Assessment of G Protein-coupled Receptors in Human Airway Smooth Muscle*

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Despite a widely accepted role of arrestins as “uncouplers” of G protein-coupled receptor (GPCR) signaling, few studies have demonstrated the ability of arrestins to affect second messenger generation by endogenously expressed receptors in intact cells. In this study we demonstrate arrestin specificity for endogenous GPCRs in primary cultures of human airway smooth muscle (HASM). Expression of arrestin-green fluorescent protein (ARR2-GFP or ARR3-GFP) chimeras in HASM significantly attenuated isoproterenol (β2-adrenergic receptor (β2-AR)-mediated) and 5’-(N-ethylcarboxamido)adenosine (A2b adenosine receptor-mediated)-stimulated cAMP production, with fluorescent microscopy demonstrating agonist-promoted redistribution of cellular ARR2-GFP into a punctate formation. Conversely, prostaglandin E2 (PGE2)-mediated cAMP production was unaffected by arrestin-GFP, and PGE2 had little effect on arrestin-GFP distribution. The pharmacological profile of various selective EP receptor ligands suggested a predominantly EP2 receptor population in HASM. Further analysis in COS-1 cells revealed that ARR2-GFP expression increased agonist-promoted internalization of wild type β2-AR and EP4 receptors, whereas EP2 receptors remained resistant to internalization. However, expression of an arrestin whose binding to GPCRs is largely independent of receptor phosphorylation (ARR2(2(R169E))-GFP) enabled substantial agonist-promoted EP2 receptor internalization, increased β2-AR internalization to a greater extent than did ARR2-GFP, yet promoted EP4 receptor internalization to the same degree as did ARR2-GFP. Signaling via endogenous EP4 receptors in CHO-K1 cells was attenuated by ARR2-GFP expression, whereas ARR2(2(R169E))-GFP expression in HASM inhibited EP2 receptor-mediated cAMP production. These findings demonstrate differential effects of arrestins in altering endogenous GPCR signaling in a physiologically relevant cell type and reveal a variable dependence on receptor phosphorylation in dictating arrestin-receptor interaction.

Signaling by G protein-coupled receptors (GPCRs) is regulated by multiple, diverse mechanisms. Among these processes is the well defined phosphorylation of GPCRs by the family of serine-threonine kinases known as G protein-coupled receptor kinases (GRKs), originally defined by their capacity to specifically phosphorylate agonist-occupied GPCRs and promote receptor desensitization (1). GRK-mediated GPCR phosphorylation induces receptor binding to arrestin molecules, which serves to sterically inhibit GPCR-heterotrimeric G protein coupling and terminate G protein activation. Four members of the arrestin family have been identified: arrestin-1 (also termed visual arrestin) and arrestin-4 (cone arrestin) are specifically expressed in the visual system and serve to regulate photoreceptors; arrestin-2 (β-arrestin1) and arrestin-3 (β-arrestin2) are more widely expressed and are involved in the regulation of nonvisual GPCRs (2). In addition to their role in GPCR desensitization, additional functions of arrestins involving GPCR internalization (3, 4) and resensitization (5), as well as roles in transducing mitogenic signals from GPCRs (6, 7), have been recently described.

Numerous studies have examined the role of arrestins in regulating heterologously expressed GPCRs in various cell lines. In such model systems, a regulatory role for arrestins has been ascribed in the agonist-dependent internalization or desensitization of numerous GPCRs, including the β2-adrenergic receptor (β2-AR) (3, 4), β1 adrenergic (8), A2b adenosine (A2bAR) (9), α1b adrenergic (10), follicle-stimulating hormone (11, 12), neurokinin-1 (13, 14), κ and δ opioid (15, 16), PAR-2 (17), and luteinizing hormone (18) receptor, and various chemokine receptors (19–22). Conversely, internalization or desensitization of the IP prostacyclin (23), µ opioid (15, 14), AT1 angiotensin (25, 26), and m2 muscarinic receptors (27) has been shown to be arrestin-independent, although such resistance may be cell type-specific (28, 29) or depend on the nature of the stimulating agonist (24–26).

