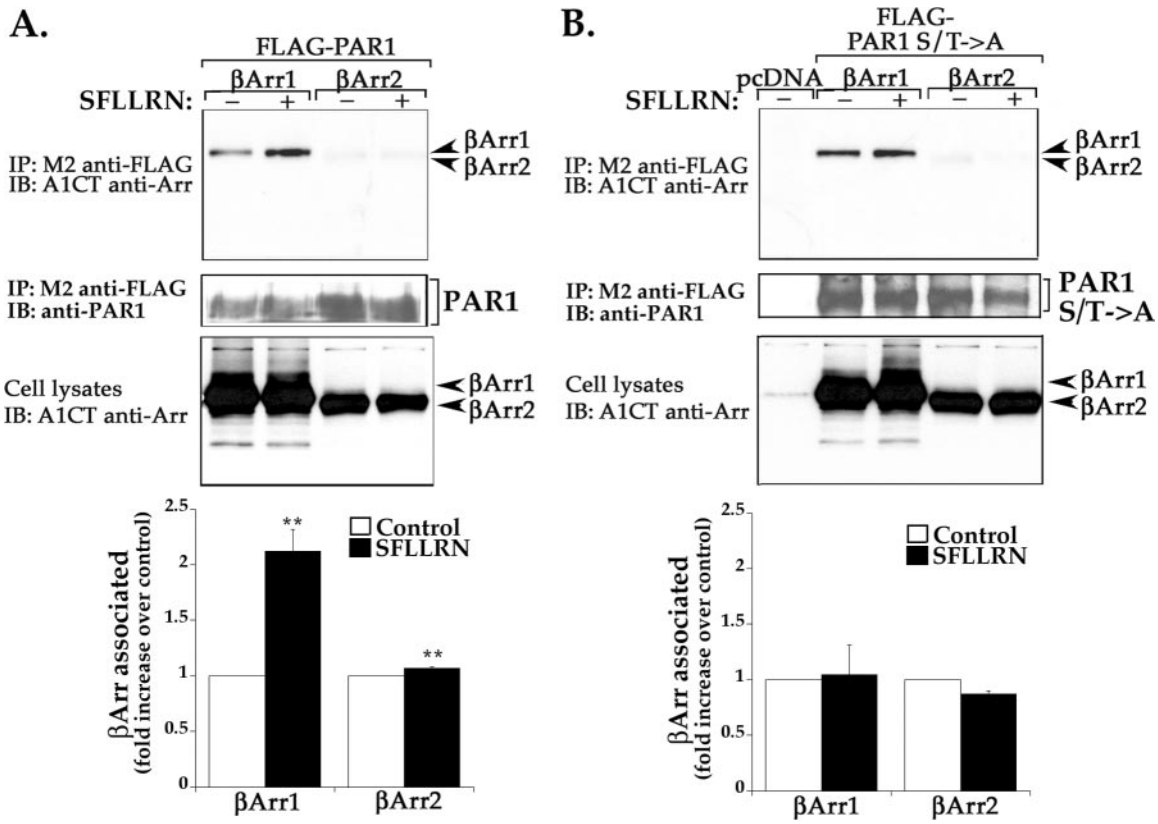

FIG. 8. **Agonist-induced association of β-arrestins with PAR1.** *A* and *B*, COS-7 cells transiently expressing PAR1 wild type or S/T→A mutant and either βarr1, βarr2, or pcDNA vector were incubated in the absence or presence of 50 μM SFLLRN for 2.5 min at 37 °C. Cells were lysed, and PAR1 was immunoprecipitated with M2 anti-FLAG antibody. Immunoprecipitates (*IP*) were resolved by SDS-PAGE and then immunoblotted (*IB*) for either β-arrestins or PAR1 using rabbit polyclonal anti-arrestin A1CT antibody or anti-PAR1 antibody, respectively. The expression of β-arrestins in total cell lysates was detected with anti-arrestin A1CT antibody. Similar findings were observed in three separate experiments. Results in the *bar graphs* represent the mean ± S.E. from three independent experiments and are shown as the -fold increase in βarr associated with PAR1 compared with untreated control. The extent of βarr1 associated with activated wild type PAR1 was significant (**, $p < 0.01$). Statistical analysis was determined using an unpaired *t* test.

