Endogenous G_s-Coupled Receptors in Smooth Muscle Exhibit Differential Susceptibility to GRK2/3-Mediated Desensitization†

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ABSTRACT: Although G protein-coupled receptor (GPCR) kinases (GRKs) have been shown to mediate desensitization of numerous GPCRs in studies using cellular expression systems, their function under physiological conditions is less well understood. In the current study, we employed various strategies to assess the effect of inhibiting endogenous GRK2/3 on signaling and function of endogenously expressed G_s-coupled receptors in human airway smooth muscle (ASM) cells. GRK2/3 inhibition by expression of a $G\beta\gamma$ sequestrant, a GRK2/3 dominant-negative mutant, or siRNA-mediated knockdown increased intracellular cAMP accumulation mediated via β-agonist stimulation of the beta-2-adrenergic receptor (β_2AR). Conversely, neither 5′-(*N*-ethylcarboxamido)-adenosine (NECA; activating the A2b adenosine receptor) nor prostaglandin E2 (PGE_2; activating EP2 or EP4 receptors)-stimulated cAMP was significantly increased by GRK2/3 inhibition. Selective knockdown using siRNA suggested the majority of PGE_2-stimulated cAMP in ASM was mediated by the EP2 receptor. Although a minor role for EP3 receptors in influencing PGE_2-mediated cAMP was determined, the GRK2/3-resistant nature of EP2 receptor signaling in ASM was confirmed using the EP2-selective agonist butaprost. Somewhat surprisingly, GRK2/3 inhibition did not augment the inhibitory effect of the β-agonist on mitogen-stimulated increases in ASM growth. These findings demonstrate that with respect to G_s-coupled receptors in ASM, GRK2/3 selectively attenuates β_2AR signaling, yet relief of GRK2/3-dependent β_2AR desensitization does not influence at least one important physiological function of the receptor.

G protein-coupled receptors (GPCRs[1]) that activate the heterotrimeric G protein G_s play an important role in regulating numerous functions of airway smooth muscle (ASM). Primarily through their activation of the cAMP-dependent protein kinase (PKA) via a G_s-adenylyl cyclase-cAMP dependent mechanism, G_s-coupled receptors regulate ASM contraction, proliferation, and autocrine/paracrine mediator synthesis (*1, 2*). β-Agonists, prostaglandin E, and adenosine are cognate ligands for the β-2-adrenergic (β_2AR), prostanoid EP2 (EP2R), and A_{2b} adenosine (A_{2b}AR) receptors, respectively. Each of these receptors has been shown to be expressed in human ASM cells (*3*−*5*). β_2ARs are the target of inhaled β-agonists used in both chronic management of asthma and rescue from life-threatening acute bronchospasm. The ability of β-agonists to regulate contractile properties of ASM has been demonstrated in *in vitro*, *ex vivo*, and *in vivo* models (reviewed in *2, 6*), and β-agonist is also known to regulate the proliferation of cultured ASM cells (*7*). The importance and role of EP2Rs and A2bARs in regulating ASM signaling and physiology have been recently suggested in studies of human ASM cultures and in both *ex vivo* and *in vivo* studies of murine ASM (*3, 5, 8*−*10*).

When heterologously expressed in cell lines such as HEK293 or COS cells, most GPCRs examined to date have been shown to rapidly lose their responsiveness to their cognate agonist via mechanisms involving GPCR kinases (GRKs) and arrestin molecules (reviewed in *11*−*13*). For example, the agonist-stimulated β_2AR is phosphorylated by members of the GRK family; this phosphorylation serves to diminish β_2AR-G_s coupling, and also promotes the association of the β_2AR with arrestin proteins. Arrestins bind the β_2AR to both sterically inhibit β_2AR-G_s interaction and initiate internalization of the β_2AR into clathrin-coated pits. Internalized β_2ARs subsequently traffic to either recycling or degradation pathways in a stimulus- and cell type-dependent manner.

By limiting the signaling capacity of both endogenous and exogenous agonists, GRK-mediated desensitization has been implicated in both disease pathogenesis and in diminishing the efficacy of therapeutics (*14*−*16*). Strategies that inhibit GRK-mediated desensitization of βARs have been shown

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[1] Abbreviations: ASM, airway smooth muscle; β_2AR, beta-2-adrenergic receptor; BSA, bovine serum albumin; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; FSK, forskolin; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; ISO, isoproterenol; NECA, 5′-*N*-ethyl-carboxamidoadenosine; PTX, pertussis toxin; PBS, phosphate-buffered saline; PGE_2, prostaglandin E2; VASP, vasolidator-stimulated phosphoprotein.

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useful in deterring chronic heart failure pathogenesis and in improving the efficacy of infused β-agonist in transgenic mouse models assessing cardiac function (*17*−*19*). The impressive efficacy of the β-agonist salmeterol in reversing airway bronchochonstriction despite its low intrinsic activity for the β_2AR may be related to its lower (relative to other β-agonists) capacity to induce β_2AR desensitization (*20*−*22*). Thus, GRK-dependent GPCR desensitization mechanisms represent an important consideration in disease management/therapy.

Although numerous GPCRs have been shown to undergo GRK-dependent desensitization using overexpression, and to a lesser extent, knockdown and knockout strategies, few studies have compared the susceptibility of multiple, endogenously expressed GPCRs to GRK-dependent desensitization in a single system. In cells such as ASM, which express multiple GPCRs, the relative susceptibility of these endogenously expressed GPCRs to agonist-induced desensitization mechanisms has clinical implications and could be important in determining the most appropriate receptor to target, the properties of the therapeutic ligand, or whether direct strategies targeting GPCR desensitization mechanisms hold any promise (*23*). In the present study, the signaling efficacy and susceptibility to GRK2/3-mediated desensitization is compared among the G_s-coupled β_2ARs, EP2Rs, and A_{2b}ARs in human ASM cells. Differences in the capacity of GRK2/3 to mediate desensitization among receptors are described, and the potential to exploit these differences to identify and develop more effective antiasthma therapies is discussed.

EXPERIMENTAL PROCEDURES

Materials. [^{125}I] cAMP was from Biomedical Technologies Inc. (Stoughton, MA). [*methyl*-^{3}H]-Thymidine (50 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). PGE_2, sulprostone, butaprost methyl ester, and other EP receptor ligands were from Cayman Chemical (Ann Arbor, MI). Phospho-p42/p44 and β-Actin antibodies were from Cell Signaling Technology (Beverly, MA). IRDye 680 or 800 secondary antibodies were from Rockland (Gilbertsville, PA). All other materials were obtained from Sigma (St. Louis, MO) or from previously identified sources (*24, 25*).