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1 The abbreviations used are: GPCR, G protein-coupled receptor; A2bAR, A2b adenosine receptor; ARR2, arrestin-2; ARR3, arrestin-3; β2-AR, β2-adrenergic receptor; FACS, fluorescence-activated cell sorting; GRK, G protein-coupled receptor kinase; GFP, green fluorescent protein; HASM, human airway smooth muscle; ISO, isoproterenol; NECA, (N-ethylcarboxamido)adenosine; PGE2, prostaglandin E2; HA, hemagglutinin; CON, control; ELISA, enzyme-linked immunosorbent assay.
Interestingly, the capacity of arrestins to regulate properties of endogenously expressed GPCRs in intact cells is relatively unexplored. Initially, Iacovelli et al. (30) demonstrated that expression of wild type arrestin-2 in FRTL5 cells could attenuate cAMP production mediated via endogenous thyrotropin receptors. Two recent studies by Mundell et al. (31, 32) have provided the only analyses of arrestin specificity for endogenously expressed GPCRs to date. Expression of antisense mRNA targeting arrestin-2 and arrestin-3 in HEK293 cells resulted in an ~50% decrease in cellular arrestin levels and an increase in signaling through endogenously expressed β2AR, A2bAR, m1 muscarinic, somatostatin, and prostaglandin E2 (PGE2) receptors, whereas signaling through P2y1 (1), P2y2 (2), and AT1 angiotensin receptors was unaffected. By demonstrating that arrestins and arrestin expression levels can be important determinants of endogenous GPCR signaling, these studies represent an important step toward establishing the relevance of arrestins in regulating receptor-mediated functions in the in vivo condition.

In this study we examined arrestin specificity for endogenously expressed GPCRs in a differentiated, physiologically relevant cell type-human airway smooth muscle (HASM), in which GPCRs regulate numerous cellular functions (33). Heterologous expression of arrestin-2- or arrestin-3-green fluorescent protein (GFP) chimeras in HASM significantly attenuated cAMP production mediated by endogenous β2ARs and A2bARs, and agonist-dependent subcellular redistribution of arrestin was consistent with a role for arrestins in mediating the internalization of activated β2ARs and A2bARs into clathrin-coated pits. Alternatively, PGE2-stimulated second messenger accumulation was independent of arrestin expression, and PGE2 failed to elicit arrestin redistribution in HASM cells. Further analysis using various cell lines expressing recombinant EP2 or EP4 PGE2 receptors revealed that internalization of the EP2 (but not of the EP4) subtype of PGE2 receptors was resistant to regulation by arrestins. This resistance could be attributed in part to EP2 receptor phosphorylation state, as a phosphorylation-independent arrestin-2 mutant was capable of promoting both EP2 internalization and desensitization. Interestingly, arrestin effects on GPCR signaling could not be observed in experimental models employing receptor overexpression, but were consistently observed in analyses of endogenous receptor signaling. These studies demonstrate the specificity of arrestins for endogenous GPCRs in a physiologically relevant cell type and reveal the disparate regulation of EP2 and EP4 receptors by arrestins that potentially contributes to the differences in receptor function.

**EXPERIMENTAL PROCEDURES**

**Materials**—[125]I-adenosine 3′,5′-cyclicphosphoric acid (2,200 Ci/mmol) was purchased from PerkinElmer Life Sciences. cAMP antibody was a gift from Mario Ascoli (University of Iowa). pEGFPN1 was purchased from CLONTECH (Palo Alto, CA). PGE2, butaprost, 11-deoxy-PGE1, 15-keto-PGE2, and SC-19220 were purchased from Cayman Chemicals (Ann Arbor, MI). Anti-HA monoclonal antibody 101R was purchased from Covance (Richmond, CA). All other reagents were purchased from Sigma or sources described previously (34, 35).

**Cell Culture**—HASM cultures were established as described by Panettieri et al. (36) from human trachea obtained from lung transplant donors, in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Characterization of these cell lines with regard to immunofluorescence of smooth muscle actin and agonist-induced changes in cytosolic calcium has been reported previously (36, 37). Third to sixth passage cells were plated at a density of 104 cells/cm2 in either 24 or 48 well (for cAMP accumulation assays in intact cells) and maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum. Confluent cells were growth-arrested by refeeding cells with Ham’s F-12 supplemented with 5 μg/ml each insulin and transferrin (IT medium) for 24 h prior to assay.

Lines of HEK293-EBNA cells stably expressing either the human EP2 receptor (HEK293EP2) or human EP4 receptor (HEK293EP4) were obtained from J. Regan (University of Arizona) and maintained in 250 μg/ml G418 and 200 μg/ml hygromycin B. COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. CHO-K1 cells were maintained in Ham’s F-12 supplemented with 10% fetal bovine serum.

**Plasmid Construction**—Construcstions encoding ARR2-GFP, ARR3-GFP, and ARR2(R169E)-GFP were generated by polymerase chain reaction amplification of the open reading frames of bovine ARR2, ARR3, and ARR2(R169E) (38) (all previously cloned into pcDNA3) and cloning in pEGFPN1 (CLONTECH, Palo Alto, CA) such that the Green Fluorescent Protein (GFP) sequence was in frame to generate the intended chimera. Plasmids encoding the EP2 and EP4 receptors fused to an N-terminal 3-HA tag were generated by polymerase chain reaction amplification of the human EP2 and EP4 open reading frames from pCEP-EP2 and pCEP-EP4 (provided by B. Ashley, Temple University) and ligation of the resultant EcoRI/XhoI digests into a pcDNA3 vector containing a 3-HA cassette immediately upstream of the EcoRI site (provided by T. Som, Thomas Jefferson University). For all constructs, orientation, in frame alignment, and sequence were confirmed by dideoxynucleotide sequencing.

