

Exploiting functional domains of GRK2/3 to alter the competitive balance of pro- and anticontractile signaling in airway smooth muscle

Deepak A. Deshpande,^{*,1} Huandong Yan,^{*,1} Kok-Choi Kong,[†] Brian C. Tiegs,[‡] Sarah J. Morgan,^{*} Tonio Pera,[‡] Reynold A. Panettieri,[§] Andrea D. Eckhart,[‡] and Raymond B. Penn^{*,†,‡,2}

^{*}Department of Medicine, Division of Pulmonary and Critical Care Medicine, University of Maryland School of Medicine, Baltimore, Maryland, USA; [†]Department of Internal Medicine, Wake Forest University Health Sciences, Winston-Salem, North Carolina, USA; [‡]Department of Medicine, Center for Translational Medicine, Jane and Leonard Korman Lung Center, Thomas Jefferson University, Philadelphia, Pennsylvania, USA; and [§]Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

ABSTRACT To clarify the potential utility of targeting GRK2/3-mediated desensitization as a means of manipulating airway smooth muscle (ASM) contractile state, we assessed the specificity of GRK2/3 regulation of procontractile and relaxant G-protein-coupled receptors in ASM. Functional domains of GRK2/3 were stably expressed, or siRNA-mediated GRK2/3 knockdown was performed, in human ASM cultures, and agonist-induced signaling was assessed. Regulation of contraction of murine tracheal rings expressing GRK2 C terminus was also assessed. GRK2/3 knockdown or expression of the GRK2 C terminus caused a significant (~30–90%) increase in maximal β -agonist and histamine [phosphoinositide (PI) hydrolysis] signaling, without affecting the calculated EC_{50} . GRK2 C-terminal expression did not affect signaling by methacholine, thrombin, or LTD4. Expression of the GRK2 N terminus or kinase-dead holo-GRK2 diminished (~30–70%) both PI hydrolysis and Ca^{2+} mobilization by every G_q -coupled receptor examined. Under conditions of GRK2 C-terminal expression, β -agonist inhibition of methacholine-stimulated PI hydrolysis was greater. Finally, transgenic expression of the GRK2 C terminus in murine ASM enabled ~30–50% greater β -agonist-mediated relaxation of methacholine-induced contraction. Collectively these data demonstrate the relative selectivity of GRKs for the β_2 AR in ASM and the ability to exploit GRK2/3 functional domains to render ASM

hyporesponsive to contractile agents while increasing responsiveness to bronchodilating β -agonist.—Deshpande, D. A., Yan, H., Kong, K.-C., Tiegs, B. C., Morgan, S. J., Pera, T., Panettieri, R. A., Eckhart, A. D., Penn, R. B. Exploiting functional domains of GRK2/3 to alter the competitive balance of pro- and anticontractile signaling in airway smooth muscle. *FASEB J.* 28, 000–000 (2014). www.fasebj.org

Key Words: asthma • β -agonist • bronchodilation • desensitization • G-protein-coupled receptor

AIRWAY SMOOTH MUSCLE (ASM) tone is regulated by agonists of G-protein-coupled receptors (GPCRs) that can cause either contraction or relaxation. Members of the GPCR superfamily that activate the heterotrimeric G protein G_q are primarily responsible for the signaling events that promote contraction, whereas GPCRs that activate G_s promote relaxation. ASM contractile state can be viewed as the sum effect of procontractile and relaxant signaling events, and many therapies for obstructive lung diseases seek to either inhibit ASM procontractile signaling *via* antagonism of ASM G_q -coupled receptors, *e.g.*, with tiotropium or montelukast, antagonists of the m3 muscarinic acetylcholine receptor (m3mAChR) and cysteinyl leukotriene type 1 receptor (CysLT1R), respectively, or stimulate relaxation through agonism of G_s -coupled receptors, *e.g.*, with β -agonists that activate the β -2-adrenoceptor (β_2 AR). Similarly, other ASM functions, such as cell growth, are stimulated by G_q -coupled receptors and inhibited by G_s -coupled receptors (1).

Abbreviations: ASM, airway smooth muscle; β_2 AR, β -2-adrenoceptor; COPD, chronic obstructive pulmonary disease; CysLT1R, cysteinyl leukotriene type 1 receptor; Epac, exchange protein directly activated by cAMP; FSK, forskolin; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; H1 HR, H1 histamine receptor; hTERT, human telomerase reverse transcriptase; ISO, isoproterenol; m3mAChR, m3 muscarinic acetylcholine receptor; MCh, methacholine; NTG, nontransgenic; PAR, protease-activated receptor; PGE₂, prostaglandin E₂; PI, phosphoinositide; PKA, protein kinase A, TP, thromboxane receptor

¹ These authors contributed equally to this work.

² Correspondence: Department of Medicine, Thomas Jefferson University, Room 317, College Bldg., 1025 Walnut St., Philadelphia, PA 19107, USA. E-mail: raymond.penn@jefferson.edu
doi: 10.1096/fj.13-240226

Many GPCRs have been shown to undergo rapid desensitization on stimulation with their cognate agonist *via* a mechanism involving receptor phosphorylation (2). A family of kinases known as GPCR kinases (GRKs) specifically recognize the agonist-occupied form of the GPCR and rapidly phosphorylate the receptor (3). This phosphorylation reduces the coupling of the receptor with G protein, and promotes the subsequent binding of arrestin proteins, which sterically hinders receptor–G-protein interaction and promotes receptor internalization *via* clathrin-coated pits (4). The extents to which GRK- and arrestin-dependent processes affect receptor responsiveness are both receptor and cell specific. However, because much of the insight into GRK and arrestin function has arisen through studies using artificial cell lines, such as HEK293 and COS, which enable overexpression of GPCRs and other regulatory molecules, our understanding of how GPCRs are regulated in primary cell types or under more physiological conditions is limited. Few studies exist employing primary, differentiated cells in which the regulation of endogenously expressed GPCRs by endogenously expressed GRKs and arrestins is examined (reviewed in ref. 5). This stems in part from the dearth of pharmacologic tools for manipulating GRK/arrestin function, the difficulty in applying molecular biology techniques to primary cell types, and the inherent difficulty of biochemical analysis of low-abundance proteins such as GRKs, arrestins, and most GPCRs.

We recently characterized the effect of inhibiting GRK-mediated desensitization on G_s -coupled GPCRs in human ASM cells. Expression of either a GRK2/3 inhibitory peptide or a kinase-dead GRK2 or siRNA-mediated knockdown of GRK2/3 resulted in increased signaling of β_2 ARs, but not of EP2 prostanoid or A2b adenosine receptors in human ASM cells (6). These findings support the notion that targeting GRK/arrestin-mediated GRK desensitization may be an effective strategy for improving the therapeutic efficacy of β -agonists for obstructive lung diseases. However, because β -agonist therapy is used to prevent or reverse the consequences of ASM G_q -coupled receptor activation, which might also be affected by GRK inhibition, the ultimate utility of this approach is unclear.

In this study, we examine the effect of diverse approaches for manipulating GRK activity to define GRK selectivity for multiple G_q -coupled receptors in ASM cells. In addition, we examine the effect of cellular GRK inhibition or knockdown on signaling events influenced by stimulation of ASM cells with various G_q -coupled-receptor agonists combined with β_2 AR activation. Our results suggest that GRKs have limited capacity to regulate most G_q -coupled receptors in ASM, and exploiting functional domains of GRK2/3 may have greater therapeutic potential than targeting GRK2/3 activity *per se*. By taking advantage of the diverse functionality of GRK2/3 conferred by their C- and N-terminal domains, ASM can be rendered hypo-

responsive to procontractile agents while β_2 AR signaling and function is enhanced.

