β-Agonist-associated Reduction in RGS5 Expression Promotes Airway Smooth Muscle Hyper-responsiveness

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Although short-acting and long-acting inhaled β2-adrenergic receptor agonists (SABA and LABA, respectively) relieve asthma symptoms, use of either agent alone without concomitant anti-inflammatory drugs (corticosteroids) may increase the risk of disease exacerbation in some patients. We found previously that pretreatment of human precision-cut lung slices (PCLS) with SABA impaired subsequent β2-agonist-induced bronchodilation, which occurred independently of changes in receptor quantities. Here we provide evidence that prolonged exposure of cultured human airway smooth muscle (HuASM) cells to β2-agonists directly augments procontractile signaling pathways elicited by several compounds including thrombin, bradykinin, and histamine. Such treated cells did not increase surface receptor amounts or expression of G proteins and downstream effectors (phospholipase CB and myosin light chain). In contrast, β-agonists decreased expression of regulator of G protein signaling 5 (RGS5), which is an inhibitor of G-protein-coupled receptor (GPCR) activity. RGS5 knockdown in HuASM increased agonist-evoked intracellular calcium flux and myosin light chain (MLC) phosphorylation, which are prerequisites for contraction. PCLS from Rgs5−/− mice contracted more to carbachol than those from WT mice, indicating that RGS5 negatively regulates bronchial smooth muscle contraction. Repetitive β2-agonist use may not only lead to reduced bronchoprotection but also to sensitization of excitation-contraction signaling pathways as a result of reduced RGS5 expression.

Asthma is a complex disorder of unknown etiology characterized by increased bronchial smooth muscle contraction, lung inflammation, and tissue remodeling. These and other abnormalities reduce airway luminal diameter and increase stiffness. A cardinal feature of asthma is “airway hyper-responsiveness” (AHR), which refers to the increased bronchial contraction to inhaled procontractile agonists such as methacholine that is observed in asthmatics relative to healthy controls. AHR manifests as reduced airflow (1–4). In allergic asthma, mediators secreted by activated inflammatory leukocytes or resident lung cells including histamine, leukotrienes, bradykinin, and thrombin promote ASM contraction by activating GPCRs linked to activation (GTP binding) of Goq (5). This initiates a signaling route culminating in generation of inositol (3, 4, 5)-trisphosphate and release of Ca2+ from endoplasmic reticulum by activation of IP3 receptors. Hydrolysis of GTP by Goq promotes pathway deactivation through formation of inactive Goq-GDP-Gβγ heterotrimeric. Goq signaling is required for AHR in mouse models of allergic airway inflammation (6).

Receptor-related events increase the frequency of intracellular Ca2+ oscillations in ASM, which induces Ca2+-calmodulin-dependent protein kinase (CaMK)-mediated activation of myosin light chain kinase (MLCK). Phosphorylation of myosin light chain (MLC) on Ser-19 by MLCK promotes actin-myosin filament interactions and muscle fiber shortening through the mechanism known as cross-bridge cycling (7). Sustained ASM contraction requires continual and/or increased MLCK activity in the presence of constant Ca2+ oscillation frequency, which is referred to as Ca2+ sensitization. This mechanism is controlled independently of Ca2+ concentration by kinases including Rho-associated kinase through inhibition of MLC phosphatase activity, resulting in prolonged MLC phosphorylation (8).

Inhaled SABA and LABA, which are commonly prescribed for the treatment of asthma, stimulate β2-adrenergic receptors (β2AR) on ASM, promoting relaxation and enhanced airflow (9). β2AR activation of Goq stimulates activity of the membrane enzyme adenylyl cyclase, which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). A primary function of cAMP is to activate protein kinase A (PKA), which in turn activates myosin light chain phosphatase, reducing MLC phosphorylation, and generating relaxation (5, 7). However, chronic administration of either of these agonists induces tolerance due to receptor desensitization (10–11). Mechanisms underlying receptor desensitization include decreased β2AR transcription, uncoupling of receptors from adenyl cyclase through down-regulation of Goq (12–13),

β2-adrenergic receptor agonists; HuASM, human airway smooth muscle; GPCR, G-protein-coupled receptor; MLC, myosin light chain; CaMK, Ca2+-calmodulin-dependent protein kinase; MLCK, myosin light chain kinase; β2AR, β2-adrenergic receptors.
internalization of uncoupled receptors, and increased phosphorylation and degradation of internalized receptors (9). Regular clinical use of these agents has been linked to heightened sensitivity to allergen-evoked AHR, exacerbation of asthma symptoms, and even death (14–15). For these reasons, the Food and Drug Administration recommends that β2AR agonists primarily be reserved for moderate to severe asthma and used in combination with inhaled corticosteroids (15–17).