**Transfection Procedures**—HASM cells seeded onto 15-cm plates were transfected as described previously (39) by addition of a HEPES-based CaPO4 mixture containing 10 μg of carrier DNA, and 30 μg of either pEGFP, pARR2GFP, pARR3GFP, or pARR2(R169E)-GFP plasmid DNA per 15-cm plate and 50 ul of lipofectamine (CLONTECH) (CLONTECH, Palo Alto, CA). After 6 h, the medium was replaced with complete growth medium, and subsequently maintained in growth-arrested for 24 h, washed with cold phosphate-buffered saline, and freshly maintained in G418. Except where noted, cells grown in 24-well plates were transfected in 60-mm plates with FuGene (Roche Molecular Biochemicals) as described previously (34). 24 h later, cells were passaged into 24-well plates (for subsequent ELISA, cAMP assay), six-well plates (immunoblot analysis of EP receptor, arrestin expression), 60-mm dishes (immunoprecipitation studies), or onto poly-l-lysine-coated coverslips (immunocytochemical localization of EP receptors, arrestin-GFP chimera).

**Stable Selection**—HASM cells transfected as described above were selected with 250 μg/ml G418 starting 48 h after transfection. Subcultures were screened for GFP or arrestin-2 expression. For one culture stably expressing AR2-GFP (ARR2-GFP(A)), unit.Arrestin-2 (immunocytochemical localization of EP receptors, arrestin-GFP chimera) from the same culture were used for control cells in studies of cAMP accumulation. In a second culture stably expressing AR2-GFP (ARR2-GFP(B)), cells stably expressing GFP (a subpopulation of the parent culture transfected with pEGFPN1, grown and passaged in parallel with ARR2-GFP(B)) served as control cells.

**CHO-K1 cells were transfected with either pEGFP, pARR2GFP, or pARR2(R169E)-GFP using FuGene to arrive as described above for COS-1 cells and subsequently maintained with 250 μg/ml G418. Ten days later cells surviving selection were sorted by FACS for GFP expression and subsequently maintained in G418.

**cAMP Assay**—Except where noted, cells grown in 24-well plates were growth-arrested for 24 h, washed with cold phosphate-buffered saline, and subsequently stimulated with 500 μl of phosphate-buffered saline containing 300 μM ascorbic acid, 1 mM RO-20-1724 (phosphodiesterase inhibitor), and either vehicle (basal), (−)-isoproterenol (ISO), PGE2, butaprost, 11-deoxy-PGE2, 15-keto-PGE2, 5′-(N-ethylcarboxamido)adenosine (NECA), or forskolin at the indicated concentrations for 10 min at 37°C. cAMP was isolated and quantified by radioimmunoassay as described previously (35). For CHO-K1 cells, to minimize promiscuous activation of receptors (other than the EP4) by PGE2, a slightly lower concentration (100 nM) of PGE2 was used, and cells were stimulated in the presence of 10 μM SC-19220, a specific EP1 receptor antagonist (41). Effects of arrestins on PGE2-mediated cAMP production in either HASM or CHO-K1 cells were similar when cells were pretreated for 8 h with 100 ng/ml pertussis toxin (data not shown). For all cAMP data, values represent cAMP generated per well in response to agonist minus background cAMP generated per well in the absence of agonist.

**Studies Involving Fluorescent Microscopy**—Visualization of the agonist-induced translocation of arrestin-GFP chimeras in cells was performed in real-time using a Nikon Eclipse E800 fluorescence microscope.
scope. Cells transfected with the arrestin-GFP chimeras were passaged onto poly-l-lysine-coated coverslips, growth-arrested, then mounted on a temperature-regulated imaging chamber (Warner Instrument Corp.) equipped with an inlet port for introduction of media/agents. Cells were observed using a Plan-Apo 60 × 1.40 NA oil immersion objective. Images were captured using QED Camera software. Agonist-induced redistribution of antibody-labeled (HA-tagged) receptors and ARR-GFP chimeras was observed in fixed COS-1 cells as described previously (9).

**Assay of Receptor Internalization—Effects of ARR2-GFP or ARR2(R169E)-GFP expression on agonist-promoted internalization of HA-tagged (βAR, EP2, and EP4) receptors were assessed in COS-1 cells by ELISA as described previously (9).** Preliminary experiments examining the kinetics of βAR, EP2, and EP4 receptor sequestration demonstrated that sequestration observed in all groups began to plateau at ~15 min after agonist addition. Subsequent experiments therefore focused on the effects of 15-min agonist treatment.