MATERIALS AND METHODS

Cell culture

Human ASM cultures were established as described previously (7) from human tracheae obtained from lung transplant donors, under procedures approved by the Wake Forest Baptist Medical Center and University of Maryland Medical Center institutional review boards. Characterization of these cells with regard to immunofluorescence of smooth muscle actin and agonist-induced changes in cytosolic calcium has been reported previously (8). 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/cm² in 24-well plates and maintained in Ham's F-12 medium supplemented with 10% FBS. Cell growth was arrested 48 h prior to stimulation by washing once in PBS and refeeding with Ham's F-12 medium supplemented with 5 μ g/ml each of insulin and transferrin, as described previously (9). For select experiments, ASM lines with stable expression of human telomerase reverse transcriptase (hTERT; a generous gift from Bill Gerthoffer, University of South Alabama, Mobile, AL, USA) were employed and maintained as described previously (10).

Retroviral infection and siRNA transfection

Stable expression of GFP, GRK2CT-GFP, GRK2NT-GFP, and GRK2K220R was achieved by retroviral infection, as described previously (6). Briefly, retrovirus for the expression of each was produced by cotransfecting GP2-293 cells with pVSV-G [encoding the pantropic (VSV-G) envelope protein] and either pLNCX2-GFP, pLNCX2-GRK2CT-GFP, pLNCX2-GRK2NT-GFP, or pLNCX2-GRK2K220R-GFP. At 48 h 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 μ g/ml G418 as described previously (6).

siRNA-mediated knockdown of GRK2/3 in ASM cells was achieved by two separate methods using siRNA duplexes (Dharmacon, Lafayette, CO, USA) targeting GRK2/3 (5'-GAUCUUCGACUCAUACAUCdTdT-3') or the scrambled (control) sequence (5'-GCGCGCUUUGUAGGAUUCGdTdT-3'). One method involved mixing of oligos with Amaxa transfection buffer (Lonza AG, Basel, Switzerland) and 5×10^6 harvested ASM cells and electroporating cells as previously reported (6). A second method involved transfection of oligos using Dharmafect (Dharmacon) as per the manufacturer's instruction. Because each method enabled comparable knockdown and elicited comparable results, as assessed by immunoblot analysis and quantification using the Odyssey system (LI-COR Biosciences, Lincoln, NE, USA), data generated using each approach were pooled and reported for each siRNA construct transfection. At 24 h post-transfection, cells were harvested from 10-cm dishes, then passaged onto 96-well plates or coverslips (Ca²⁺ mobilization), 24-well plates [phosphoinositide (PI) hydrolysis or cAMP accumulation], or 6-well plates (immunoblot analysis of GRK2/3 knockdown as per ref. 6) for subsequent assays performed 72 h later, corresponding to the period of peak GRK2/3 knockdown.

cAMP accumulation

Cells expressing GFP chimeric constructs as described above, or cells transfected with scrambled/GRK2/3 siRNA, were plated into 24-well plates and analyzed for agonist-induced cAMP accumulation as described previously (9). Briefly, cells were growth arrested, washed, and stimulated with PBS containing 300 μ M ascorbic acid, 1 mM RO-20-1724, and either vehicle, isoproterenol (ISO; 1 nM–1 μ M), 1 μ M prostaglandin E₂ (PGE₂), or 100 μ M forskolin (FSK), for 10 min at 37°C. Reactions were quenched by buffer aspiration and addition of 400 μ l 100% cold ethanol. Isolated cAMP was subsequently quantified by radioimmunoassay (9).

Measurement of PI generation

Human ASM cells were grown to confluence on 24-well plates and loaded with 2 μ Ci/ml myo-[³H]inositol for 18 h. After washing with PBS, the cells were incubated with DMEM containing 5 mM LiCl for 15 min, then stimulated with vehicle or the indicated agonist for 30 min. In select experiments, cells were also pretreated with vehicle or 1 μ M ISO for 5 min prior to addition of vehicle or indicated agonist. Excepting those experiments examining the concentration-dependent effect of agonist, maximal concentrations of agonists employed were either >10-fold the EC₅₀ value known for the target receptor, or those previously shown by our laboratory to maximally stimulate the target receptor (11–15). Reactions were quenched with 20 mM formic acid and inositol, and PI fractions were separated by anion exchange chromatography as described previously (15). PI production was calculated by dividing PI fraction by total (PI+inositol) inositol fraction, and the data were reported as fold basal (agonist-stimulated/vehicle).

Intracellular calcium ([Ca²⁺]_i) measurements

Cells were plated onto collagen-coated 96-well plates, grown to confluence, and loaded with 2 μ M Fluo-4 AM (BD Biosciences, San Jose, CA, USA) and probenecid for 1 h. Indicated agonists were added by an automated pipetting system in triplicate, and the 525-nm signals were generated by excitation at 485 nm with a Flex Station II (Molecular Devices, Sunnyvale, CA, USA) as previously reported (11). In select experiments, cells were pretreated with vehicle or 1 μ M isoproterenol 5 min prior to stimulation with agonists. The net peak Ca²⁺ response was calculated as (maximum agonist-induced fluorescence units) – (basal fluorescence units).

Ex vivo ASM tension development

Because gene ablation of GRK2 is embryonically lethal (16), studies employed heterozygote C57BL/6 mice expressing GRK2CT under the smooth muscle-specific SM22 α promoter (GRK2CT-TG; littermates not expressing the transgene [non-transgenic (NTG)] served as controls) as previously reported (17) as a potential means of GRK2/3 inhibition *in vivo*. Mice were euthanized at 12 wk of age, and their tracheal segments were excised and either digested for immunoblot analysis of GRK2CT expression or shipped overnight for subsequent analysis of ISO-mediated relaxation of methacholine (MCh)-induced contraction the next day. Tracheal ring preparations were mounted into a multiwire myograph chamber in Krebs-Henseleit solution (pH 7.40) maintained at 37°C with 5% CO₂ and 95% O₂ with frequent changing of the solution. The chambers were mounted onto the Myo-Interface (model 610 M) and connected to the transducer and PowerLab (AD Instruments, Sydney, NSW, Australia) for data transferring and record-

ing. Chart5 software for Windows (AD Instruments) was used to collect and analyze the data. A basal tension of 0.5 g was set, and the tracheal rings were stimulated with 10 μ M MCh for 5 min. The rings were washed, preload was reset to 0.5 g for 30 min, and then rings were stimulated with 60 mM KCl. After stabilization, the tracheal rings were again washed, preload was reset to 0.5 g, and then rings were stimulated with 10 μ M MCh and allowed to contract for 10 min. Stimulation of the rings with 10 μ M MCh produces ~80% maximum tension in the rings, as determined previously (18). Subsequently, the precontracted rings were stimulated with increasing concentrations of ISO (10, 100, and 1000 nM) for 5 min, tension was recorded, and maximal inhibition was calculated as previously reported (19). Studies involving the use of mouse tissue were approved by the Animal Care and Use Committee of Wake Forest Baptist Medical Center.

Data analysis

Data are presented as mean $\pm$ SE values. Statistically significant differences among groups were assessed by analysis of variance, and Tukey *post hoc* tests for specific pairwise differences, or *t* test where appropriate, with values of *P* < 0.05 sufficient to reject the null hypothesis.