type and mutant β-arrestins to regulate signaling by wild type PAR1 in transiently transfected COS-7 cells. Compared with control cells lacking β-arrestins, agonist-stimulated PI hydrolysis was decreased by ~35 and ~40% in cells expressing either βarr1 wild type or βarr1 R169E mutant, respectively (Fig. 9*A*). Thus, both wild type and mutant R169E βarr1 are equally effective at decreasing signaling by wild type PAR1. Consistent with a lack of βarr2 effectiveness at desensitizing PAR1, neither βarr2 wild type nor βarr2 R170E mutant significantly decreased signaling by activated PAR1 (Fig. 9*B*). Together, these results indicate that desensitization of activated PAR1 is equally sensitive to wild type and mutant β-arrestins. Since mutant β-arrestins are capable of binding to activated receptors independent of phosphorylation, these findings suggest that phosphorylation of activated PAR1 is not essential for β-arrestin binding.

Next, we examined the ability of wild type and mutant β-arrestins to desensitize PAR1 S/T→A mutant signaling. In cells expressing PAR1 S/T→A mutant alone, a significant increase in basal signaling was consistently observed compared with cells expressing comparable amounts of wild type receptor (Fig. 9). These findings suggest that the PAR1 S/T→A phosphorylation-defective mutant is at the least partially constitutive active. Interestingly, expression of either βarr1 or βarr1 R169E caused a significant ~50% decrease in both basal and agonist-induced signaling by PAR1 S/T→A mutant (Fig. 9*C*). These findings suggest that βarr1 is able to uncouple activated PAR1 from signaling independent of phosphorylation. βarr2 and βarr2 R170E mutant also modestly decrease both basal and agonist-induced signaling by PAR1 S/T→A mutant but were clearly less effective than βarr1 (Fig. 9, *C* and *D*). Surprisingly, however, compared with wild type β-arrestins, βarr1 R169E and βarr2 R170E fail to significantly attenuate signaling of either PAR1 wild type or S/T→A phosphorylation-defective mutant.

A cluster of three serine residues residing in the third intracellular loop (IC_3) of PAR1 could potentially contribute to β-arrestin binding and desensitization of PAR1 signaling. To assess whether these residues are important for termination of PAR1 signaling, the IC_3 serine residues ($S^{297}SS^{299}$) of both PAR1 wild type and S/T→A mutant were mutated to alanines. COS-7 cells expressing PAR1 wild type or IC_3 $S^{297}SS^{299}$ mutant and either βarr1 or βarr1 R169E mutant were exposed to agonist for 30 min, and IP accumulation was assessed. The mutation of the IC_3 serine cluster failed to effect the ability of either βarr1 or βarr1 R169E mutant to terminate PAR1 signaling (Fig. 10*A*). Interestingly, mutation of the three serine residues in the IC_3 loop of PAR1 S/T→A mutant also failed to effect desensitization of signaling by either βarr1 or βarr1 R169E (Fig. 10*B*). Both βarr2 wild type and βarr2 R170E mutant also failed to alter signaling by PAR1 wild type or S/T→A mutant in which the IC_3 serine cluster was mutated (data not shown). Together, these findings support the distinct possibility that phosphorylation-independent β-arrestin binding contributes to PAR1 desensitization.

To determine whether the βarr1 R169E and βarr2 R170E mutants display enhanced activity at desensitizing GPCRs in COS-7 cells as reported in other cell types (27, 28), we examined their effects on desensitization of the serotonin 5-HT_{2A}