Cell Culture. Human ASM cultures were established from human tracheae as described previously (*25*). Third to sixth passage cells or fifth to eighth passage cells stably selected after retroviral infection as described below were plated at a density of 10^4 cells/cm^2 in either 24-well (cAMP accumulation, [^{3}H]-thymidine incorporaton assay) or 12-well plates (immunoblots) and maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum. Twenty-four hours prior to stimulation, cells were growth-arrested by washing once in PBS and refeeding with Ham's F-12 media supplemented with 0.1% bovine serum albumin (BSA). In select experiments, cells were pretreated with 50 ng/mL pertussis toxin (PTX) or vehicle (control) for 12 h prior to stimulation with agonists.

Generation of Constructs. GRK2-(495−689) (GRK2CT (*26*)), GRK2-(45−178) (GRK2NT (*27*)), or GRK2K220R (*28*) were subcloned into pEGFPN1 by PCR cloning. *Hin*dIII/NotI fragments, encoding GFP, GRK2CT-GFP, GRK2NT-GFP, or GRK2K220R-GFP were cloned into the retrovirus expression vector pLNCX2 as described previously (*24*).

Retroviral Infection. Stable expression of GFP, GRK2CT-GFP, GRK2NT-GFP, or GRK2K220R was achieved by retroviral infection as described previously (*29*). Briefly, retrovirus for the expression of each construct was produced by cotransfecting GP2−293 cells with pVSV-G vector (encoding the pantropic (VSV-G) envelope protein) and pLNCX2-GFP, pLNCX2-GRK2CT-GFP, pLNCX2-GRK2NT-GFP, or pLNCX2-GRK2K220R-GFP. Forty-eight hours after transfection, supernatants were harvested and used to infect human ASM cultures, with effective virus concentrations established by immunoblot analysis. Cultures were selected to homogeneity with 250 μg/mL G418 as described previously (*29*).

siRNA-Mediated Knockdown of GRK2/3. siRNA-mediated knockdown of GRK2/3 in ASM cells was achieved using a modification of the procedure we recently employed in Iwata et al. (*30*) for GRK2/3 knockdown in HEK293 cells. Two micrograms of siRNA duplexes (Dharmacon, Lafayette, CO) for GRK2 (5′-GAU CUU CGA CUC AUA CAU CdTdT-3) or the scrambled (control) sequence (5′-GCG CGC UUU GUA GGA UUC GdTdT-3′) were mixed with Amaxa transfection buffer and 5×10^6 harvested ASM cells and electroporated using the proprietary program designed for smooth muscle cells. As an additional control, cells were transfected with siRNA targeting arrestin2/3 as described previously (*31*). This same siRNA protocol was employed using On-TARGETplus SMARTpool oligos (Dharmacon, #L-005712-00-0005) to knockdown the EP2 receptor (EP2R). Cells were then plated in 10 cm dishes, recovered for 24 h, then passaged into 24 well (cAMP accumulation assay) or 6 well (real-time PCR analysis, immunoblot analyses) plates for subsequent assays performed 72 h later, corresponding to the period of peak GRK2/3 or EP2R knockdown.

Real-Time PCR Analysis of EPR mRNA Levels. Total RNA was isolated by standard procedures using Trizol (Invitrogen, Carlsbad, CA), then 1 μg of RNA was converted to cDNA using TAQman reverse transcription reagents with Multiscribe Reverse Transcriptase (Applied Biosystems TaqMan reverse transcription reagents Cat# N8080234). Human EP1, EP2, EP3, and EP4 receptor and *GAPDH* primers and probes (Assay on Demand, Applied Biosystems) were used in a standard real-time PCR reaction using an ABI PRISM 7700 sequence detection system. Briefly, a 50 μL reaction containing the TAQman RT reagents and 1 μg of RNA was incubated at 25 °C for 10 min, 48 °C for 60 min, 95 °C for 5, then 4 °C overnight in a Perkin-Elmer Gene Amp PCR 2400 thermal cycler. Five microliters of the cDNA reaction is then added to a PCR plate with the primer set and amplified: 50 °C × 2 min, 95 °C × 10 min, followed by 40 PCR cycles at 95 °C (15 s) and 60 °C (1 min). Results were normalized to *GAPDH* using the comparative C_t method. The threshold cycle C_t is defined as the cycle number at which the ΔRn crosses a software-generated threshold defined as 10 standard deviations above baseline (during cycles 3−15). C_t is linearly proportional to the logarithm of the input copy number. Negative controls included GAPDH amplification using RT reactions in which reverse transcriptase was omitted.

Immunoblot Analyses. Cells were grown to near confluence in 12-well plates and growth-arrested for 24 h in serum-free Ham's F-12 supplemented with 0.1% BSA as described above, then stimulated with the indicated agents for 0−3 h.

Cell lysates were subsequently harvested and agonist-stimulated changes in phospho-Thr202/Tyr204p42/p44 and induction of vasodilator-stimulated phosphoprotein (VASP) mobility shift were assessed as described previously (*29, 32*) employing the referenced primary antibodies and infrared-conjugated secondary antibodies. For analysis of GRK2/3 and arrestin2/3 expression in cells transfected with control, GRK2/3, or arrestin2/3 -targeting siRNA, blots were probed with anti-GRK2/3 and anti-arrestin2/3 antibodies as described previously (*4, 31*). Bands were visualized and signals (infrared emission) quantified directly using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

Assay of cAMP Accumulation. For assay of cAMP accumulation, cells plated in 24 well plates were grown to near confluence in Ham's F12/10% fetal bovine serum, washed, and fed Ham's F12/0.1% bovine serum albumin (BSA) for 24 h, then stimulated with PBS containing 300 μM ascorbic acid, 1 mM RO-20-1724 (phosphodiesterase inhibitor), and either vehicle (basal), 1 μM ISO, 1 μM PGE_2, 10 μM butaprost, 50 μM 5′-*N*-ethyl-carboxamidoadenosine (NECA), or 100 μM forskolin (FSK) for 0−10 min at 37 °C. cAMP was isolated and quantified by radioimmunoassay as described previously (*5*).