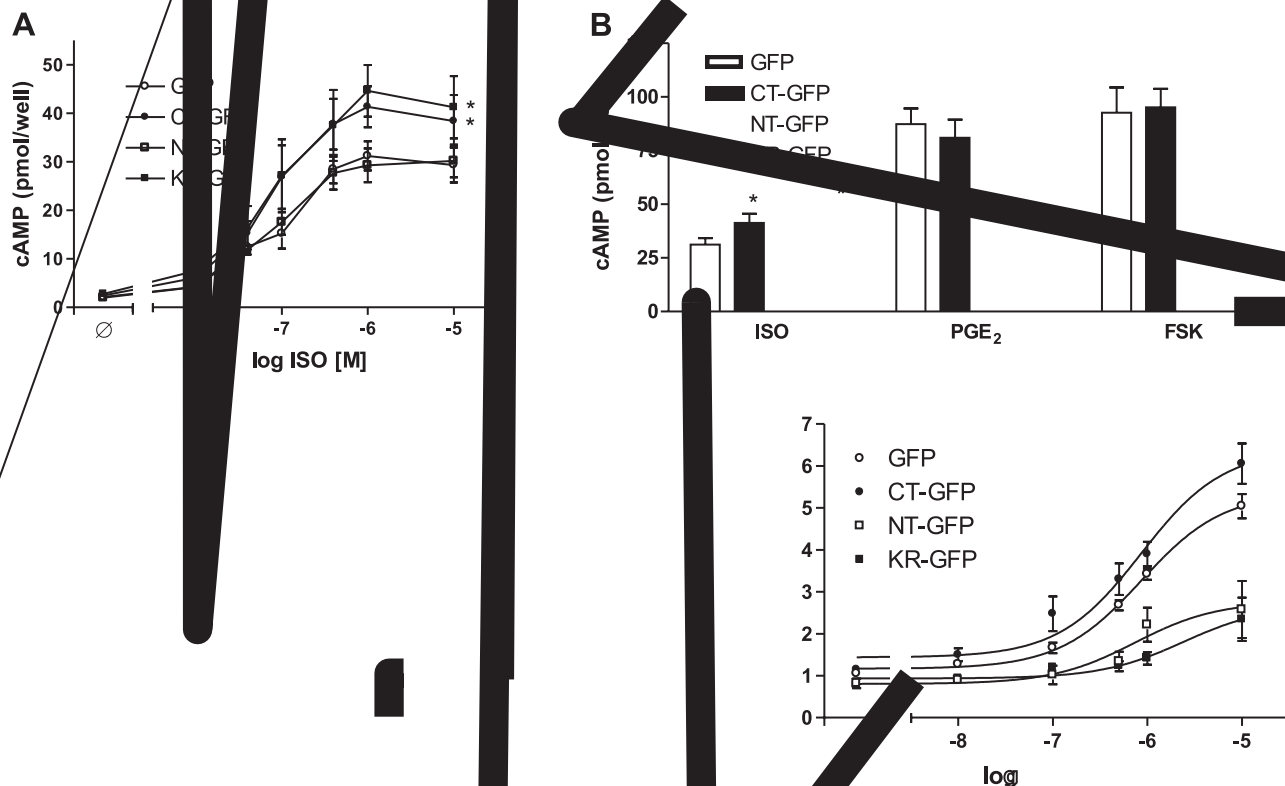
RESULTS

Regulatory effect of expressed GRK2/3 functional domains

To examine the capacity of GRK2/3 to regulate GPCRs in ASM, cells were infected with retrovirus enabling expression of GFP chimeras of GRK2/3 regulatory constructs GRK2CT, GRK2K220R, and GRK2NT and selected with 250 μ g/ml G418 to establish stable expression as per Kong *et al.* (6). Consistent with our previous studies, expression of GRK2CT-GFP or GRK2K220R-GFP, both previously shown to inhibit GRK2/3 activity *via* sequestration of G β γ (20), increased cAMP accumulation in human ASM cells stimulated with maximal concentrations of ISO (activating the G_s-coupled β_2 AR in ASM) but not PGE₂ or FSK (Fig. 1A, B). Conversely, expression of GRK2NT-GFP had no effect on maximal cAMP accumulation stimulated by ISO. No significant effect of any of the constructs was observed on calculated EC₅₀ values.

With respect to procontractile GPCRs known to be expressed in human ASM, expression of GRK2CT-GFP caused a modest (24 $\pm$ 11%) but statistically significant increase in PI hydrolysis stimulated by 10 μ M histamine [activating the H1 histamine receptor (H1 HR)], while significantly inhibiting (49 $\pm$ 11%) PI hydrolysis stimulated by 1 μ M sulprostone (EP3 receptor) (Fig. 1C). No effect of GRK2CT-GFP expression was observed on PI hydrolysis stimulated by maximal (*i.e.*, concentrations >10 $\times$ EC₅₀ values reported for their cognate receptors) concentrations of thrombin–protease-activated receptor-1 (PAR-1), U46619–thromboxane receptor (TP), MCh-m3mAChR, or LTD4-CysLT1R.

The most profound effect was observed in cells expressing GRK2NT-GFP or GRK2K220R-GFP (which retains the functional N terminus of GRK2), as maximal PI hydrolysis stimulated by each of the agonists,



excepting sulprostone, was significantly decreased relative to that observed in GFP-expressing control cells. Analysis of construct effect on the dose-dependent effect of histamine demonstrated the augmenting effect of GRKCT-GFP, as well as the inhibitory effect of GRKNT-GFP and GRKK220R throughout the range of histamine concentrations tested (Fig. 1D). No significant effect of construct expression was observed on calculated EC_{50} values.

Regulatory effect of siRNA-mediated GRK2/3 knockdown

As a second means of regulating GRK activity, human ASM cells were transiently transfected with siRNA enabling knockdown of GRK2/3 protein by >80% (Fig. 2A), as demonstrated in our previous study (6). Consistent with our previous findings, GRK2/3 knockdown was associated with an increase in cAMP accumulation in ASM cells stimulated by saturating concentrations of ISO. The dose-dependent response (Fig. 2B) suggests that the augmenting effect occurs at all ISO concentrations ≥ 10 nM; the calculated EC_{50} values were not significantly different. Similar to the effects of expression of GRK2CT-GFP, GRK2/3 knockdown significantly

increased PI hydrolysis stimulated by histamine but had no effect on PI hydrolysis stimulated by any of the other GPCR-mediated agonists (Fig. 2C).

Effects of GRK2/3 functional domains and GRK2/3 knockdown on GPCR-mediated Ca²⁺ mobilization

To further examine regulation of ASM procontractile GPCRs, effects of both GRK2/3-regulatory constructs and GRK2/3 knockdown on agonist-induced Ca²⁺ mobilization were examined. Interestingly, expression of GRK2CT-GFP had no effect on Ca²⁺ mobilization stimulated by any of the agonists except sulprostone, where it was significantly lower ($26 \pm 8\%$, $n=7$) than that measured in control cells (Fig. 3A). However, expression of GRK2NT-GFP or GRK2K220R-GFP significantly reduced Ca²⁺ mobilization stimulated by each of the agonists, the one exception being U46619, which exhibited a weak and variable ability to mobilize Ca²⁺. siRNA-mediated GRK2/3 knockdown had no effect on Ca²⁺ mobilization stimulated by any agonist (Fig. 3B). GRK2/3 expression and responsiveness in ASM cells is known to rapidly wane in ASM cells with culture passage. To exclude potential confounding effects of