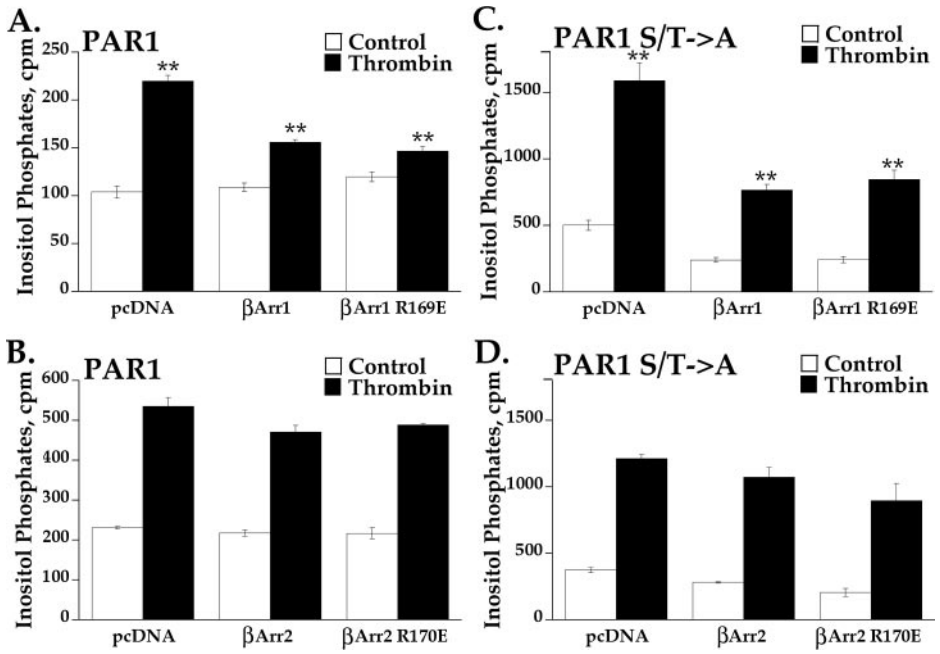

FIG. 9. **β-Arrestin wild type and constitutively active mutants are equally effective at desensitizing PAR1 signaling.** *A* and *C*, COS-7 cells transiently transfected with PAR1 wild type or S/T→A mutant and either pcDNA, βarr1, or βarr1 R169E. Cells were labeled with *myo*-[^{3}H]inositol and incubated in the absence or presence of 10 nM α-thrombin for 30 min at 37 °C, and [^{3}H]IPs were then measured. The data shown in *bar graphs* are the mean ± S.D. of one experiment with triplicate samples and are representative of six individual experiments. The initial levels of PAR1 wild type and S/T→A mutant surface expression (mean ± S.D.; $n = 3$) co-transfected with pcDNA, βarr1, or βarr1 R169E were 0.139 ± 0.002, 0.131 ± 0.003, or 0.136 ± 0.004 and 0.153 ± 0.002, 0.152 ± 0.012, or 0.168 ± 0.004, respectively. The difference in PAR1 wild type or S/T→A mutant signaling co-expressed with pcDNA compared with either βarr1 or βarr1 R169E was significant (**, $p < 0.01$). Statistical significance was determined using an unpaired *t* test. *B* and *D*, COS-7 cells transiently transfected with PAR1 wild type or S/T→A mutant and either βarr2 or βarr2 R170E were labeled with *myo*-[^{3}H]inositol and processed as described above. The initial levels of PAR1 wild type and S/T→A mutant co-transfected with pcDNA, βarr2 or βarr2 R170E were 0.141 ± 0.012, 0.146 ± 0.013, or 0.144 ± 0.012 and 0.151 ± 0.003, 0.157 ± 0.004, or 0.147 ± 0.002, respectively. The data shown in *bar graphs* are the mean ± S.D. of one experiment with triplicate samples and are representative of six individual experiments.

receptor. In COS-7 cells expressing FLAG-tagged 5-HT_{2A} receptor in the absence of β-arrestins, the addition of selective agonist 2,5-dimethoxy-4-iodophenylisopropylamine stimulated a robust ~4-fold increase in IP accumulation measured after 30 min of agonist exposure (Fig. 11*A*). Agonist-stimulated PI hydrolysis was markedly inhibited in cells expressing 5-HT_{2A} receptor and either wild type βarr1 or βarr2 (Fig. 11*A*), an ~48% decrease was caused by both βarr1 and βarr2, respectively. Interestingly, however, both βarr1 R169E and βarr2 R170E mutants were significantly more effective than wild type β-arrestins and caused virtually complete inhibition in activated 5-HT_{2A} receptor signaling compared with control cells lacking β-arrestin expression (Fig. 11*A*). The differential ability of β-arrestins to desensitize 5-HT_{2A} receptor signaling is not due to differences in expression of 5-HT_{2A} receptor at the cell surface (Fig. 11*B*). These findings are consistent with published studies demonstrating that mutant βarr1 R169E and βarr2 R170E are able to bind to activated GPCRs with high affinity and decrease signaling responses more effectively than wild type arrestins.

DISCUSSION

PAR1 is proteolytically irreversibly activated, and thus mechanisms that control PAR1 signaling determine the magnitude and duration of thrombin cellular responses. In this study, we demonstrate that β-arrestins bind to activated PAR1 independent of phosphorylation and promote termination of receptor signaling. Moreover, βarr1 is more effective than βarr2 at uncoupling activated PAR1 from signaling, suggesting that β-arrestins can differentially regulate PAR1 signaling independent of receptor phosphorylation. Consistent with these results, activated PAR1 associated with βarr1, whereas PAR1 interaction with βarr2 was virtually undetectable. By contrast, both βarr1 and βarr2 were equally effective at desensitizing the classic reversibly activated SPR. Together, these findings suggest that PAR1 signaling is regulated by multiple independent mechanisms including receptor phosphorylation itself and the binding of β-arrestins independent of phosphorylation.