To minimize the effects of within-group variance of absolute agonist-stimulated cAMP accumulation, cAMP accumulation was calculated and reported as a percentage of cAMP accumulation stimulated by 100 μM FSK for each group as described previously (*5*). The various inhibitory strategies (recombinant construct or siRNA expression) did not affect FSK-stimulated cAMP accumulation (not shown).

[^{3}H] Thymidine Incorporation. [^{3}H] Thymidine incorporation in human ASM cultures was assessed as per ref *29*. Briefly, cells expressing GFP, GRK2CT-GFP, GRK2K220R-GFP, or GRK2NT-GFP were grown in 24 well plates to near confluence, then serum starved in 0.1% BSA media for 24 h. Cells were pretreated with vehicle, 1 μM ISO, 1 μM PGE_2, or 50 μM NECA for 10 min, then stimulated with vehicle or 10 nM epidermal growth factor (EGF). After 16 h of stimulation, cells were labeled with 3.0 μCi [methyl-^{3}H] thymidine (1 μCi/mL) and incubated for an additional 24 h. Cells were then washed with PBS, lysed with 20% trichloroacetic acid, aspirated onto filter paper, and counted in scintillation vials.

Calcium Mobilization. Primary cultures of ASM were plated on coverslips, loaded with FURA-2 AM, pretreated 5 min with vehicle, and then stimulated with either 1 μM PGE_2 or 1 μM sulprostone. Cells were subsequently washed with HBSS, then pretreated with vehicle or 1 μM SC19220, and stimulated again with agonist-stimulated Ca^{2+} mobilization assessed using a dual excitation fluorescence photomultiplier system (IonOptix, Milton, MA) as described previously (*33*).

Statistical Analysis. Retroviral-infected or siRNA-transfected cultures were generated from multiple cultures derived from different donor tracheae such that group effects could be analyzed as paired observations, and data from a given line contributed to an *n* of 1. Similarly, transfection of siRNA was performed on multiple (*n*) cultures to generate *n* paired observations. Data analysis was performed using GraphPad Prism and data expressed as the mean ± SE values. Group comparisons were performed using two-way ANOVA or Student's *t*-test where appropriate, with a p value of <0.05 sufficient to reject the null hypothesis.

RESULTS

To examine the selectivity of GRKs for G_s-coupled receptors in ASM, cultures of human ASM were infected with GFP-chimeras of either the C-terminus of GRK2 (CT-GFP), the N-terminus of GRK2 (NT-GFP), a kinase dead mutant of GRK2 (KR-GFP), or GFP alone. The GRK2 C-terminus contains a pleckstrin homology domain previously shown to be capable of sequestering $G\beta\gamma$ subunits important in GRK activation (*34, 35*). The GRK2 N-terminus has been previously shown to function as an RGS protein capable of inhibiting $G\alpha_q$-mediated signaling (*27*). A lysine to arginine mutation at amino acid 220 in GRK2 has been previously show to ablate phosphotransferase activity and render the mutant capable of blocking endogenous GRK activity in multiple cells types (*28, 36*). As shown in Figure 1A, stable expression of either CT-GFP or KR-GFP in ASM caused a significant increase in time-dependent ISO-stimulated cAMP accumulation, whereas NT-GFP expression had no effect. In CT-GFP- compared to GFP- expressing cells, cAMP accumulation was 27−40% higher throughout the time points examined. KR-GFP expression had an effect similar to that of CT-GFP. In PGE_2-stimulated cells (Figure 1B), CT-GFP caused a small reduction, and NT-GFP and KR-GFP a small increase, in cAMP accumulation, although none of the effects was statistically significant. NECA-stimulated cAMP accumulation (Figure 1C) was modestly increased (16−38% at the various time points examined) by CT-GFP or KR-GFP expression; these differences did not achieve statistical significance, although trends were observed (GFP vs CT-GFP, $p = 0.44$; GFP vs KR-GFP, $p = 0.32$). FSK-stimulated cAMP accumulation was not significantly affected by CT-GFP, KR-GFP, or NT-GFP expression relative to values observed in the control GFP cells (not shown), suggesting that effects of construct expression reflected a regulation of the receptor locus.

To further examine the regulatory effects of endogenous GRKs in ASM, cultures of human ASM were transiently transfected with siRNA oligos that target both GRK2 and GRK3 (*30*). As shown in Figure 2A, selective and significant knockdown (>70% in all experiments, ranging from 72−90%, without associated effects on arrestin2/3 expression) of endogenous GRK2/3 was achieved 96 h post transfection. Knockdown of GRK2/3 was associated with significant increases in ISO-, but not PGE_2- or NECA-stimulated cAMP accumulation (Figure 2B−D). As with the above studies assessing effects of GRK inhibitory constructs, a modest effect of GRK2/3 knockdown was observed for NECA-stimulated cAMP accumulation, but failed to achieve statistical significance ($p = 0.28$).

To explore the possibility that a lack of effect of GRK-inhibiting strategies on PGE_2-stimulated cAMP accumulation might be attributed to the presence of multiple (and possibly differentially regulated) EP receptor isoforms in human ASM, we performed additional experiments in an attempt to isolate and characterize EP isoform-specific signaling and regulation in ASM. Although our previous studies suggested that the EP2 receptor is the functionally dominant EP isoform in human ASM, expression of low levels of either EP1 or