**Immunoprecipitation Studies—**COS-1 cells transiently transfected to express GFP, ARR2-GFP, or ARR3-GFP, and either HA-tagged EP2 or EP4 receptor, were stimulated for 5 min with vehicle or 1 μM PGE2. Cells were then scraped in lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and transferred to microcentrifuge tubes, centrifuged at 30,000 rpms for 30 min in a TLA45 rotor, and the resultant supernatant was incubated overnight at 4 °C with purified anti-arrestin-2 antibody 178. Samples were then incubated with protein A-agarose for 2.5 h at 4 °C, washed in lysis buffer, and resuspended in SDS-sample buffer for immunoblot analysis of co-precipitated receptors using anti-HA antibody 10IR.

**Data Presentation and Statistical Analysis—**Data points from individual assays represent the mean values from duplicate or triplicate measurements. Data are presented as mean ± S.E. Statistically significant differences among groups were assessed by either analysis of variance with Fisher’s post-hoc analysis (Statview 4.5, Abacus Concepts, Berkeley, CA) or by t test for paired samples, with p values < 0.05 sufficient to reject the null hypothesis.

**RESULTS**

To assess the role of arrestins in GPCR signaling in HASM cells, cultures were transiently transfected with plasmids encoding GFP chimeras of arrestin-2 (ARR2-GFP) or arrestin-3 (ARR3-GFP) or with pEGFP (GFP). Because transfection efficiency in HASM cultures is low (presently optimized to 20–30%) (39), GFP-expressing cells were subsequently isolated by FACS, resulting in homogeneous populations of cells expressing the construct of interest (Fig. 1A). Immunoblot analysis of sorted populations demonstrated high ARR2-GFP expression relative to the (low) endogenous arrestin-2 levels (Fig. 1B). Similarly, ARR3-GFP expression was high, but no endogenous arrestin-3 could be detected in HASM cells. In addition, two separate cultures were established under G418 selection to express ARR2-GFP with repeated passage. One culture exhibited a low level of ARR2-GFP expression (~3–4-fold that of endogenous arrestin-2), whereas the other exhibited high ARR2-GFP expression (Fig. 1C).

As shown in Fig. 2, A and C, expression of ARR2-GFP or ARR3-GFP significantly inhibited cAMP accumulation elicited by either ISO or NECA, implicating both arrestin-2 and arrestin-3 as effective uncouplers of βAR and A2bAR signaling in HASM cells. However, neither ARR2-GFP nor ARR3-GFP inhibited PGE2-stimulated cAMP accumulation (Fig. 2B). A significantly lower level of ISO- and NECA-stimulated cAMP generation was also observed in two separate lines of HASM stably expressing ARR2-GFP when compared with matched control values (Fig. 2, D and F), whereas levels elicited by PGE2 remained unaffected (Fig. 2E). Interestingly, the reductions (relative to matched control levels) in cAMP accumulation exhibited in the two stable ARR2-GFP lines were similar, despite the difference in ARR2-GFP expression (Fig. 1C). Arrestin-GFP expression did not significantly alter cAMP accumulation stimulated by forskolin (a receptor-independent activator of adenylyl cyclase; data not shown).

Subsequent experiments were performed to analyze ARR2-GFP translocation in transiently transfected HASM cells. Cells expressing ARR2-GFP were plated onto poly-l-lysine-coated coverslips and mounted in a temperature-regulated imaging chamber for observation of ARR2-GFP localization in real-time. Upon addition of ISO, ARR2-GFP underwent rapid redistribution into punctate vesicles (Fig. 3A), suggesting a rapid internalization of ASM βARs with arrestin into clathrin-coated pits or early endosomes (42). A less profound but still clear redistribution of ARR2-GFP was observed upon addition of NECA (Fig. 3B), suggesting an arrestin-mediated internalization of A2bARs. However, PGE2 failed to promote any redistribution of
ARR2-GFP (Fig. 3C); subcellular ARR2-GFP localization remained unchanged in cells observed up to 2 h following PGE2 introduction. Cells stimulated with PGE2 remained responsive to subsequent stimulation with ISO (Fig. 3C, far right panel).