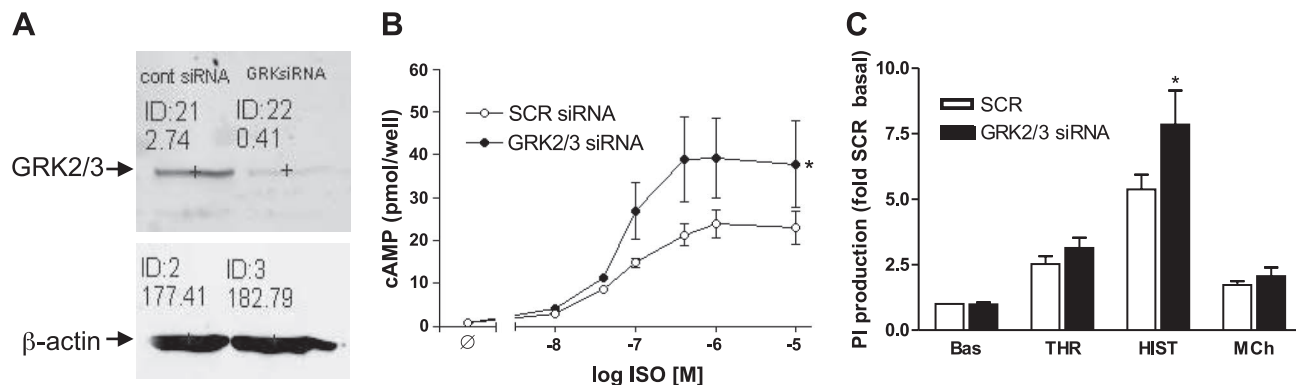


Figure 2. Regulation of agonist-stimulated cAMP accumulation and PI production in ASM by GRK2/3 knockdown. *A*) Human ASM cells were transfected with either a scrambled siRNA or siRNA targeting GRK2/3 as previously reported (6). Representative immunoblot demonstrating GRK2/3 knockdown; numbers associated with bands represent quantification of emission signal from secondary antibody by the Odyssey system (44). *B*) Effect of GRK2/3 knockdown on ISO dose-dependent effect on intracellular cAMP accumulation; $n = 4$. *C*) Effect of GRK2/3 knockdown on agonist-stimulated PI hydrolysis; $n = 5$. Data depict mean $\pm$ SE values. * $P < 0.05$ vs. SCR siRNA group.

this phenomenon, we analyzed m3mAChR responsiveness in hTERT-expressing human ASM cell lines, which maintain m3mAChR expression and signaling. In hTERT lines subject to siRNA-mediated knockdown of GRK2/3 ($93 \pm 1\%$, $n=4$), MCh-induced $[Ca^{2+}]_i$ flux was not affected by GRK2/3 knockdown (Fig. 3C). Similarly, GRK2/3 knockdown did not affect MCh-induced PI hydrolysis in hTERT ASM cells (data not shown).

Effects of targeting GRK2/3 under conditions of combined G_q -coupled-receptor and β_2 AR activation in ASM

We previously demonstrated that either expression of GRK2CT-GFP or GRK2/3 knockdown augments β_2 AR- but not EP2R- mediated signaling in ASM cells (6). To determine whether GRK2/3 inhibition translates into improved β_2 AR-mediated functional antagonism

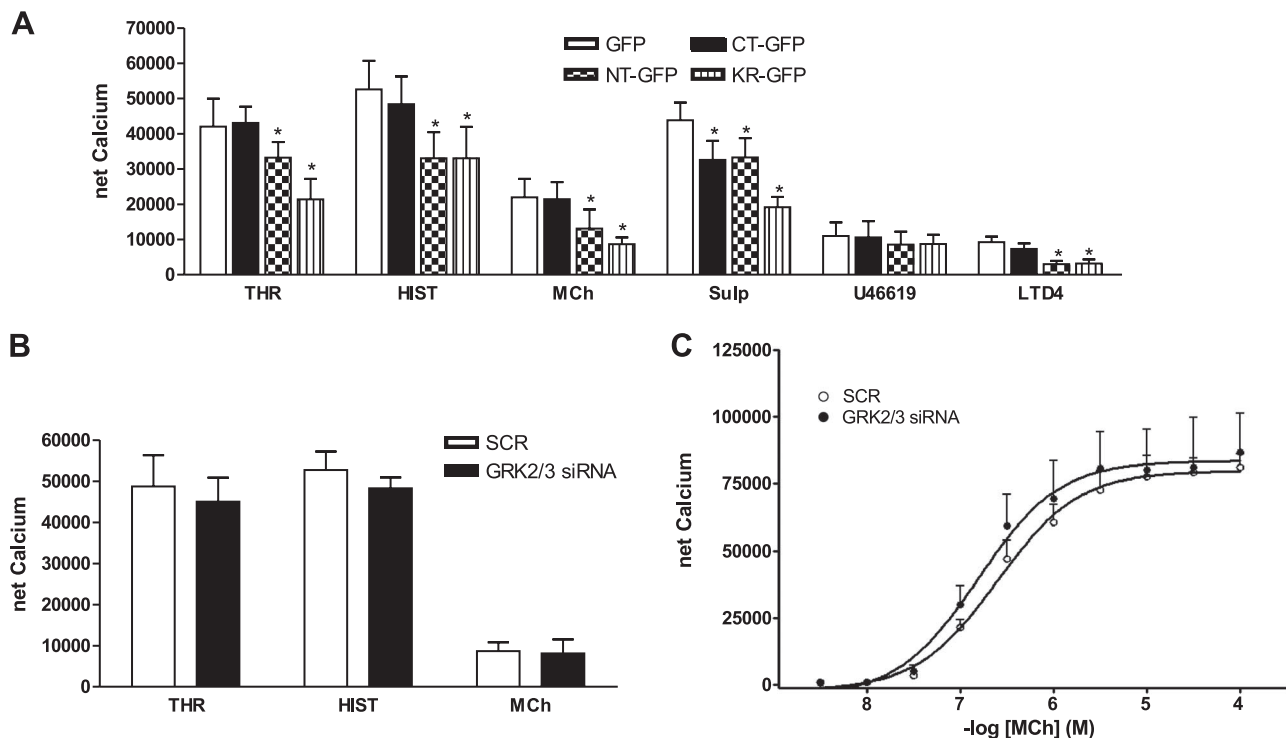


Figure 3. Regulatory effect of GRK domains and GRK2/3 knockdown on agonist-stimulated Ca^{2+} mobilization in ASM. *A*) ASM cultures expressing GFP, GRK2CT-GFP (CT-GFP), GRK2NT-GFP (NT-GFP), or GRK2K220R-GFP (KR-GFP) plated in 96-well plates were loaded with Fluo-4 AM, and Ca^{2+} mobilization in response to the indicated agonists was assessed as previously reported (11); $n = 5-7$ paired observations. * $P < 0.05$ vs. GFP (control). *B*) Same analysis of agonist-induced Ca^{2+} mobilization using cells transfected with SCR or GRK2/3 siRNA [96 h post-transfection; $n=6$ thrombin (THR) and MCh stimulation, $n=8$ histamine (HIST)]. *C*) Ca^{2+} mobilization in response to increasing concentrations of MCh in hTERT-immortalized human ASM cells transfected with SCR or GRK2/3 siRNA (96 h post-transfection; $n=4$).