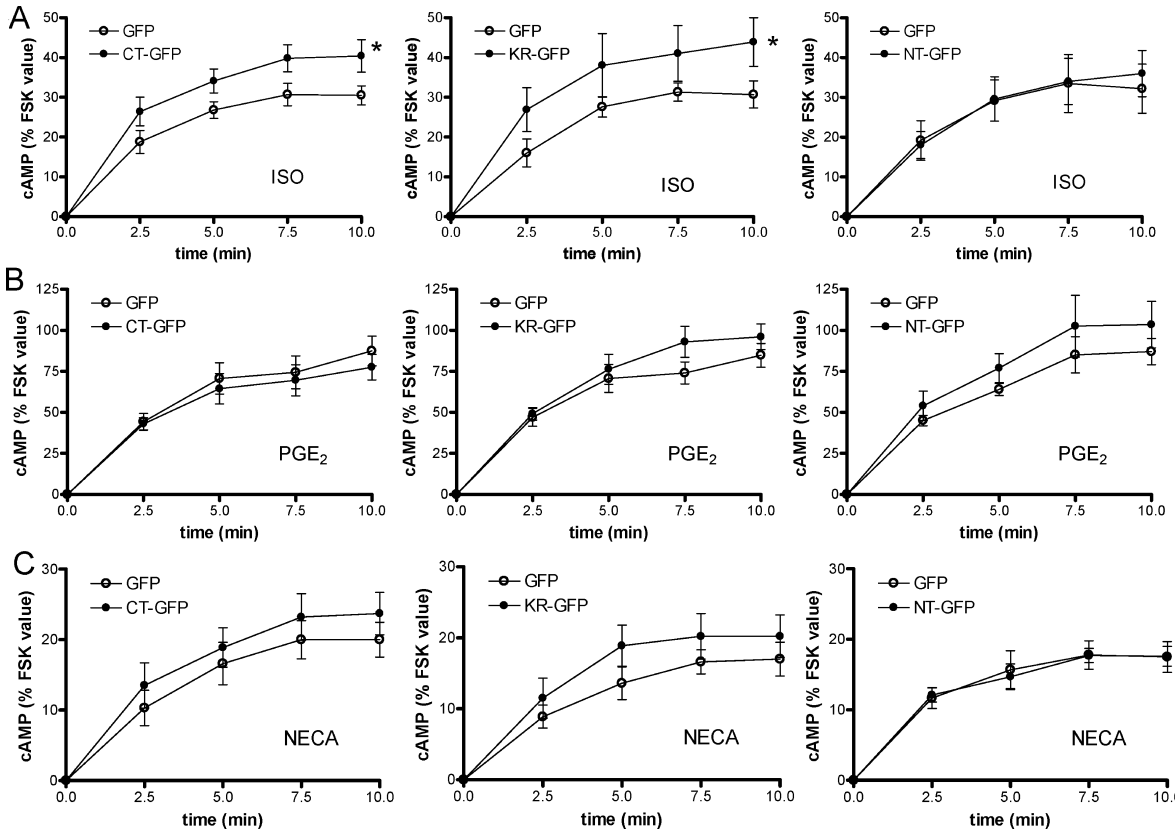

FIGURE 1: Effects of GRK mutants on G_s-coupled receptor-mediated cAMP accumulation in ASM. Primary ASM cultures were infected with retrovirus and selected with 250 μg/mL G418 to generate lines stably expressing GFP, GRK2CT-GFP (CT-GFP), GRK2K220R-GFP (KR-GFP), or GRK2NT-GFP (NT-GFP). Cells grown to near-confluence in 24 well plates were stimulated with 1 mM RO-20-1724 and 1 μM ISO (A), 1 μM PGE_2 (B), or 50 μM NECA (C) for 0−10 min, or 100 μM FSK for 10 min. cAMP was isolated and quantified by RIA as described in Experimental Procedures. Data represent the mean ± SE values from 6−9 experiments, with each experiment employing a different culture derived from a unique donor. *$p < 0.05$, 2-way ANOVA.

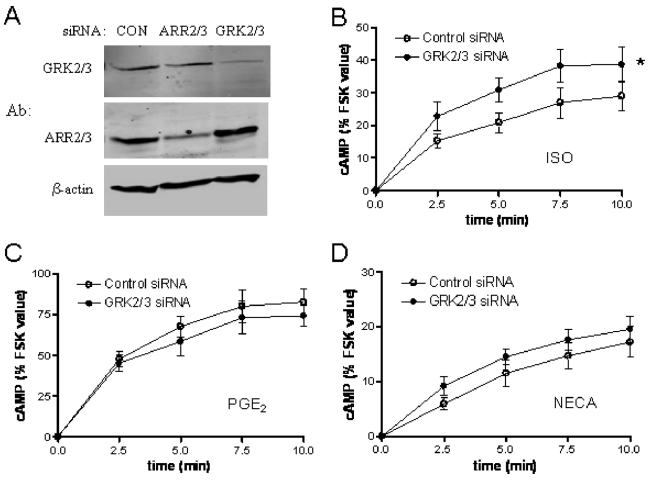

FIGURE 2: Effects of GRK2/3 knockdown on G_s-coupled receptor-mediated cAMP accumulation in ASM. (A) Immunoblot analysis of effects of transfection of control (CON) or GRK2/3 siRNA on GRK2/3 expression in ASM cultures. (B) Cultures transfected as in (A) were plated in 24 well plates and stimulated with 1 mM RO-20-1724 and 1 μM ISO (A), 1 μM PGE_2 (B), or 50 μM NECA (C) for 0−10 min, or 100 μM FSK for 10 min. cAMP was isolated and quantified by RIA as described in Experimental Procedures. Data represent the mean ± SE values from 6 experiments, using 6 sets of cultures each derived from a unique donor. *$p < 0.05$, 2-way ANOVA.

EP3 receptors was suggested by effects of various EP ligands on cAMP accumulation (*5*). EP1 and EP3 receptors can signal through G_q and G_i heterotrimeric proteins, and thus could influence adenylyl cyclase activity via PKC- and G$\beta\gamma$-dependent mechanisms (*11*). Moreover, regulation of EP1/EP3 receptor responsiveness by CT-GFP, KR-GFP, or NT-GFP could indirectly regulate EP2R signaling independent of direct effects of these constructs on the EP2R. In order to clarify whether EP1 or EP3 receptors are expressed and clarify the functional consequence in human ASM cultures, multiple approaches were taken. First, mRNA levels for each receptor subtype were assessed by reverse transcriptase PCR and real-time PCR. In naïve human ASM cultures, reverse transcriptase PCR amplified mRNA for each of the EP receptors (Figure 3A). Analysis of EtBr-stained PCR products generated at 28, 34, and 40 cycles suggests that mRNA abundance in ASM is greatest for EP2R mRNA, followed by EP3 = EP4 > EP1. Characterization of 6 cultures using real-time PCR analysis suggested again that EP2 and EP3 mRNA were relatively abundant on the basis of a relatively lower C_t (threshold) value. (Table 1). Attempts to characterize protein expression using subtype-selective antibodies of each of the EP R subtypes failed to detect expression of any of the subtypes (data not shown), a result likely attributable to the lack of sensitivity of currently available antibodies. Next, receptor subtype-selective ligands were employed to selectively inhibit/activate EP1 or EP3 receptors and characterize activation of downstream signaling events typically associated with G_i and G_q G-protein signaling. As shown in Figure 3B−D, PGE_2 only weakly stimulated a calcium flux in human ASM, yet the EP3R-selective agonist sulprostone elicited a strong response. Pretreatment with the EP1R-