The two β-arrestin isoforms appear to have redundant functions in regulating desensitization of most classic GPCRs (18). However, their capacity to differentially regulate GPCR internalization suggests that these molecules are not absolutely functionally redundant. Indeed, our finding that βarr1 is more effective than βarr2 at decreasing thrombin signaling responses (Figs. 1 and 5), implies that β-arrestins differentially regulate PAR1 signaling even in the absence of receptor phosphorylation. These results are consistent with our previous studies in which desensitization of PAR1 signaling is markedly impaired in mouse embryonic fibroblasts that lack βarr1 but retain βarr2 expression (15). Moreover, we demonstrate that neither βarr1 nor βarr2 enhances PAR1 internalization in COS-7 cells (Figs. 6 and 7), suggesting that receptor trafficking is not responsible for differential effects of β-arrestins on PAR1 signaling. The molecular basis for the differential ability of the individual isoforms of β-arrestin to regulate GPCR signaling is not known. It is possible that the individual β-arrestin isoforms have distinct determinants for binding to PAR1. It is also possible that post-translational modifications of either βarr1 or βarr2 differentially regulate their ability to desensitize or internalize PAR1. Phosphorylation and ubiquitination regulate the endocytic functions of arrestins (29, 30); however, whether these changes modulate the ability of β-arrestins to desensitize PAR1 signaling is not known.

Previous studies have shown that arrestins interact preferentially with the third cytoplasmic loop of certain GPCRs (31,