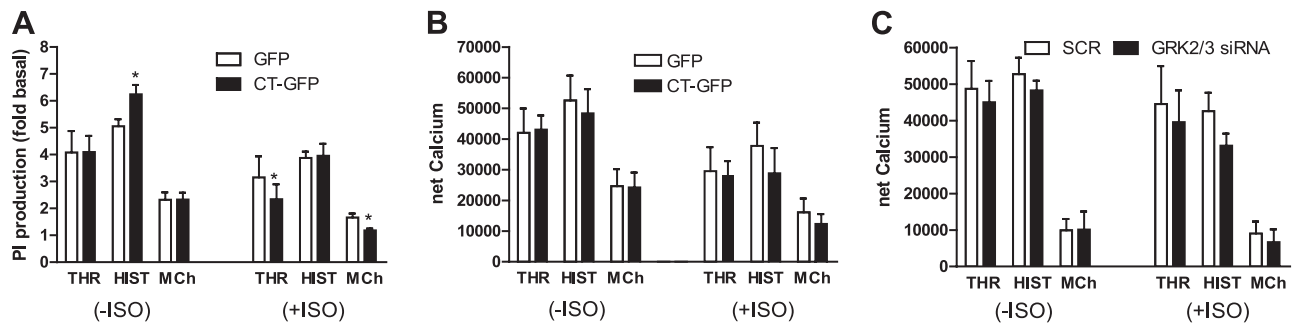


Figure 4. Effect of GRK2CT and GRK2/3 knockdown on β -agonist regulation of contractile agonist signaling in ASM. *A*, *B*) PI hydrolysis (*A*) or net calcium mobilization (*B*) stimulated by thrombin (THR), histamine (HIST), or MCh under condition of 5 min pretreatment with either vehicle (–ISO) or 1 μ M ISO (+ISO) in ASM cultures expressing GFP or GRK2CT-GFP (CT-GFP); $n = 7$ for all groups (*A*); $n = 4$ (THR), 5 (MCh), and 6 (HIST) (*B*). * $P < 0.05$ vs. GFP. *C*) Similar design as *B*, comparing cells transfected with control or GRK2/3 siRNA; $n = 4$ (THR), 5 (MCh), and 6 (HIST).

of ASM procontractile GPCRs, ASM cultures stably expressing GRK2CT-GFP were examined for PI hydrolysis and Ca^{2+} mobilization under conditions of combined G_q -coupled-receptor agonist (thrombin, histamine, or MCh) and $\beta_2\text{AR}$ (ISO) stimulation. GRK2CT-GFP expression resulted in a significantly greater percentage inhibition of thrombin- and MCh-stimulated PI hydrolysis (**Fig. 4A**). PI production under conditions of concomitant histamine and ISO treatment was similar in cultures expressing GFP and GRK2CT-GFP, although histamine-stimulated PI generation was significantly greater in GRK2CT-GFP-expressing cells. With respect to regulation of ISO inhibition of stimulated calcium mobilization, analysis of net peak calcium mobilization failed to reveal a statistically significant regulatory effect of either GRK2CT-GFP expression (**Fig. 4B**) or GRK2/3 siRNA-mediated knockdown (**Fig. 4C**). ISO inhibition of thrombin-, histamine-, or MCh-stimulated PI hydrolysis was also not significantly affected by GRK2/3 siRNA-mediated knockdown (not shown).

Functional effect of GRK2CT expression in ASM

To examine the potential role of GRK2/3 inhibition on regulation of ASM contraction, tracheae were isolated from transgenic mice expressing GRK2CT under the control of the smooth muscle-specific SM22 α promoter (17). As shown in **Fig. 5A**, lysates of ASM tissue from heterozygote GRK2CT-TG mice exhibit GRK2CT expression whereas those generated from littermate controls (NTG) do not. Tracheal segments were suspended in a myograph for analysis of regulation of MCh-induced tension development. Although KCl-mediated tension development did not differ between groups (**Fig. 5B**), that elicited by 10 μ M MCh was modestly but significantly lower (169 ± 11 vs. $209 \pm 19\%$ for NTG, $P < 0.05$, $n = 6$, % KCl-stimulated values; **Fig. 5C**) in segments from GRK2CT-TG mice. Progressive addition of ISO (10 nM–1 μ M) to MCh-contracted tracheal segments reduced tension development, and this reduction was significantly greater at each ISO dose in segments from GRK2CT-TG mice (**Fig. 5D**).

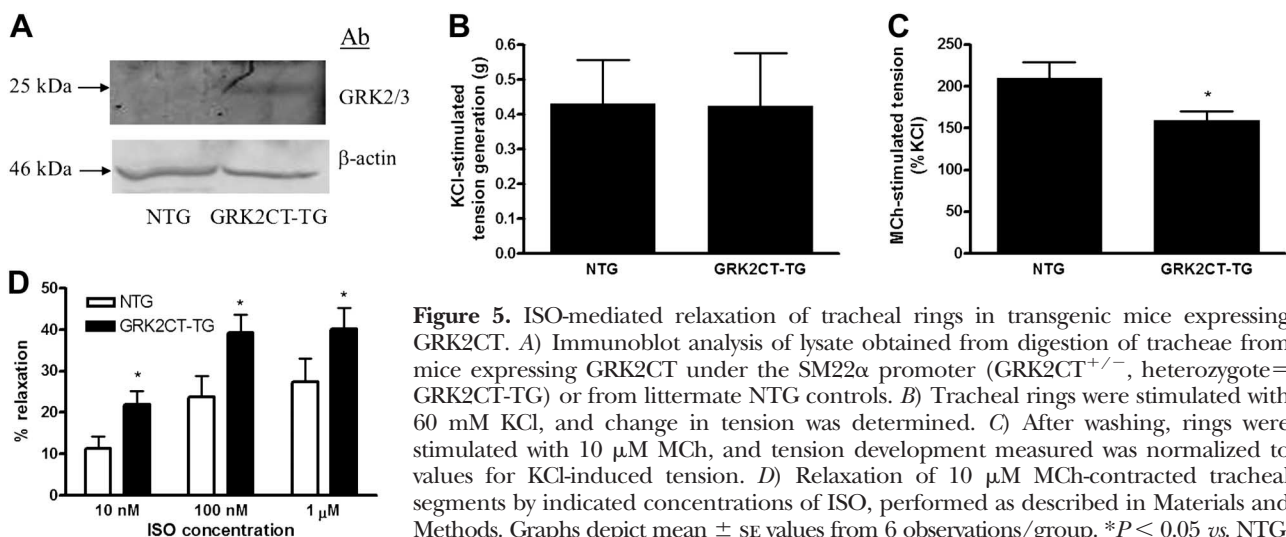


Figure 5. ISO-mediated relaxation of tracheal rings in transgenic mice expressing GRK2CT. *A*) Immunoblot analysis of lysate obtained from digestion of tracheae from mice expressing GRK2CT under the SM22 α promoter (GRK2CT $^{+/-}$, heterozygote = GRK2CT-TG) or from littermate NTG controls. *B*) Tracheal rings were stimulated with 60 mM KCl, and change in tension was determined. *C*) After washing, rings were stimulated with 10 μ M MCh, and tension development measured was normalized to values for KCl-induced tension. *D*) Relaxation of 10 μ M MCh-contracted tracheal segments by indicated concentrations of ISO, performed as described in Materials and Methods. Graphs depict mean $\pm$ SE values from 6 observations/group. * $P < 0.05$ vs. NTG.

DISCUSSION

Signaling through GPCRs mediates numerous ASM functions, including contraction, growth, and synthetic functions that orchestrate airway inflammation and promote remodeling of the airway (1). The significance of GPCRs in regulating ASM function, and consequently airway function, is evidenced by the fact that almost every drug used in the treatment of obstructive airway diseases directly or indirectly targets either GPCRs, GPCR signaling, or processes that produce GPCR agonists.

GPCR ligands are useful asthma and chronic obstructive pulmonary disease (COPD) therapies but have limitations. One widely recognized limitation is the loss of β_2 AR responsiveness that occurs with chronic administration of inhaled β -agonist. This loss of responsiveness is reflected in a loss of bronchoprotective effect (21–24), deterioration of asthma control, and susceptibility to exacerbations (25). It is presumed that ASM β_2 AR desensitization is mechanistically linked to these problems, but to date only association and not causality has been demonstrated. In murine models of heart failure, elevated levels of circulating catecholamines induce increased expression of GRK2 and promote β_2 AR desensitization; inhibition of GRK-mediated β_2 AR desensitization by transgenic expression of the C terminus of GRK2 (GRK2CT) reverses β AR desensitization, improves β -agonist-stimulated cardiac function, and reverses disease phenotype (reviewed in ref. 5). Interestingly, Finney *et al.* (26) recently observed that lung GRK2 levels are elevated in rats treated chronically with β -agonist, suggesting that the “catecholamine hypothesis” (5, 27–29) may also apply to ASM.