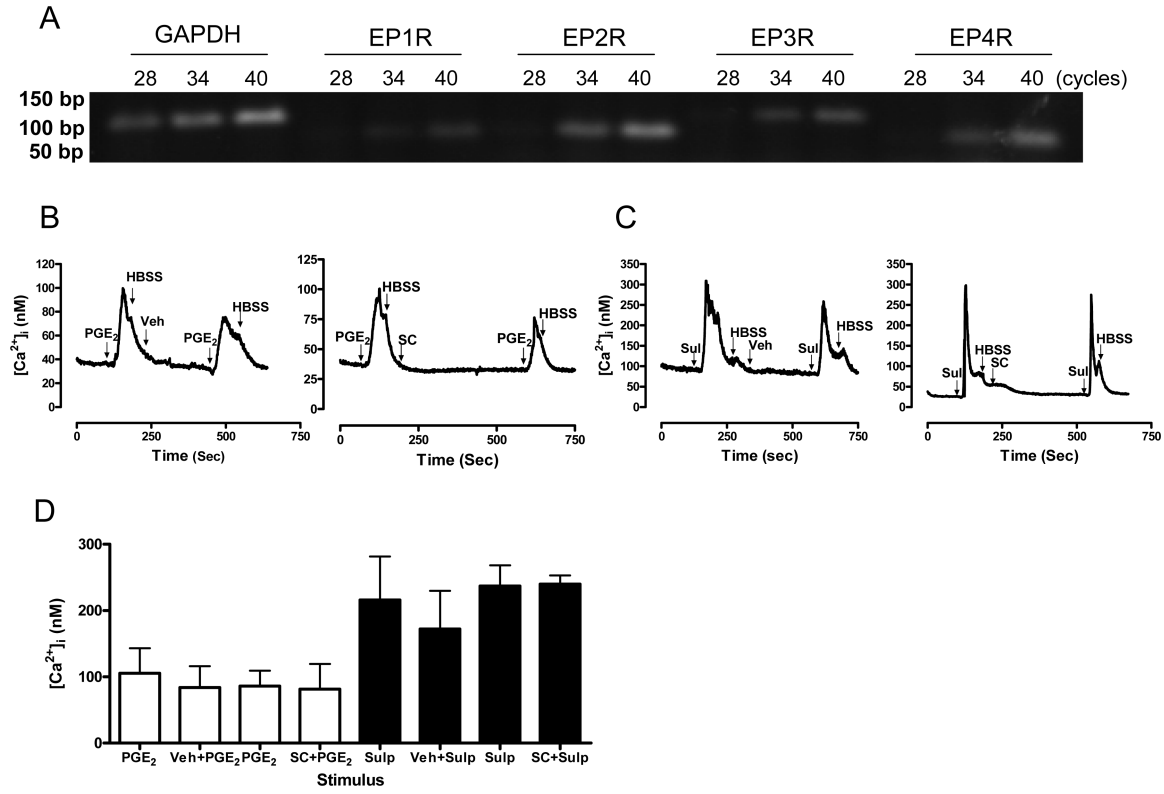

FIGURE 3: Characterization of EP receptor mRNA expression and EPR-mediated Ca^{2+} mobilization in ASM cells. (A) Reverse transcriptase PCR analysis of EP receptor subtype mRNA expression in ASM cultures. mRNA of each of the EPR subtypes as well as GAPDH was amplified for the indicated number of cycles, and products were purified and electrophoresed in a 2% agarose, EtBR-containing gel. (B−D) Primary cultures of ASM were plated on coverslips, loaded with FURA-2 AM, pretreated 5 min with vehicle, and then stimulated as indicated. Cells were subsequently washed with HBSS, then pretreated with vehicle or 1 μM SC19220, and stimulated again, with agonist-stimulated Ca^{2+} mobilization assessed as described in Experimental Procedures. B and C are representative traces depicting Ca^{2+} mobilization stimulated by 1 μM PGE_2 (B) or 1 μM sulprostone (C) in the presence/absence of 1 μM SC19220; (D) is the graphical representation of mean ± SE values of 3 (PGE_2) or 4 (sulprostone) independent experiments.

Table 1: Real-Time PCR Analysis of EP Receptor Subtype mRNA Expression[a]

	GAPDH	EP1	EP2	EP3	EP4
mean	21.40	32.87	29.21	29.68	31.35
SE	2.13	1.86	0.70	0.95	1.05

[a] Threshold cycle C_t values determined from 6 human ASM cultures.

selective antagonist SC19220 had no effect on sulprostone-stimulated calcium mobilization.

To further clarify the EP receptor subtype function in ASM, agonist-stimulated p42/p44 activation was assessed. Both PGE_2 and sulprostone, but not the EP2R-selective ligand butaprost methyl ester (*37*), stimulated a rapid phosphorylation of p42/p44 (Figures 4A−C), which was PTX-sensitive. However, p42/p42 phosphorylation by PGE_2 was relatively weak and transient, compared to the more efficacious and sustained phosphorylation induced by sulprostone (Figure 4D). Of note, PGE_2 elicited a strong and sustained shift in VASP, reflecting PKA activation, which likely accounts for the rapid waning of the p-p42/p44 signal. Pretreatment of cells with 10 μM SC19220 had no effect on PGE_2-stimulated p42/p44 phosphorylation in 3 of the cultures examined, while causing a small (~20%) inhibition in 3 other cultures (data not shown). However, sulprostone-stimulated p42/p44 phosphorylation was similarly inhibited by SC19220 in these cultures (data not shown). Pertussis toxin pretreatment of ASM cells caused a small (~30%), statistically significant increase in PGE_2-stimulated cAMP accumulation (Figure 4E), and siRNA-mediated knockdown of the EP2R (69 ± 15% knockdown of EP2R mRNA levels) resulted in profound loss of PGE_2- and butaprost-stimulated cAMP accumulation (73% and 85% decrease, respectively)(Figure 4F). These data suggest that concomitant EP2R activation has a large inhibitory effect on PGE_2-stimulated calcium mobilization and p42/p44 phosphorylation mediated primarily by the EP3R, in contrast to the small inhibitory effect of EP3R receptor activation on the PGE_2-stimulated cAMP accumulation that is mediated primarily by the EP2R.