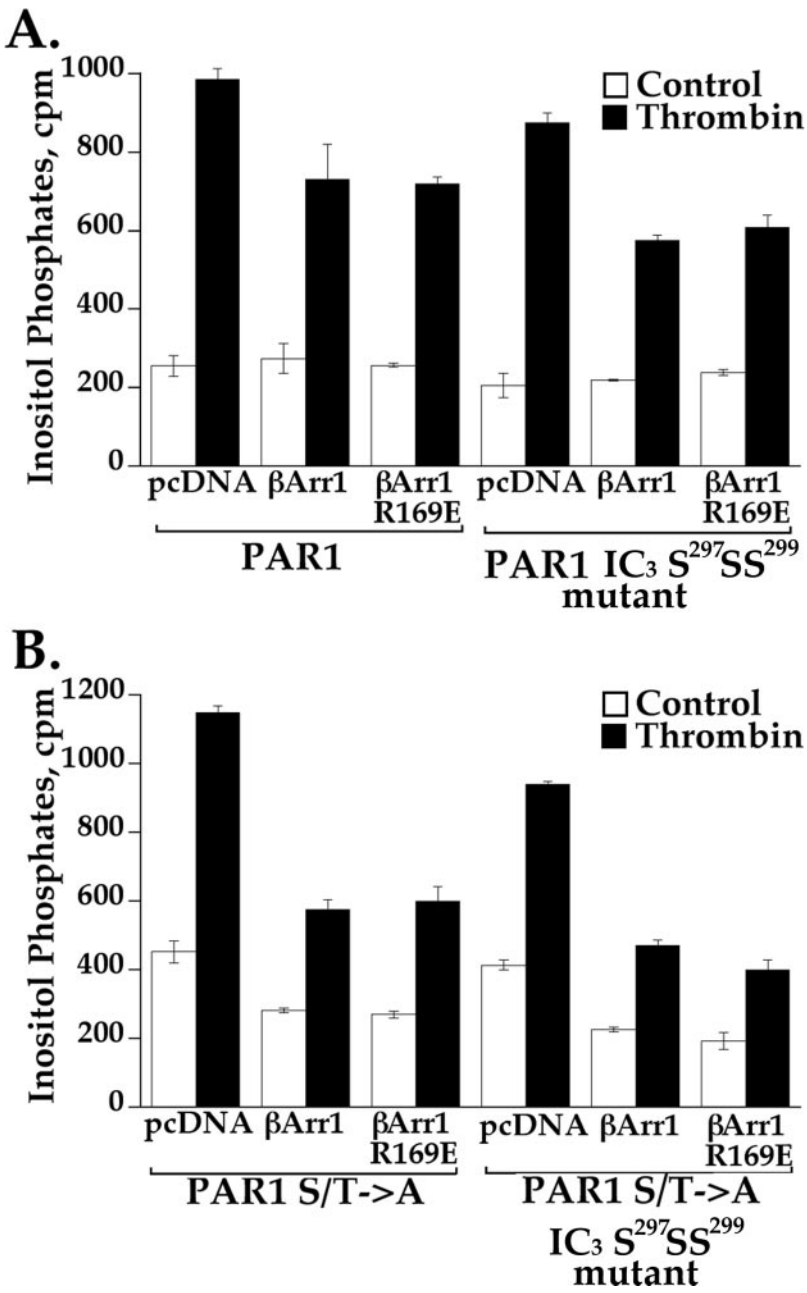

FIG. 10. **PAR1 wild type and IC_3 $S^{297}SS^{299}$ mutants are regulated similarly by β-arrestins.** *A* and *B*, COS-7 cells transiently co-transfected with PAR1, PAR1 S/T→A, PAR1 IC_3 $S^{297}SS^{299}$ mutant, or PAR1 S/T →A IC_3 $S^{297}SS^{299}$ mutant and either pcDNA, βarr1, or βarr1 R169E were stimulated with 10 nM α-thrombin for 30 min at 37 °C, and [^{3}H]IP accumulation was determined. The *bar graph* results are the mean ± S.D.; $n = 3$ of one experiment representative of three independent experiments. The initial surface expression (mean ± S.D.; $n = 3$) of PAR1 wild type and IC_3 $S^{297}SS^{299}$ mutant co-expressed with pcDNA, βarr1, or βarr1 R169E were 0.196 ± 0.002, 0.205 ± 0.026, or 0.216 ± 0.003 and 0.297 ± 0.006, 0. 316 ± 0.003, or 0.306 ± 0.010, respectively. PAR1 S/T→A and S/T→A IC_3 $S^{297}SS^{299}$ mutant initial surface expressions were 0.107 ± 0.006, 0.118 ± 0.041, or 0.103 ± 0.002 and 0.095 ± 0.007, 0.084 ± 0.001, or 0.087 ± 0.009, respectively. The signaling responses by receptors co-expressed with pcDNA were significantly different ($p < 0.01$) compared with receptor responses in cells co-expressing βarr1 or βarr1 R169E in all cases. Statistical significance was determined using an unpaired *t* test.

32). More recent *in vivo* studies suggest that the C-tails of many classic GPCRs are also involved in determining β-arrestin interaction (33). In the latter case, β-arrestin binding promotes GPCR internalization. It is possible that the binding of β-arrestins to different domains on a GPCR could confer differential functions (*i.e.* desensitization *versus* internalization). The C-tail of PAR1 is the major site of phosphorylation and is involved in desensitization (11, 13). However, it is unlikely that PAR1 C-tail phosphorylation is solely responsible for β-arrestin interaction, since β-arrestins bind to PAR1 S/T→A phosphorylation-defective mutant and promote desensitization (Figs. 1 and 8). Moreover, we also found that βarr1 binds to an activated PAR1 truncation mutant lacking the entire C-tail domain (data not shown), suggesting that the C-tail is not essential for β-arrestin binding. Although there is currently no evidence to suggest that other residues besides those residing in the C-tail of PAR1 are major sites of phosphorylation, a cluster of three serine residues residing in the third cytoplasmic loop of PAR1 could potentially contribute to β-arrestin binding. However, in both PAR1 wild type and S/T→A mutant in which the serines ($S^{297}SS^{299}$) were converted to alanines, we observed no difference in the ability of β-arrestins to regulate thrombin-induced signaling responses (Fig. 10). Together, these findings raise the distinct possibility that C-tail phosphorylation and phosphorylation-independent β-arrestin binding both contribute to PAR1 desensitization.