Unfortunately, the mechanism(s) responsible for the seemingly paradoxical effects of SABA and LABA on bronchial contractility/AHR remain unresolved. Several studies have demonstrated that chronic application of β2-agonists to whole animals (12), mammalian tracheal smooth muscle strips ex vivo (18–20), or cultured ASM (21) increased contraction or Ca2+ responses to agonists (histamine, methacholine, or endothelin-1). In some cases, hyper-responsiveness was accompanied by up-regulation of cognate receptors for the procontractile ligand (18–19). Transgenic overexpression of β2AR in mice has also been utilized to evaluate chronic, persistent receptor activity. Surprisingly, tracheas isolated from β2AR transgenic mice contracted more than wild-type counterparts, and cultured smooth muscle cells had enhanced IP3 accumulation in response to contractile agonists (22). Mechanisms underlying these responses included up-regulation of PLCβ in smooth muscle cells from β2AR-overexpressing mice. These and other studies demonstrate that in addition to its acute effect on signaling pathways, prolonged β2-receptor activation may also elicit transcriptional changes in ASM (20).

Here, we investigated the importance of transcriptional adaptations for the activity of excitation-contraction coupling in ASM. We found that exposure of ASM to the high-affinity β2-agonist isoproterenol enhanced Ca2+ responses to several GPCR ligands independently of changes in receptor levels by triggering decreased expression of RGS5. RGS proteins inhibit GPCR responses downstream of receptor-ligand interactions by accelerating the intrinsic GTPase activity of Gαi and Gαq. G protein deactivation leads to enhanced formation of inactive Gαβγ heterotrimers and reduced signal output (23). Knockdown of RGS5 phenocopied the effects of isoproterenol on ASM, resulting in increased intracellular Ca2+ flux and myosin light chain phosphorylation. These studies suggest that β2-agonists directly sensitize the contractile pathways of ASM through mechanisms that include a reduction in RGS5 transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human ASM cells were isolated from lung transplant donors as previously described in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings (11). The cells were routinely maintained in Ham’s F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM HEPES buffer. Cultured medium was replaced every 72 h. Cells in subculture during passage 2–4 were used. HEK293T cells were cultured in complete Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% (v/v) fetal bovine serum, 1% penicillin, and streptomycin at 37 °C with 5% CO2.

**PCLS Contraction**—Lung slice studies were performed as described (24). Rgs5−/− mice (25) were backcrossed onto a C57BL/6 background for more than 5 generations. WT littermates were used as controls. All mouse experiments were performed in accordance with Animal Study Protocol LAD-3E, approved by the NIAID Animal Care and Use Committee, NIH.

**RNA Isolation, RT-PCR, and Quantitative RT-PCR**—RNA was isolated using Trizol reagent (Invitrogen) per the manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed into cDNA using the SuperScript III first strand synthesis kit (Invitrogen). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed using 35 cycles consisting of a 30-s denaturation step at 94 °C, followed by annealing for 30 s at 56 °C, and extension for 30 s at 72 °C. The sequences of the forward and reverse primers used for identification of human RGS mRNAs are shown in supplemental Table S1. Quantitative real-time PCR (qPCR) was performed with TaqMan gene expression assay probes for each specific RGS using an ABI 7500 HT thermal cycler (Applied Biosystems). The 20-μl reaction contained 10 μl of 2× TaqMan Universal Master Mix, 1 μl of specific RGS or GAPDH gene expression assay probe, and 1 μl of cDNA. The reactions were run for 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The average cycle threshold (Ct) value from each RGS probe was applied to calculate specific gene expression and normalized by the human GAPDH Ct value. Results represent values from six individual donors assayed in duplicate.

**RNA Interference**—Scrambled, non-targeting, and RGS5-specific siRNAs were purchased from Dharmacon (scrambled: catalogue no. AM4611-7, sequence AGUACUGCUUACGAUACGG; RGS5: catalogue no. D-008384, sequence CCAAGGA-GAUUGAAGAUCAAU). An additional RGS5 siRNA was purchased from Ambion (catalogue no. 4392420 (siRNA ID# s224973), sequence AGUUUGGAUUGCCUGUGATT). siRNA duplexes (160 nM) were transfected into HuASM by nucleofection (Lonza), program U-25. After 48–96 h, RGS5 knockdown was evaluated by quantitative RT-PCR and immunoblotting.