Collectively, the data in Figures 3 and 4 suggest a small but potentially confounding effect of EP3R activation on PGE_2-stimulated cAMP accumulation in ASM cells. In order to selectively activate and assess GRK-dependent regulation of the EP2R in ASM, we therefore assessed the effect of CT-GFP/KR-GFP expression or GRK2/3 knockdown in cells stimulated with butaprost. CT-GFP expression (Figure 5A), KR-GFP (Figure 5B) expression, or GRK2/3 knockdown (Figure 5C) did not affect the time-dependent stimulation of cAMP accumulation by butaprost.

The functional consequences of GRK2/3 inhibition were examined by assessing the effect of CT-GFP or KR-GFP expression on the inhibitory effect of ISO, NECA, or PGE_2 on EGF-stimulated [^{3}H] thymidine incorporation in ASM cultures (Figure 6). NECA did not significantly inhibit ASM growth in GFP-, CT-GFP-, of KR-GFP-expressing

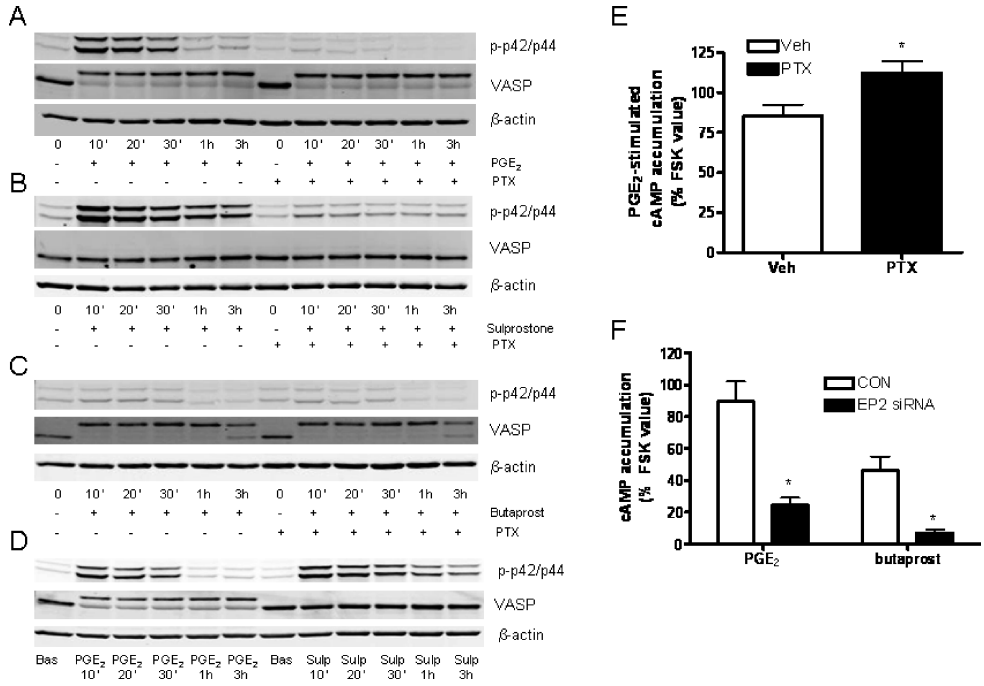

FIGURE 4: EPR-mediated p42/p44 phosphorylation and cAMP accumulation. (A−D) ASM cells plated in 12 well plates were pretreated for 12 h with vehicle or 50 ng/mL PTX then stimulated for 0−3 h with 1 μM PGE_2, 1 μM sulprostone, or 10 μM butaprost. Cell lysates were harvested, and phospho-p42/p44, VASP, and β-actin levels were assessed by immunoblotting. Data depicted are representative of experiments performed using 4 separate cultures. (E) ASM cells plated in 24 well plates were pretreated for 12 h with vehicle or 50 ng/mL PTX then stimulated for 10 min with vehicle, 1 μM PGE_2, or 100 μM FSK. (F) ASM cells were transfected with either scrambled (CON) or EP2R siRNA oligonucleotides, plated in 24 well plates, and stimulated 96 h later with vehicle, 1 μM PGE_2, 10 μM butaprost, or100 μM FSK. Knockdown of EP2R mRNA (69 + 15%) was assessed by real-time PCR using cells plated in parallel in 6 well plates. Neither PTX treatment nor EP2R siRNA affected FSK-stimulated cAMP accumulation, and data were normalized to this value determined for each group. Data represent the mean ± SE values from 6 (E) and 4 (F) experiments. *$p < 0.05$, Student's *t*-test.

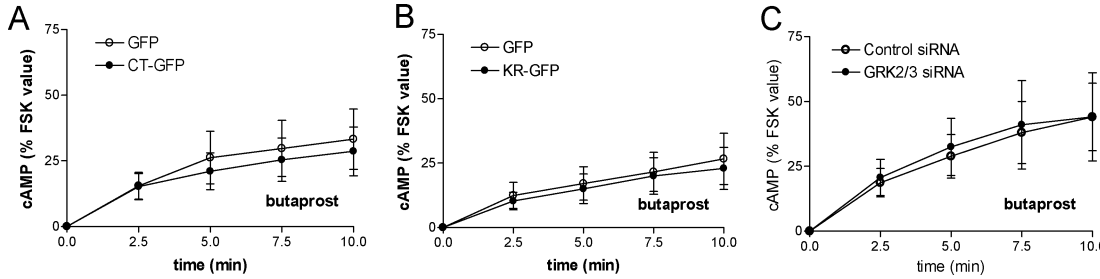

FIGURE 5: Effects of GRK inhibition on selective EP2 R signaling. Time-dependent, butaprost-stimulated cAMP accumulation in GFP- vs GRK2CT-GFP (A)- and GFP- vs GRK2K220R-GFP (B)-expressing cells, and in cells transfected with control siRNA vs GRK2/3 siRNA (C), assessed as per experiments described in Figure 1. (C) Data represent the mean ± SE values from 6 (A), 5 (C), or 4 (B) paired observations.

ASM cells. PGE_2 caused a large (67 ± 8%) inhibition of EGF-stimulated growth, which was unaffected by CT-GFP or KR-GFP expression (Figure 6). ISO significantly inhibited (21 ± 5%) EGF-stimulated [^{3}H] thymidine incorporation in GFP-expressing cells. However, this inhibitory effect was also not augmented by CT-GFP or KR-GFP expression. These findings suggest that relief of GRK2/3-dependent desensitization of the β_2AR demonstrated in analyses of acute signaling does not translate into a greater ability of the β_2AR to modulate this functional outcome in ASM.