Most activated GPCRs require phosphorylation for β-arrestin binding and consequent receptor desensitization. In contrast, β-arrestins bind to activated PAR1 independent of phosphorylation to promote uncoupling from G protein signaling. The mutant arrestins, βarr1 R169E and βarr2 R170E, which bind with high affinity to activated GPCRs independent of phosphorylation (20, 27), are equally effective at promoting desensitization of both PAR1 wild type and S/T→A mutant. These findings suggesting that PAR1 phosphorylation *per se* is not critical for β-arrestin binding. Moreover, agonist-induced enhanced association of β-arrestins with activated PAR1 (Fig. 8*A*) supports the idea that β-arrestins recognize the active conformation of the receptor. Thus, activation of PAR1 may expose negatively charged residues or another critical domain

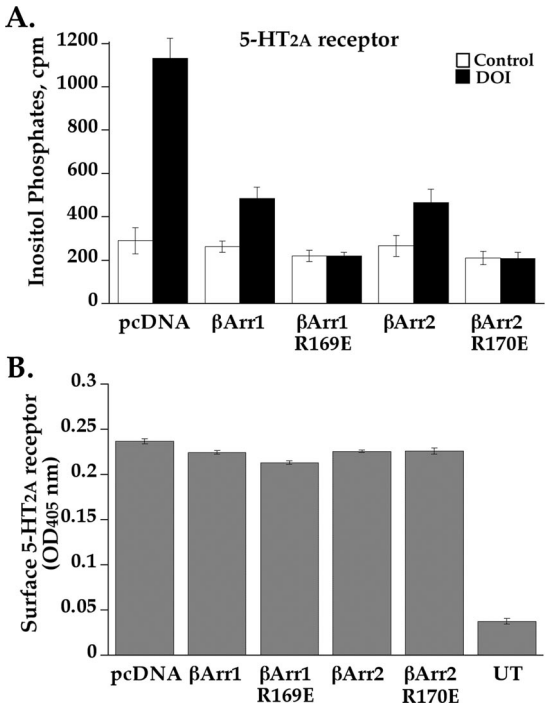

FIG. 11. **Constitutively active β-arrestin mutants display enhanced ability at desensitizing the 5-HT_{2A} receptor.** *A* and *B*, COS-7 cells were transiently transfected with FLAG-tagged 5-HT_{2A} receptor and either pcDNA, βarr1, βarr1 R169E, βarr2, or βarr2 R170E. Cells were then labeled with *myo*-[^{3}H]inositol and incubated in the presence or absence of 10 μM 2,5-dimethoxy-4-iodophenylisopropylamine for 30 min at 37 °C, and [^{3}H]IPs were then measured. The data are expressed as the mean ± S.D. of an experiment performed in triplicate and are representative of three independent experiments. The initial levels of 5-HT_{2A} receptor expressed on the cell surface were similar in each transfection condition as assessed by cell surface ELISA. The data are shown as the mean ± S.D. of triplicates of one experiment representative of three independent experiments. The amount of antibody binding to untransfected cells (*UT*) is shown. Co-expression of either βarr1 or βarr2 caused a significant ($p < 0.01$) decrease in activated 5-HT_{2A} signaling response compared with vector control. The expression of mutant βarr1 R169E and βarr2 R170E induced a further significant decrease ($p < 0.01$) in 5-HT_{2A} signaling response compared with wild type β-arrestins. Statistical analysis was performed with an unpaired *t* test.

residing on the cytoplasmic face of the receptor that perhaps mimic phosphorylation and thereby promotes binding of β-arrestins. Consistent with these findings, wild type and mutant arrestins are equally effective at desensitizing the luteinizing hormone/choriogonadotropin receptor (34). This receptor is desensitized in a phosphorylation independent manner and requires a conserved negatively charged Asp-564 residue localized to the third intracellular loop for β-arrestin binding and desensitization.

In conclusion, we examined the contribution of phosphorylation *versus* β-arrestin binding to the termination of PAR1 signaling in COS-7 cells. In these studies, we demonstrate that β-arrestins can bind to activated PAR1 independent of phosphorylation and promote termination of receptor signaling. We also demonstrate that βarr1 is more effective than βarr2 at desensitizing both PAR1 wild-type and S/T→A phosphorylation-defective mutant. These findings suggest that the individual isoforms of β-arrestins can differentially regulate GPCR desensitization independent of receptor phosphorylation. PAR1 couples to $G\alpha_q$ as well as $G\alpha_i$ and $G\alpha_{12/13}$, and whether arrestins differentially regulate PAR1 coupling to distinct G protein subtypes is not known. Thus, desensitization of PAR1 signaling is regulated by multiple independent mechanisms including C-tail phosphorylation itself and binding of β-arrestins independent of phosphorylation. The precise mechanisms by which β-arrestins bind to and desensitize activated PAR1 remain to be determined. These findings bring new insight into how signaling by irreversibly proteolytically activated GPCRs is regulated.

Acknowledgments—We thank Dr. David P. Siderovski and members of our laboratory for comments and helpful discussions.

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