Two different receptor subtypes of the PGE2 receptor family, EP2 and EP4, are known to couple to Gs and stimulate adenylyl cyclase (43). These subtypes are distinguished by their structural, pharmacological, and functional features. The human EP2 receptor possesses a short third intracellular loop and a short C-terminal tail, is responsive to the agonist butaprost, and is resistant to agonist-induced short term desensitization (44, 45). The EP4 receptor possesses a long third intracellular loop and a long C-terminal tail, is unresponsive to the agonist butaprost, and undergoes rapid agonist-induced desensitization (45, 46). We characterized EP subtype expression in HASM cultures by examining cAMP accumulation stimulated by PGE2 (equally selective for EP2 and EP4), butaprost (selective for EP2), 11-deoxy-PGE1 (EP2/EP4-selective), and 15-keto-PGE2 (EP2-selective with slight activity at the EP4 receptor) (43) (Fig. 4). The dose-dependent response of HASM cells to these compounds is consistent with a predominately EP2 population of receptors, with the response to butaprost and 11-deoxy-PGE1 relative to that to PGE2 similar to that observed in HEK293 cells expressing recombinant human EP2 receptors (44). However, the relative efficacy of 15-keto-PGE2 is less than that reported for EP2 receptors (45), suggesting that a low level of other PGE2-responsive receptors may be expressed in HASM. PGE2 did not stimulate phosphoinositide production or a calcium transient in HASM (data not shown), suggesting a lack of EP1 receptors. Forskolin-stimulated cAMP generation was virtually unaffected by sulprostone (an EP3 receptor agonist) (data not shown). However, PGE2-stimulated cAMP generation in HASM is significantly increased by pertussis toxin pretreatment, and chronic sulprostone treatment caused a small Gi-dependent sensitization of adenylyl cyclase,2 suggesting that EP3 receptors or possibly other Gi-coupled receptors responsive to PGE2 are expressed in HASM.

These data suggest that the EP2 receptor is largely responsible for PGE2-mediated signaling in HASM, and a lack of agonist-promoted arrestin binding explains in part the resistance of this receptor to rapid homologous desensitization. To

\[2 \text{ R. M. Pascual and R. B. Penn, unpublished observations.} \]
further establish the relationship between EP receptor signaling/internalization and arrestin-2, we analyzed heterologously expressed EP2 and EP4 receptors in two separate cell lines. In HEK293 cells stably expressing the human EP2 (HEK293EP2), PGE₂ failed to promote punctate formation of expressed ARR2-GFP (Fig. 5A). Conversely, in HEK293 cells expressing the EP4 receptor (HEK293EP4) PGE₂ was observed to induce ARR2-GFP redistribution, although the kinetics varied among cells (Fig. 5B). To further explore the selectivity of arrestins for EP2 and EP4 receptors, we generated constructs encoding the human EP2 or EP4 receptor, each containing an N-terminal 3-HA tag. These constructs were transiently expressed in COS-1 cells with either ARR2-GFP or the arrestin mutant ARR2(R169E)-GFP. The R169E mutant of arrestin-2 has been previously characterized to bind to agonist-activated β2ARs in a phosphorylation-independent manner (38). Prior to stimulation with PGE₂, both the EP2 and EP4 receptors were primarily localized to the plasma membrane, whereas ARR2-GFP and ARR2(R169E)-GFP were more diffusely distributed with a tendency toward nuclear/perinuclear localization (Fig. 6). Following stimulation with PGE₂, both the EP2 and EP4 receptors were primarily localized to the plasma membrane, whereas ARR2-GFP and ARR2(R169E)-GFP were more diffusely distributed with a tendency toward nuclear/perinuclear localization (Fig. 6). Following stimulation with PGE₂, both the EP2 and EP4 receptors were primarily localized to the plasma membrane, whereas ARR2-GFP and ARR2(R169E)-GFP were more diffusely distributed with a tendency toward nuclear/perinuclear localization (Fig. 6). Following stimulation with PGE₂, both the EP2 and EP4 receptors were primarily localized to the plasma membrane.

In parallel experiments, ARR2-GFP and ARR2(R169E)-GFP effects on β2AR, EP2, and EP4 receptor internalization in COS-1 cells were assessed by ELISA. Both the β2AR and EP4 receptor exhibited significant agonist-mediated internalization in the absence of heterologously expressed arrestins (i.e. in control cells expressing GFP), whereas the EP2 receptor did not internalize (Fig. 7). ARR2-GFP significantly increased internalization of both the β2AR and the EP4 receptor, but did little to promote EP2 receptor internalization. Expression of ARR2(R169E)-GFP increased β2AR internalization to an even greater extent than did ARR2-GFP, but had essentially the same effect as ARR2-GFP on EP4 receptor internalization. Interestingly, ARR2(R169E)-GFP dramatically promoted EP2 receptor internalization, suggesting that a lack of EP2 receptor phosphorylation limits the capacity of the EP2 receptor to interact with and be regulated by arrestins.