DISCUSSION

The current study examines, for the first time, the selectivity of GRK2/3 for endogenously expressed G_s-coupled receptors in a primary cell type. Signaling by endogenous β_2ARs in ASM cells is shown to be subject to GRK-dependent desensitization, whereas EP2R and A_{2b}ARs signaling are not. Our previous studies demonstrated that overexpression of GRK2 could enhance desensitization of the β_2AR and that overexpresssion of arrestin2 or arrestin3 could attenuate β_2AR- and A_{2b}AR-, but not PGE_2-mediated cAMP accumulation in human ASM cultures (*4, 5*). However, these overexpression strategies, while informative, have the potential to promote nonphysiological protein−protein interactions. For example, in our recent study (*5*) arrestin2/3 overexpression was shown to attenuate signaling while augmenting internalization of the cysteinyl leukotriene type 1 receptor (CysLT1R). However, neither expression of a dominant−negative arrestin nor arrestin gene ablation affected CysLT1R signaling or internalization, suggesting that although overexpressed arrestin could associate with the CysLT1R, endogenous arrestins did not. Thus strategies designed to target the interaction of endogenous regulatory molecules with GPCRs afford greater insight into physiologic regulation.

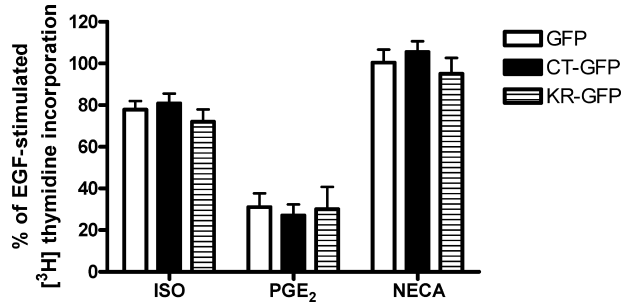

FIGURE 6: Effects of GRK mutant expression on ISO- and PGE_2-mediated inhibition of ASM growth. ASM cultures expressing GFP, CT-GFP, or KR-GFP were grown to near-confluence in 24 well plates then growth arrested in 0.1% BSA serum-free media for 24 h. Cell were then pretreated 10 min with vehicle, 1 μM ISO, 1 μM PGE_2, or 50 μM NECA, then stimulated with 10 nM EGF. [^{3}H] thymidine incorporation was subsequently assessed as described in Experimental Procedures. Data represent the mean ± SE values from 6 paired observations from cultures of GFP vs CT-GFP cells and 4 paired observations from cultures of GFP vs KR-GFP cells.

The failure of GRK inhibition or knockdown to influence PGE_2-stimulated cAMP accumulation is consistent with our previous studies suggesting that the EP2 receptor is relatively resistant to agonist-specific desensitization. cAMP accumulation and measures of PKA activation, under conditions of either acute or chronic agonist treatment, are significantly greater with PGE_2 than with β-agonist (*4, 5, 32, 38* and unpublished observations), and such signaling is associated with greater efficacy in modulating ASM functions such as inhibition of ASM growth (current study and ref *25*) and migration (*39*), induction of IL-6 synthesis, and inhibition of RANTES synthesis (*40*)). As mentioned above, overexpression of arrestin2 or arrestin3 in ASM cells could not reduce PGE_2-mediated cAMP accumulation (*5*). Arrestin overexpression had minimal effect on PGE_2-induced internalization of either endogenous EP2Rs in ASM cells or recombinant EP2R expressed in CHO cells. However, overexpression of an arrestin mutant capable of binding GPCRs regardless of their phosphorylation status was effective in increasing agonist-induced EP2R internalization in both cells types, suggesting that the inability of GRKs to phosphorylate agonist-occupied EP2R accounts for EP2R resistance to GRK/arrestin-mediated desensitization. The inability of either GRK2/3 inhibition or GRK2/3 knockdown to augment PGE_2-mediated cAMP accumulation demonstrated herein further supports this interpretation that the EP2R is a poor substrate for GRKs, and its signaling is not limited by classical GRK-mediated desensitization.

The current study also helps clarify the cross-regulation of EP receptor subtypes in human ASM. On the basis of the efficacy of the EP2R-selective agonist butaprost and the profound effect of EP2R knockdown on PGE_2-stimulated cAMP accumulation, the EP2R can be concluded to be most responsible for the effects of PGE_2 on cAMP accumulation in human ASM. This conclusion is consistent with our previous study demonstrating the EP2R as mediating the relaxation of contracted murine ASM *ex vivo* (*10*), with Norel et al. (*41*), which concluded EP2R, but not EP4R-mediated relaxation of human bronchial preparations, and with Sheller et al. (*9*), which demonstrated that PGE_2 inhibited methacholine-stimulated bronchoconstriction in wild type mice, yet increased methacholine-stimulated bronchoconstriction in EP2R knockout mice. In the current study, the efficacy of the EP3R-selective agonist sulprostone in stimulating both calcium mobilization and p42/p44 phosphorylation in ASM cells, and the regulatory effects of PTX treatment on both PGE_2-stimulated p42/p44 phosphorylation and cAMP accumulation suggest that EP3R is the other EP receptor of consequence in ASM. Although other studies have implicated roles for the EP1R in murine ASM (*42*), and the possibility that the "selective" ligand sulprostone may possess some affinity for the EP1R, the collective data from our study strongly point to the EP3R as the mediator of PGE_2-stimulated calcium mobilization and p42/p44 phosphorylation. EP3R activation does also appear to limit the magnitude of PGE_2-stimulated cAMP accumulation, albeit to a small extent, perhaps via either kinase-dependent phosphorylation of the EP2R or $G_{\alpha i}$-mediated inhibition of adenylyl cyclase. The more compelling evidence for EPR cross-regulation occurs in the EP2R-mediated suppression of EP3R signaling. The capacity of PGE_2 to stimulate G_q/G_i-dependent Ca^{2+} mobilization or p42/p44 phosphorylation in naïve ASM was observed to be minimal. However, selective activation of EP3R with sulprostone resulted in significantly greater Ca^{2+} mobilization and p42/p44 activation.