The current work extends our previous finding regarding the effectiveness of GRK inhibition to enhance β_2 AR signaling in ASM cells and reveals the ability of different GRK2 domains to regulate the complement of GPCRs in ASM in a therapeutically advantageous manner.

Early studies from our group established that overexpressed GRK2 (originally termed β ARK for β -adrenergic receptor kinase) could effect greater desensitization of the β_2 AR in human ASM cells (9), and that desensitization of the β_2 AR and A2b adenosine receptor, but not the EP2 receptor, could be enhanced by overexpression of arrestin-2 or arrestin-3 (30). This latter study was the first to provide clues as to the specificity of arrestins for G_q -coupled receptors and identified the mechanism underlying the relative desensitization-resistant nature of the EP2 receptor. More recently, we determined that inhibition of GRK2/3, *via* (as in the current study) either expression of GRK2CT or GRK2K220R, or siRNA-mediated knockdown of GRK2/3, augmented signaling of the β_2 AR but not that of the EP2R or A2b adenosine receptor in human ASM (6).

However, given the potential role of GRKs to regulate responsiveness of G_q -coupled receptors in ASM, whether augmentation of β_2 AR signaling by targeting

GRK-dependent desensitization mechanisms would serve to improve the ability of β -agonists to inhibit procontractile signaling in ASM remained unclear. Indeed, previous studies employing recombinant receptors in cellular expression systems or cell-free assays implicate a role for GRKs in regulating numerous G_q -coupled receptors. Expression of GRK3, and to a lesser extent GRK2, augmented thrombin-stimulated phosphorylation of wild-type PAR-1 expressed in Rat 1 cells, while quenching thrombin-stimulated Ca^{2+} mobilization (31). When expressed in HEK293 cells, the β isoform of the TP internalizes in a GRK-dependent manner (32), and the H1 HR undergoes agonist-induced phosphorylation and desensitization dependent on GRK2/3 (33). The m3mAChR has been shown to be a substrate for GRK2 (34), although other kinases also appear capable of phosphorylating and regulating the m3mAChR, in a cell-specific manner (35, 36). Interestingly, Luo *et al.* (37) recently demonstrated that Ca^{2+} mobilization by endogenous m3mAChR in HEK293 cells is increased by knockdown of GRK2, arrestins, or casein-kinase-1 α .

To examine the selectivity of GRK2/3 for G_q -coupled receptors in human ASM, we employed two strategies: expression of recombinant proteins (GRK2CT and, GRK2K220R) capable of inhibiting GRK activity, and GRK2/3 knockdown using siRNA. GRK2CT represents the C-terminal domain of GRK2, which contains a pleckstrin-homology domain capable of sequestering $G_{\beta\gamma}$; $G_{\beta\gamma}$ has been previously shown to be important for GRK2 activation (38). Kinase-dead GRK2 (GRK2K220R) lacks phospho-transfer ability and also contains the C terminus of GRK2 and was the first construct used to effectively inhibit GRK activity and demonstrate the role of GRK2 in homologous desensitization of the β_2 AR in intact cells (20). Both ectopic expression and siRNA approaches increased histamine-stimulated PI hydrolysis, but failed to affect PI hydrolysis by thrombin, the TXA2 analog U46619, LTD4, or MCh. These findings suggest that among these G_q -coupled receptors in ASM cells, only the H1HR appears regulated significantly by GRK2/3.

However, consistent with the ability of the N-terminal domain of GRK2/3 to interdict G_q -PLC coupling (39), expression of either GRK2NT or GRK2K220R significantly decreased both PI hydrolysis and Ca^{2+} mobilization stimulated by each G_q -coupled-receptor agonist.

Human ASM expresses PAR-1 and PAR-2 (40), receptors that can couple to both G_q and G_i . Our previous studies of mitogenic signaling (11, 41) suggest that thrombin-mediated signaling in ASM cells is mediated primarily by G_q but also has a G_i component. Given that G_i utilizes $G_{\beta\gamma}$ to activate PLC, the effects of both GRK2CT or GRK2K220R may be complex. Whereas $G_{\beta\gamma}$ sequestration may mitigate GRK-mediated desensitization of PAR-1 or PAR-2, it may also inhibit $G_{\beta\gamma}$ -dependent PLC activation by G_i , thus offsetting any relief of PAR desensitization. However, the lack of effect of GRK2/3 knockdown suggests that PARs in ASM are largely insensitive to GRK2/3, and the major-

ity of PI and Ca^{2+} mobilization mediated by thrombin is dependent on G_q .

Similarly, human ASM expresses mAChR subtypes m3, which couples to G_q , and m2, which couples to G_i . Consistent with our previous studies (11, 41) and those examining the role of mAChR subtypes in ASM contraction (42), MCh-stimulated PLC activation resulting in PI hydrolysis and Ca^{2+} mobilization in ASM appears mediated primarily by the m3mAChR and is significantly inhibited by constructs containing the GRK2 N-terminal domain.

One interesting observation was the capacity of GRK2CT-GFP to significantly reduce PI hydrolysis stimulated by sulprostone in ASM cells. Sulprostone is an agonist for the EP3 subtype of EP family GPCRs, and we have previously noted the capacity of sulprostone to stimulate p42/p44 mitogen-activated protein kinase in human ASM cells in a pertussis toxin-sensitive manner (18). Presumably, the ability of GRK2CT to inhibit sulprostone-mediated PI hydrolysis reflects inhibition of EP3 receptor-, $\text{G}_{\beta\gamma}$ -dependent PLC activation. Thus, the EP3 receptor is distinct from most GPCRs capable of promoting PLC signaling in ASM, which appear to do so *via* G_q in a $\text{G}_{\beta\gamma}$ -independent manner.

Interestingly, we failed to observe an effect of siRNA-mediated GRK2/3 knockdown on calcium mobilization by any of the agonists tested, including histamine. Potential confounding effects of loss of m3mAChR signaling in cultured primary ASM cells were excluded by using hTERT-immortalized human ASM cells that retain m3mAChR responsiveness. Similarly, we could not observe a regulatory effect of GRK2/3 knockdown on the ability of ISO to inhibit (G_q -coupled-receptor-stimulated) calcium mobilization or PI hydrolysis. Several factors likely contribute to this lack of effect. One possible contributing factor is the data variability within and between groups in these experiments. Perhaps more important, however, is the relatively short time frame in which peak calcium mobilization is achieved (typically <20 s), which may limit the capacity of GRKs to affect this signal against the backdrop of other established regulatory mechanisms affecting calcium release and sequestration by the sarcoplasmic reticulum. Of note, GRK2/3 knockdown was effective in regulating histamine-stimulated PI hydrolysis, which reflects 30 min of H1 histamine receptor activation, and GRK2CT-GFP expression also augmented histamine-stimulated PI hydrolysis but also has no effect on calcium mobilization.

Within the context of concomitant stimulation of ASM with both G_q -coupled-receptor agonists and β -agonist, ISO was able to inhibit both thrombin- and MCh-stimulated PI hydrolysis to a greater extent in GRK2CT-GFP-expressing relative to control (GFP-expressing) cells. Whereas a greater percentage inhibition of histamine-stimulated PI hydrolysis was also observed for GRK2CT-GFP cells, the absolute PI hydrolysis was similar between groups under conditions of combined histamine and ISO stimulation, due to the

augmenting effect of GRK2CT-GFP under conditions of histamine treatment alone.