To explore the relative effects of ARR2-GFP and ARR2(R169E)-GFP on signaling via the EP2 and EP4 receptors, we examined agonist-mediated cAMP production in: 1) HEK293EP2 and HEK293EP4 cell lines transfected with either of the arrestin constructs at up to 70% efficiency or 2) COS-1 cells co-transfected with HA-tagged β2AR, EP2, or EP4 receptor, and ARR2-GFP or ARR2169EGFP. Despite demonstrated effects of ARR2-GFP on β2AR signaling in HASM (Fig. 2), ARR2-GFP on β2AR and EP4 internalization (Fig. 7), and of ARR2(R169E)-GFP on internalization of the β2AR, EP2, or EP4 receptors (Fig. 7), we observed no effect of either ARR2-GFP or ARR2(R169E)-GFP on cAMP production mediated by any of the overexpressed receptors (data not shown). We therefore explored alternative models to examine signaling regulation of the EP2 and EP4 receptor. PGE₂-mediated cAMP production in CHO cells has been previously attributed to endogenously expressed EP4 receptors (47). Moreover, CHO cells are known to express relatively low levels of arrestins (48), rendering them a suitable system for examining the effects of heterologously expressed arrestins. In CHO-K1 cells we observed a significant cAMP response to 100 nM PGE₂ (~3-fold basal levels at 10 min), yet no response to butaprost. In these cells, cAMP production mediated via endogenous EP4 receptors was significantly decreased by expression of ARR2-GFP (22 ± 7%, p < 0.05) or ARR2169EGFP (18 ± 6%, p < 0.05) (Fig. 8A), and PGE₂ promoted a punctate formation of ARR2-GFP (Fig. 8B) as well as of ARR2(R169E)-GFP (not shown).

To assess the ability of ARR2(R169E)-GFP to regulate EP2 receptor signaling, we revisited our model of EP2 receptor signaling in HASM cells. Expression of ARR2(R169E)-GFP, but not of ARR2-GFP, caused a small (21 ± 5%, p < 0.05) but significant decrease in PGE2-mediated cAMP production (Fig. 9A). ARR2(R169E)-GFP caused a slightly greater decrease in ISO-mediated cAMP production than did ARR2-GFP. Of note, PGE₂-induced punctate formation of ARR2(R169E)-GFP was more readily observed in HASM cells (Fig. 9B) than in COS-1 cells expressing wild type EP2 receptor, perhaps reflecting lower levels of endogenous arrestins in HASM cells.

Given the large disparity in the effects of ARR2(R169E)-GFP on the EP receptors, we examined the interaction of ARR2-GFP and ARR2(R169E)-GFP with EP2 or EP4 receptor in intact cells. After 5-min stimulation with vehicle or 1 μM PGE₂, ARR2-GFP or ARR2(R169E)-GFP was immunoprecipitated from COS-1 cell lysates in which HA-tagged EP2 or EP4 receptor had been co-expressed (Fig. 10). Interestingly, for EP2 receptor we failed to observe a significant effect of activation on the amount of receptor that co-precipitated with arrestins, whereas only a small effect in ARR2-GFP expressing cells (~2-fold greater amount with activation) was observed with
EP4 receptor activation (discussed below). However, clear differences between the EP2 and EP4 receptor were observed with respect to the relative effects of ARR2-GFP and ARR2(R169E)-GFP in stimulated cells. Although co-precipitation of EP2 receptor was observed with immunoprecipitation of ARR2-GFP, a 5–8-fold greater (densitometry analysis of duplicate experiments) amount of EP2 receptor co-precipitated with ARR2(R169E)-GFP. In contrast, the difference in the amount of EP4 receptor co-precipitating with ARR2-GFP versus ARR2(R169E)-GFP was minimal. Whereas a greater amount of the EP4 receptor migrating at ~55 kDa was observed to co-precipitate with ARR2(R169E)-GFP, aggregates of the receptor (migrating at ~100–150-kDa oligomers (49)) that co-precipitated with ARR2-GFP were more abundant, such that the difference in total co-precipitating EP4 receptor was insignificant (less than 20% in duplicate experiments). Thus, the relative degree of interaction between EP receptors and the arrestin chimeras suggested by co-precipitation parallels the effects of the two arrestins on agonist-promoted internalization and desensitization.

**DISCUSSION**

The present study demonstrates that alterations in arrestin levels can differentially affect GPCR signaling in a physiologically relevant cell type. By emphasizing cellular models enabling analyses of endogenously expressed GPCRs, we discerned a capacity of arrestins to inhibit signaling of the β2AR and EP4 receptor. Additional studies examining trafficking of epitope-tagged receptors determined that arrestins similarly promote internalization of the EP4 receptor. Conversely, our data suggest that arrestins are not involved in regulating either EP2 receptor signaling or internalization. This failure of arrestins to regulate the EP2 receptor appears to be related to a lack of EP receptor phosphorylation, given that a phosphorylation-independent mutant of arrestin-2 is capable of promoting significant EP2 internalization and desensitization. Moreover, differences in the ability of phosphorylation-independent arrestin-2 to affect internalization of the β2AR, EP2, and EP4 receptor suggest that receptor phosphorylation is of varied importance among GPCRs in determining arrestin-receptor interactions.