Our findings that GRK2-CT expression (and GRK2/3 knockdown) caused a slight inhibition of PGE_2-stimulated cAMP accumulation, whereas GRK2K220R or GRK2NT expression caused a slight increase, suggested a possible subtle effect of EP1/3Rs on EP2R signaling. With GRK inhibition, the null effect on EP2Rs combined with slight enhancement of EP1/3R signaling may cause a small inhibition of EP2R-mediated cAMP accumulation. Effects of $G\beta\gamma$ sequestration with GRK2-CT and GRK2K220R, and $G\alpha_q$ inhibition by GRK2K220R or GRK2NT are difficult to control and interpret in studies of PGE_2-mediated cAMP accumulation. EP1/3R regulation of adenylyl cyclase and PLC (which could in turn regulate EP2R via activation of PKC) may occur in a $G\beta\gamma$-dependent manner. With GRK2K220R or GRK2NT expression, EP1/3R signaling via $G\alpha_q$ activation could be inhibited by the RGS function of the N-terminal domain of GRK2 (*27*), thus inhibiting a possible constraining effect of EP1/3Rs on cAMP accumulation. Although these modes of regulation and impact on EP2R signaling in ASM are intriguing, our data suggest that they are small, and clarifying them and their biological relevance is hampered by the small mean effect and the biologic variability observed in ASM cultures.

We have previously reported that the A2b adenosine receptor is the principal adenosine receptor subtype expressed in human ASM cultures and is subject to agonist-specific desensitization (*3*). Our finding that neither GRK2CT expression nor GRK2/3 knockdown could significantly increase NECA-stimulated cAMP accumulation was somewhat surprising, given that we previously determined that overexpression of arrestins in human ASM significantly decreases this A2bAR signaling, and a prior study (*43*) demonstrated that expression of a GRK dominant−negative mutant increased adenylyl cyclase activity in NG108-15 cells, which express both A2a and A2b adenosine receptors. However, as mentioned above, mass action effects associated with overexpression are capable of producing nonphysiological protein−protein interactions. Alternatively, because a trend toward statistical significance was observed with both

GRK2CT and GRKK220R expression and GRK2/3 knockdown, it is possible that GRKs exert a true but small effect on A2bAR signaling and that our analysis reflects a type 2 statistical error. In addition, it is also possible that the presence of A1 or A3 adenosine receptors, which are expressed at low levels in human ASM cultures (*3*), could have confounded our analysis. Unfortunately, no useful subtype-selective ligands are currently available to enable selective A2b AR activation. In any event, our findings suggest that GRK2/3 has at most a minimal effect in regulating A2bAR signaling in human ASM cells.

It should be noted that the present study does not account for the potential regulatory role of GRKs 4−6. Although GRK2 is the most abundantly expressed GRK in human ASM, GRK5/6 proteins can be detected at low levels (*4*), and their roles in regulating Gs-coupled receptors in ASM cannot be excluded. Future studies will clarify the extent to which these GRK isoforms contribute to agonist-specific desensitization.

Findings from the current study hold important clinical implications. First, a superior therapeutic efficacy of selective EP2R agonists is suggested, attributable to the desensitization-resistant nature of the receptor and the high potency/efficacy of PGE_2 in inhibiting ASM growth. Although to date the use of PGE_2 as a bronchodilator has been confounded by the presence of multiple EPR subtypes expressed in multiple airway cell types (contributing to problematic cough), our data suggest that selective targeting of ASM EP2Rs could provide optimal bronchodilation and prophylaxis against ASM hyperplasia. In a similar vein, current drug discovery approaches could focus on identifying novel β-agonists with high efficacy for stimulating $G_{\alpha s}$ and adenylyl cyclase yet minimal ability to invoke receptor desensitization; such functional diversity of GPCR ligands is now appreciated (*23*) and has been specifically demonstrated for βAR ligands in a recent study by Wisler et al. (*44*).

Although our findings suggest that the GRK-resistant nature of the EP2 receptor may contribute to its superior functional efficacy, we were unable to demonstrate that GRKs modulate the ability of the β_2AR to regulate at least one important ASM function, proliferation. GRK inhibition via either expression of GRK2-CT or GRK2K220R failed to alter the ISO-mediated inhibition of EGF-stimulated ASM growth. The most likely explanation of this finding is that inhibition of GRKs enables an increase in β_2AR signaling for a limited duration, after which other mechanisms of desensitization compensate and effectively quench signaling. That GRK2/3 regulation of the β_2AR is of limited duration is suggested by our data in Figures 1 and 2, in which the rate of cAMP accumulation after the first 2.5 min of agonist stimulation does not appear to be significantly affected by GRK2/3 inhibition. After an initial period of GKR2/3 mediated desensitization, PKA-mediated phosphorylation of the receptor, in cooperation and synergistic with induction of phosphodiesterase activity (*45*−*47*), is predicted to contribute to β_2AR desensitization, reduction of cellular cAMP levels, and presumed antimitogenic signaling attributable to PKA activity. Indeed, our recent study has suggested an important role for PKA in β-agonist-induced desensitization of the β_2AR in ASM (*32*). Should inhibition of ASM growth by G_s-coupled receptors be dependent on sustained signaling (presumably linked to PKA activation) throughout the cell cycle, transient relief of homologous desensitization may very well be insufficient to impact any inhibitory effect of receptor activation.

Future studies employing genetic strategies to inhibit or reduce GRK expression *in vivo* will be necessary to determine whether targeting GRK desensitization can alter β_2AR-mediated regulation of other ASM functions such as contraction. Unfortunately, GRK2 knockout in the mouse is lethal (*48*); thus, alternative genetic strategies are required. However, we have recently reported that mice in which the arrestin3 gene is ablated exhibit an augmented relaxant effect of β-agonists on ASM contracted either *in vivo* or *ex vivo* (*31*), suggesting that GRKs may play a role in constraining the efficacy of β_2ARs with respect to this function. These findings and those of the current study suggest that the relevance of GRK-mediated GPCR regulation may be restricted to not only certain receptors but also to specific events of perhaps short duration, in which the rapid effects of GRKs are not obscured or rendered redundant by other regulatory mechanisms.

A widely recognized limitation of asthma management with inhaled β-agonist therapy is the loss of β_2AR responsiveness reflected in a loss of bronchoprotective effect (*49*−*52*), deterioration of asthma control, and susceptibility to exacerbations (*53*) that occurs with chronic administration of the drug. A therapeutic strategy that inhibits GRK-mediated phosphorylation of ASM β_2ARs could conceivably improve the efficacy and extend the duration of activity of β-agonists in the asthmatic airway. The utility of either mitigating mechanisms of agonist-specific desensitization or selectively targeting the desensitization-resistant EP2R would be further enhanced should allergic inflammation of the human lung be associated with upregulated GRK activity, as has been demonstrated in rat lungs (*54*).

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