Consistent with the ability of GRK2CT expression to augment $\beta_2\text{AR}$ but not m3mAChR signaling, we observed a greater β -agonist-mediated relaxation of MCh-contracted tracheal rings isolated from transgenic mice expressing GRK2CT (Fig. 5). What specific pharmacomechanical coupling processes are affected, as a consequence of augmented $\beta_2\text{AR}$ signaling, are unclear. Our inability to detect an effect of GRK inhibition on β -agonist inhibition of whole-cell calcium mobilization suggests that inhibition of calcium mobilization may not be a critical mechanism by which cAMP [and effector protein kinase A (PKA)/exchange protein directly activated by cAMP (Epac)] activity stimulated by $\beta_2\text{AR}$ activation antagonizes ASM contraction. Indeed, the capacity of ISO to inhibit peak intracellular calcium mobilization by various agonists in our Flex Station analyses is only ~15%; it is possible that the regulatory effect of cAMP/PKA/Epac on ASM contraction is more dependent on regulation of compartmentalized calcium (affecting calmodulin activation or ryanodine receptor regulation), myosin light chain kinase and phosphatase, and HSP20 (43).

Although GRK2/3 knockdown was able to increase $\beta_2\text{AR}$ - but not MCh-mediated signaling in ASM, we were unable to demonstrate an augmentation of $\beta_2\text{AR}$ -mediated inhibition of MCh-mediated signaling. Thus, although the ability of GRK2CT expression to increase ISO-mediated inhibition of MCh-mediated signaling and contraction suggests a functional consequence of GRK2/3 inhibition, definitive proof of such is lacking. Future studies, perhaps employing inducible ablation of the GRK2 gene in ASM to escape the embryonic lethality of the global GRK2 knockout, will be required to clarify this issue.

From a therapeutic standpoint, findings from the present study suggest that peptides mimicking the N-terminal domain of GRK2/3 could serve as an effective global inhibitor of those G_q -coupled GPCRs that mediate ASM contraction. Although several small-molecule antagonists for different G_q -coupled receptors are currently marketed for the treatment of asthma and COPD, the effectiveness of these drugs tends to be limited, in part due to the fact that airway inflammation associated with these diseases promotes elevated levels of numerous procontractile agonists in the airway. Thus, inhibition of just one receptor is often insufficient to antagonize bronchoconstriction, except for those patients in which bronchoconstriction is dominated by one particular agonist/receptor.

In addition, peptides that mimic both N- and C-terminal domains of GRK2 would have added therapeutic value. C-terminal domain peptides could preferentially relieve desensitization of the $\beta_2\text{AR}$, rendering either endogenous or exogenous β -agonists more efficacious in reversing or preventing bronchoconstriction. Moreover, any $\text{G}_{\beta\gamma}$ -dependent procontractile signaling, mediated possibly by G_i activation, would also be antagonized by such a construct. Future studies clarifying the

utility of these approaches *in vivo* could help usher in a new approach to the management of obstructive airway disease by nonspecific targeting of G_q signaling and mitigation of β_2 AR desensitization. **[F]**

This work was funded by U.S. National Institutes of Health grants HL58506 (R.B.P.) and AG041265 and HL087560 (D.A.D.). S.J.M. is supported by U.S. National Institute of Arthritis and Musculoskeletal and Skin Disease grant T32 AR07592.

REFERENCES

- Deshpande, D. A., and Penn, R. B. (2006) Targeting G protein-coupled receptor signaling in asthma. *Cell. Signal.* **18**, 2105–2120
- Penn, R. B., and Benovic, J. L. (1998) Regulation of G protein-coupled receptors. In *Handbook of Physiology*, Vol. 1 (Conn, P. M., ed), pp 125–164, Oxford University Press, Oxford, UK
- Benovic, J. L., De Blasi, A., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989) β -Adrenergic receptor kinase: primary structure delineates a multigene family. *Science* **246**, 235–240
- Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the β_2 -adrenergic receptor. *Nature* **383**, 447–450
- Penn, R. B., Pronin, A. P., and Benovic, J. L. (2000) Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc. Med.* **10**, 81–89
- Kong, K. C., Gandhi, U., Martin, T. J., Anz, C. B., Yan, H., Misior, A. M., Pascual, R. M., Deshpande, D. A., and Penn, R. B. (2008) Endogenous Gs-coupled receptors in smooth muscle exhibit differential susceptibility to GRK2/3-mediated desensitization. *Biochemistry* **47**, 9279–9288
- Panettieri, R. A., Murray, R. K., DePalo, L. R., Yadavish, P. A., and Kotlikoff, M. I. (1989) A human smooth muscle cell line that retains physiological responsiveness. *Am. J. Physiol. Cell Physiol.* **256**, C329–C335
- Murray, R. K., Fleischmann, B. K., and Kotlikoff, M. I. (1993) Receptor-activated Ca²⁺ influx in human airway smooth muscle: use of Ca²⁺ imaging and perforated patch-clamp techniques. *Am. J. Physiol. Cell Physiol.* **264**, 485–490
- Penn, R. B., Panettieri, R. A., Jr., and Benovic, J. L. (1998) Mechanisms of acute desensitization of the β_2 AR-adenyl cyclase pathway in human airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.* **19**, 338–348
- Gosens, R., Stelmack, G. L., Dueck, G., McNeill, K. D., Yamasaki, A., Gerthoffer, W. T., Unruh, H., Gounni, A. S., Zaagsma, J., and Halayko, A. J. (2006) Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **291**, L523–L534
- Deshpande, D. A., Wang, W. C., McIlmoyle, E. L., Robinett, K. S., Schillinger, R. M., An, S. S., Sham, J. S., and Liggett, S. B. (2010) Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nat. Med.* **16**, 1299–1304
- Billington, C. K., Kong, K. C., Bhattacharyya, R., Wedegaertner, P. B., Panettieri, R. A., Chan, T. O., and Penn, R. B. (2005) Cooperative regulation of p70S6 kinase by receptor tyrosine kinases and G protein-coupled receptors augments airway smooth muscle growth. *Biochemistry* **44**, 14595–14605
- Deshpande, D. A., Pascual, R. M., Wang, S. W., Eckman, D. M., Riemer, E. C., Funk, C. D., and Penn, R. B. (2007) PKC-dependent regulation of the receptor locus dominates functional consequences of cysteinyl leukotriene type 1 receptor activation. *FASEB J.* **21**, 2335–2342
- Krymskaya, V. P., Penn, R. B., Orsini, M. J., Scott, P. H., Plevin, R. J., Walker, T. R., Eszterhas, A. J., Amrani, Y., Chilvers, E. R., and Panettieri, R. A., Jr. (1999) Phosphatidylinositol 3-kinase mediates mitogen-induced human airway smooth muscle cell proliferation. *Am. J. Physiol.* **277**, 65–78
- Naik, S., Billington, C. K., Pascual, R. M., Deshpande, D. A., Stefano, F. P., Kohout, T. A., Eckman, D. M., Benovic, J. L., and Penn, R. B. (2005) Regulation of cysteinyl leukotriene type 1 receptor internalization and signaling. *J. Biol. Chem.* **280**, 8722–8732
- Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. A., Sulik, K. K., Ross, J., Jr., Lefkowitz, R. J., Caron, M. G., and Giros, B. (1996) Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 12974–12979
- Cohn, H. I., Harris, D. M., Pesant, S., Pfeiffer, M., Zhou, R. H., Koch, W. J., Dorn, G. W., and Eckhart, A. D. (2008) Inhibition of vascular smooth muscle G protein-coupled receptor kinase 2 enhances α_1 D-adrenergic receptor constriction. *Am. J. Physiol. Heart Circ. Physiol.* **295**, H1695–H1704
- Guo, M., Pascual, R. M., Wang, S., Fontana, M. F., Valancius, C. A., Panettieri, R. A., Tilley, S. L., and Penn, R. B. (2005) Cytokines regulate beta-2-adrenergic receptor responsiveness in airway smooth muscle via multiple PKA- and EP2 receptor-dependent mechanisms. *Biochemistry* **44**, 13771–13782
- Tilley, S. L., Hartney, J. M., Erikson, C. J., Jania, C., Nguyen, M., Stock, J., McNeish, J., Valancius, C., Panettieri, R. A., Jr., Penn, R. B., and Koller, B. H. (2003) Receptors and pathways mediating the effects of prostaglandin E2 on airway tone. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**, 599–606
- Kong, G., Penn, R., and Benovic, J. L. (1994) A beta-adrenergic receptor kinase dominant negative mutant attenuates desensitization of the β_2 -adrenergic receptor. *J. Biol. Chem.* **269**, 13084–13087
- Abisheganaden, J., and Boushey, H. A. (1998) Long-acting inhaled beta 2-agonists and the loss of “bronchoprotective” efficacy [editorial; comment]. *Am. J. Med.* **104**, 494–497
- Bhagat, R., Kalra, S., Swystun, V. A., and Cockcroft, D. W. (1995) Rapid onset of tolerance to the bronchoprotective effect of salmeterol. *Chest* **108**, 1235–1239
- Cheung, D., Timmers, M. C., Zwinderman, A. H., Bel, E. H., Dijkman, J. H., and Sterk, P. J. (1992) Long-term effects of a long-acting beta 2-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *N. Engl. J. Med.* **327**, 1198–1203
- Peters, S. P., and Fish, J. E. (1999) Prior use of long-acting beta-agonists: friend or foe in the emergency department? *Am. J. Med.* **107**, 283–285
- Israel, E., Chinchilli, V. M., Ford, J. G., Boushey, H. A., Cherniack, R., Craig, T. J., Deykin, A., Fagan, J. K., Fahy, J. V., Fish, J., Kraft, M., Kunselman, S. J., Lazarus, S. C., Lemanske, R. F., Jr., Liggett, S. B., Martin, R. J., Mitra, N., Peters, S. P., Silverman, E., Sorkness, C. A., Szefer, S. J., Wechsler, M. E., Weiss, S. T., and Drazen, J. M. (2004) Use of regularly scheduled albuterol treatment in asthma: genotype-stratified, randomised, placebo-controlled cross-over trial. *Lancet* **364**, 1505–1512
- Finney, P. A., Belvisi, M. G., Donnelly, L. E., Chuang, T. T., Mak, J. C., Scorer, C., Barnes, P. J., Adcock, I. M., and Giembycz, M. A. (2000) Albuterol-induced downregulation of Gsalpha accounts for pulmonary beta(2)-adrenoceptor desensitization in vivo. *J. Clin. Invest.* **106**, 125–135
- Choi, D. J., Koch, W. J., Hunter, J. J., and Rockman, H. A. (1997) Mechanism of beta-adrenergic receptor desensitization in cardiac hypertrophy is increased beta-adrenergic receptor kinase. *J. Biol. Chem.* **272**, 17223–17229
- Gros, R., Chorazyczewski, J., Meek, M. D., Benovic, J. L., Ferguson, S. S., and Feldman, R. D. (2000) G-Protein-coupled receptor kinase activity in hypertension: increased vascular and lymphocyte G-protein receptor kinase-2 protein expression. *Hypertension* **35**, 38–42
- Koch, W. J., and Rockman, H. A. (1999) Exploring the role of the beta-adrenergic receptor kinase in cardiac disease using gene-targeted mice. *Trends Cardiovasc. Med.* **9**, 77–81
- Penn, R. B., Pascual, R. M., Kim, Y.-M., Mundell, S. J., Krymskaya, V. P., Panettieri, R. A., Jr., and Benovic, J. L. (2001) Arrestin specificity for G protein-coupled receptors in human airway smooth muscle. *J. Biol. Chem.* **276**, 32648–32656
- Ishii, K., Chen, J., Ishii, M., Koch, W. J., Freedman, N. J., Lefkowitz, R. J., and Coughlin, S. R. (1994) Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase: functional specificity among G-protein coupled receptor kinases. *J. Biol. Chem.* **269**, 1125–1130
- Parent, J. L., Labrecque, P., Driss, R. M., and Benovic, J. L. (2001) Role of the differentially spliced carboxyl terminus in