Numerous studies employing cell-free assays or cellular models of receptor overexpression have established a broad specificity for arrestins in regulating GPCR internalization and signaling. Although these studies have revealed much about the capacity of arrestins to regulate GPCRs, the relative importance of arrestins among the numerous elements of control systems that regulate GPCR signaling under true physiological conditions remains unknown. Under such conditions it is unclear whether arrestins are required, facilitating, or redundant in the process of GPCR desensitization. Moreover, it is also uncertain whether cellular arrestin levels can limit the rate and magnitude of desensitization (and thus signaling) of a given GPCR, as has been proposed for cellular GRK levels (50).

We have recently begun to address this issue by examining arrestin specificity for endogenously expressed GPCRs in a given cell type. In two previous studies (31, 32) we characterized arrestin specificity among various Gi, Go, and Gq-coupled receptors in HEK293 cells utilizing an antisense approach to reduce cellular arrestin levels by ~50%. In the present study we were able to demonstrate that increasing cellular levels of arrestins by as little as ~3–4-fold significantly attenuated cAMP production elicited by either ISO or NECA, suggesting that in HASM cells, the level of arrestin expression is an important determinant of agonist-specific desensitization of the β2AR and A2bAR. However, our finding that PGE2-mediated signaling was not inhibited by increased arrestin expression seemed to contradict the recent findings of Mundell et al. (31) in which PGE2-mediated cAMP production was increased by arrestin antisense expression in HEK293 cells. We therefore examined potential differences in arrestin effects on PGE2 re-
ceptor subtypes by analyzing trafficking of heterologously expressed EP2 and EP4 receptors. Co-expression of ARR2-GFP was shown to increase agonist-promoted internalization of the EP4 receptor, but had little effect on the EP2 receptor, which failed to sequester after exposure to agonist. In addition, agonist treatment caused a rapid redistribution of the EP4 receptor into punctate vesicles that co-localized with ARR2-GFP, but was largely ineffective in promoting the association of the EP2 receptor with ARR2-GFP.

To explore potential mechanisms underlying the observed arrestin specificity, we examined the effect of expressing ARR2(R169E)-GFP. The ARR2(R169E) mutant was generated based on a previously characterized mutation in visual arrestin (51) to test the validity of the model which proposes two primary binding sites of arrestins: an activation-recognition site that recognizes the agonist-activated conformation of the receptor and the phosphorylation-recognition site that interacts with GRK-phosphorylated residues of the receptor (52, 53). Generation of the R169E mutation in arrestin-2 reverses the charge of the phosphorylation-sensitive trigger in arrestin-2, resulting in the ability of ARR2(R169E) to bind and desensitize activated β2AR regardless of its phosphorylation status (38). Because the EP2 receptor has a short C-tail, one potential explanation for its desensitization- and internalization-resis-

FIG. 8. Effects of arrestin expression on endogenous EP4 receptor-mediated signaling in CHO-K1 cells. A, stable cell lines of CHO-K1 expressing GFP, ARR2-GFP, or ARR2(R169E)-GFP were passaged onto 24-well plates and subsequently stimulated with 100 nM PGE2 in the presence of 10 μM SC-19220 and 1 μM RO-20-1724 for 10 min at 37 °C. cAMP was isolated and quantified as described under "Experimental Procedures." Data represent mean ± S.E. from seven to eight paired observations. *, p < 0.05, ARR2-GFP group versus GFP group; **, p < 0.05, ARR2(R169E)-GFP versus ARR2-GFP group. B, stable cell lines of CHO-K1 cells expressing ARR2-GFP were grown on poly-1-lysine-coated coverslips and stimulated with 100 nM PGE2 for 15 min, resulting in redistribution of ARR2-GFP into a punctate formation. Similar effects of PGE2 were observed in CHO-K1 cells expressing ARR2(R169E)-GFP (not shown).

FIG. 9. Effects of ARR2(R169E)-GFP expression on agonist-stimulated cAMP production in HASM cells. A, HASM cells expressing GFP, ARR2-GFP, or ARR2(R169E)-GFP (transfected then enriched by FACS as described under "Experimental Procedures") were stimulated in 48-well plates with vehicle, 1 μM ISO, or 1 μM PGE2 in the presence of 1 μM RO-20-1724 for 10 min at 37 °C. Intracellular cAMP was isolated and quantified by radioimmunomassay as described under "Experimental Procedures." Data represent mean ± S.E. from 6–7 paired observations. *, p < 0.05, ARR2-GFP group versus GFP group. B, HASM cells expressing ARR2(R169E)-GFP seeded onto poly-1-lysine-coated coverslips were stimulated with 1 μM PGE2 for 15 min, resulting in redistribution of ARR2(R169E)-GFP into a punctate formation.