**Lentiviral Amplification and Transduction**—The human RGS5 coding sequence was amplified by PCR using pEGFP-RGS5 plasmid as a template and subcloned into pLenti7.3/V5 TOPO (negative control) was transfected into HEK293T cells together with a lentiviral packaging mix (Sigma) using Lipofectamine 2000. After 72 h virus was harvested in the supernatant and concentrated 10-fold by centrifugation. Concentrated virus was added directly to HuASM cells, and gene expression and function were assayed 48–72 h after transduction.

**Flow Cytometry**—HuASM cells were suspended in FACS buffer (PBS plus 0.2% BSA) and incubated for 30 min at 4 °C with Alexa Fluor 647-conjugated anti-thrombin receptor antibody (Santa Cruz Biotechnology sc-13503) or mouse IgG1 as a control. Cells were washed several times followed by analysis on a FACSCalibur flow cytometer (BD Biosciences).

**Subcellular Fractionation**—Cells lysates were prepared in TE buffer and homogenized by passage through a 25-gauge
syringe. Cells were centrifuged at 500 \times g to obtain postnuclear supernatants, followed by ultracentrifugation at 100,000 \times g to separate membrane and cytosolic fractions.

**Measurement of Intracellular Ca^{2+} Mobilization**—HuASM cells (2.5 \times 10^4 cells/well) were seeded in Biocat poly-lysine 96-well plates (BD Biosciences), allowed to attach overnight and serum-starved in Ham’s F-12 medium plus 0.5% BSA for 48 h prior to measurement of intracellular Ca^{2+} concentration. Ca^{2+} indicator dye (FLIPR Calcium 3 Assay Component A (Molecular Devices)) was added to each well followed by incubation for 1 h at 37 °C with 5% CO_2. Serial dilutions of agonists (bradykinin, thrombin, endothelin-1, and histamine (Sigma-Aldrich)) were prepared in a separate plate at 10× concentrations for robotic addition to cells by the FLEX Station II fluorimeter (Molecular Devices). Relative fluorescence units (RFUs) were measured every 2 s for a period of 180 s after agonist addition. Results were plotted as the maximum-minimum signal for each agonist concentration over this time period. Graphs represent results (mean ± S.E.) of three or more independent experiments using cells derived from separate individual donors.

**Protein Phosphorylation and Immunoblotting**—ASM cells were plated in 6 well tissue culture plates (1 \times 10^6 cells/well) and serum-starved for 48 h prior to stimulation with agonist for the times indicated. Reactions were terminated by addition of cold PBS containing protease-phosphatase inhibitor mix (Roche). Lysates were prepared by adding buffer containing 20 mM Tris, pH 7.5, 300 mM NaCl, 10 mM β-ME, 10% glycerol, 1% Triton X-100, a protease-phosphatase inhibitor mix (Roche) and 1× NuPAGE LDS sample buffer (Invitrogen). Proteins were separated by SDS-PAGE and immunoblotted with specific antibodies purchased from the following sources: PAR1 (R & D Systems); Histamine H1 receptor (H1R), Bradykinin B2 receptor (BK2R), and Goq (Santa Cruz Biotechnology); PLC/β1 (BD Biosciences); pErk, Erk 1/2, pAkt, Akt, pMLC, caveolin, and MLC20 (Cell Signaling); RGS5 (ProSci); GAPDH (Abcam); β-actin (Sigma). Rabbit polyclonal RGS4 antibody U1079 was the kind gift of Suzanne Mumbry (University of Texas, Southwestern School of Medicine) (26). Blots were developed with Infrared IRDye-labeled secondary antibodies (Li-Cor Biosciences). Signals were detected and quantified using the Li-Cor Odyssey Imaging System.

**Immunofluorescence**—HuASM cells were plated into chamber wells at 0.1 \times 10^6/well overnight to allow cell adherence, followed by fixation in neutral buffered formalin. Fixed cells were permeabilized briefly in PBS containing 0.5% Triton X-100 followed by a blocking step in Ready-to-Use blocking buffer (DakoCytomation). RGS5 was detected with anti-RGS5 primary antibody (Prosci, 1:250) followed by incubation with AlexaFluor 594-conjugated goat anti-chicken antibody (Invitrogen). Cells were washed with PBS/0.5% Triton X-100 and fluorescence visualized with a Leica DMI 4000B confocal microscope.