- thromboxane A2 receptor trafficking: identification of a distinct motif for tonic internalization. *J. Biol. Chem.* **276**, 7079–7085
33. Iwata, K., Luo, J., Penn, R. B., and Benovic, J. L. (2005) Bimodal regulation of the human H1 histamine receptor by G protein-coupled receptor kinase 2. *J. Biol. Chem.* **280**, 2197–2204
 34. Wu, G., Bogatkevich, G. S., Mukhin, Y. V., Benovic, J. L., Hildebrandt, J. D., and Lanier, S. M. (2000) Identification of Gbetagamma binding sites in the third intracellular loop of the M(3)-muscarinic receptor and their role in receptor regulation. *J. Biol. Chem.* **275**, 9026–9034
 35. Budd, D. C., McDonald, J. E., and Tobin, A. B. (2000) Phosphorylation and regulation of a Gq/11-coupled receptor by casein kinase 1alpha. *J. Biol. Chem.* **275**, 19667–19675
 36. Willets, J. M., Challiss, R. A., Kelly, E., and Nahorski, S. R. (2001) G protein-coupled receptor kinases 3 and 6 use different pathways to desensitize the endogenous M3 muscarinic acetylcholine receptor in human SH-SY5Y cells. *Mol. Pharmacol.* **60**, 321–330
 37. Luo, J., Busillo, J. M., and Benovic, J. L. (2008) M3 muscarinic acetylcholine receptor-mediated signaling is regulated by distinct mechanisms. *Mol. Pharmacol.* **74**, 338–347
 38. Kim, C. M., Dion, S. B., and Benovic, J. L. (1993) Mechanism of b-adrenergic receptor kinase activation by G proteins. *J. Biol. Chem.* **268**, 15412–15418
 39. Carman, C. V., Parent, J. L., Day, P. W., Pronin, A. N., Sternweis, P. M., Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999) Selective regulation of Gq/11alpha by an RGS domain in the G protein-coupled receptor kinase GRK2. *J. Biol. Chem.* **274**, 34483–34492
 40. Hauck, R. W., Schulz, C., Schomig, A., Hoffman, R. K., and Panettieri, R. A., Jr. (1999) alpha-Thrombin stimulates contraction of human bronchial rings by activation of protease-activated receptors. *Am. J. Physiol.* **277**, 22–29
 41. Kong, K. C., Billington, C. K., Gandhi, U., Panettieri, R. A. Jr., and Penn, R. B. (2006) Cooperative mitogenic signaling by G protein-coupled receptors and growth factors is dependent on G(q/11). *FASEB J.* **20**, 1558–1560
 42. Struckmann, N., Schwering, S., Wiegand, S., Gschnell, A., Yamada, M., Kummer, W., Wess, J., and Haberberger, R. V. (2003) Role of muscarinic receptor subtypes in the constriction of peripheral airways: studies on receptor-deficient mice. *Mol. Pharmacol.* **64**, 1444–1451
 43. Penn, R. B. (2008) Embracing emerging paradigms of G protein-coupled receptor agonism and signaling to address airway smooth muscle pathobiology in asthma. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **378**, 149–169
 44. Bond, D., Primrose, D. A., and Foley, E. (2008) Quantitative evaluation of signaling events in *Drosophila* S2 cells. *Biol. Proc. Online* **10**, 20–28

Received for publication August 2, 2013.

Accepted for publication October 3, 2013.