FIG. 10. Association of arrestins with EP receptors in immunoprecipitates. Cells were stimulated for 5 min with vehicle or 1 μM PGE2, and ARR2-GFP (ARR2) or ARR2(R169E)-GFP (ARR2/R169E) was immunoprecipitated from cell lysates incubated with anti-arrestin-2 polyclonal antibody 178 as described under "Experimental Procedures." Blots of immunoprecipitated proteins were probed with an anti-HA antibody to assess cellular interaction of arrestin chimeras with each of the receptors.
tant nature could be its inability to be phosphorylated by GRKs, which may in turn limit its affinity for arrestins. Based on the demonstrated ability of ARR2(R169E) to rescue the homologous desensitization of a truncated δ opioid receptor lacking GRK phosphorylation sites (38), we hypothesized that ARR2(R169E)-GFP would be able to associate with the EP2 receptor and promote its agonist-dependent internalization. Indeed, this was the case as ARR2(R169E)-GFP could bind activated EP2 receptor (Fig. 10), promote its internalization (Fig. 7), and attenuate EP2 receptor signaling (Fig. 9). The relative inability of ARR2-GFP to promote any of these effects suggests that the lack of phosphorylation recognition minimizes arrestin-2-EP2 receptor interaction and thus the role of arrestin in EP2 receptor regulation.

Interestingly, we also observed disparate effects of ARR2(R169E)-GFP on the β2AR and EP4 receptor. For the β2AR, ARR2(R169E)-GFP promoted a greater degree of agonist-dependent internalization than did ARR2-GFP. This finding is consistent with previous findings demonstrating that in the presence of GRK3, phosphorylation-independent arrestin-2 induced a more rapid desensitization of the β2AR than did wild type arrestin-2, suggesting that ARR2(R169E) actually binds more readily to the phosphorylated β2AR (38). However, this property of ARR2(R169E) does not appear to extend to the EP4 receptor, as we found ARR2(R169E)-GFP was essentially equal to ARR2-GFP in promoting its internalization. Moreover, ARR2(R169E)-GFP was only marginally better than ARR2-GFP in associating with EP4 receptor based on co-precipitation analysis. Previous studies have demonstrated agonist-promoted phosphorylation of the EP4 receptor (49) and a requirement of C-tail phosphorylation sites in EP4 receptor homologous desensitization (54). However, a recent study by Desai et al. (55) demonstrated that mutation of numerous potential GRK phosphorylation sites in the EP4 receptor C-tail did not alter agonist-induced internalization, suggesting that phosphorylation may be unimportant in EP4 receptor internalization. In light of this finding, our data suggest that arrestins cannot make use of or do not require the phosphorylation-sensitive trigger in its interaction with the EP4 receptor and that other determinants dictate arrestin-EP4 interaction. Alternatively, changes in arrestin conformation that occur as a consequence of phosphorylation recognition and are important in sequential multisite arrestin binding (52, 53) may nevertheless occur with arrestin-EP4 interaction regardless of receptor phosphorylation state.

A somewhat curious finding of the present study was our inability to deduce any effects of arrestin expression on signaling in cells overexpressing the β2AR or EP4 receptor. However, we did observe cAMP production via endogenously expressed β2AR (in HASM) and EP4 receptors (in CHO-K1) to be significantly decreased by increased arrestin expression. This latter finding suggests that the effects of reduced arrestin expression on PGE2-mediated signaling in arrestin antisense-expressing HEK293 cells (31) reflected altered EP4 receptor signaling. Our inability to detect arrestin effects on signaling via overexpressed GPCRs suggests that possibly spare receptors may overwhelm some effects of desensitization mechanisms on GPCR-Gi-adenyl cyclase signaling, or perhaps some critical compartmentalization effect may be obscured in receptor overexpression models.

Of additional interest is the largely agonist-independent co-precipitation of the arrestin-GFP chimera observed with the EP2 and EP4 receptors (Fig. 10). This contrasts with the arrestin-promoted co-localization (Figs. 5 and 6) and internalization (Fig. 7) of EP2 and EP4 receptors that appeared to be largely agonist-dependent. These differences suggest that arrestins might weakly interact with EP2 and EP4 receptors in an agonist-dependent manner and that this interaction might be stabilized by the co-immunoprecipitation conditions. Agonist-independent interaction of arrestins with the EP receptors may reflect an ability of arrestins to stabilize the activated conformation as shown for the β2AR and M2AChR (56) or the ability of constitutively active arrestins to interact in an agonist-independent manner as observed recently with the M2AChR (57).

In summary, the differing susceptibilities of the β2AR, EP2, and EP4 receptor to arrestin and a phosphorylation-independent arrestin mutant suggest that arrestin-receptor interaction is determined by multiple factors that are of varied importance among GPCRs. With respect to the EP2 receptor, resistance to arrestin-promoted desensitization and internalization appears attributed in part to a lack of EP2 receptor phosphorylation. Last, the demonstrated capacity to assess arrestin-GPCR specificity in a differentiated cell type offers the opportunity to explore the role of arrestins in regulating discrete cellular functions modulated by GPCRs.
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