**Immunohistochemistry**—Frozen human lung sections were fixed in cold acetone and blocked with hydrogen peroxidase followed by incubation with anti-RGS5 primary antibody (ProSci, 1:750) overnight. Slides were then washed extensively with Tris-buffered saline plus 0.05% Tween followed by incubation with biotinylated anti-chicken IgY (Abcam ab6876, 1:500) and horseradish peroxidase-conjugated streptavidin (DakoCytomation, 1:400) for 30 min. each. Staining was detected with 3, 3’-diaminobenzidine (DAB) followed by hematoxylin counterstaining. Slides were dehydrated in graded ethanol and cleared in xylene prior to mounting with coverslips. Image shown is representative of lung sections derived from 8 individual donors.

**Statistical Analysis**—Data were analyzed by one- or two-way ANOVA or Student’s t test using PRISM software (GraphPad, CA). We considered a p value < 0.05 statistically significant.

**RESULTS**

**β2-Agonist Exposure Augments Agonist-evoked Ca^{2+} Responses of ASM**—To address the molecular mechanisms underlying enhanced contraction of bronchi after chronic β2-agonist exposure, we evaluated excitation-contraction coupling pathways in HuASM cells treated for 48 h with the high affinity β2-agonist isoproterenol. We loaded cells with Ca^{2+}-sensing fluorescent dye and measured accumulation of intracellular Ca^{2+} after stimulation with a range of concentrations of the GPCR ligands bradykinin, thrombin, and histamine, which activate the Goq signaling pathway (27). Treatment of ASM cells with these compounds evoked a transient increase in cytosolic calcium lasting >120 s, with a peak increase at ~10–20 s, suggesting an IP3-mediated release of Ca^{2+} from intracellular stores (Fig. 1, A–C). Although baseline Ca^{2+} levels were similar in untreated and isoproterenol-treated cells, peak intracellular Ca^{2+} concentrations in response to bradykinin and thrombin were significantly higher in cells incubated with isoproterenol than in control cells in response to several different concentrations of agonist (Fig. 1, A–C and Table 1). Although we observed an increased histamine-evoked E_{max} in isoproterenol-treated cells, it did not achieve statistical significance, which we attribute to reduced membrane expression of H1 receptors (see Fig. 2C). We also noted that basal phosphorylation of MLC (i.e. without re-stimulation) was higher in cells exposed to isoproterenol than in untreated cells (Fig. 1D). These results support the hypothesis that procontractile signaling pathways are amplified in β2-agonist-experienced ASM cells.

To determine whether up-regulation of GPCRs by β2-agonist could account for sensitization of Ca^{2+} responses, we evaluated receptor expression in the presence or absence of isoproterenol by immunoblotting. Drug treatment had no effect on the total cellular expression of thrombin (PAR1), bradykinin (B2), or histamine (H1) receptors (Fig. 2A). Surface expression of PAR1 was also similar in untreated and isoproterenol-treated cells by flow cytometry (Fig. 2B). As we were unable to assess surface levels of B2 or H1 receptors by flow due to lack of available reagents, we performed cell fractionation and immunoblotting. The subcellular distribution of PAR1 and B2 receptors was similar in untreated and isoproterenol-treated cells (Fig. 2C). In contrast, membrane levels of H1 receptor decreased somewhat after isoproterenol treatment. We also determined whether isoproterenol exposure affected expression of intracellular signaling components downstream of GPCRs. The levels of Goq, PLC/β1, and MLC were similar in isoproterenol-treated
and untreated HuASM cells (Fig. 2A). Thus, exposure of HuASM cells to β₂-agonist does not substantially influence expression of key signaling components yet induces amplified Ca²⁺ responses to pro-contractile agonists.

Expression of RGS Proteins in ASM—We noted that isoproterenol pretreatment primarily altered the efficacy of the agonists to induce intracellular Ca²⁺ flux (i.e. maximal signal at high doses, $E_{\text{max}}$) rather than their potency ($EC_{50}$) (Fig. 1 and

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**TABLE 1**

| Agonist | Basal-control | Basal-iso | $p$ value | $EC_{50}$-control | $EC_{50}$-iso | $p$ value | $E_{\text{max}}$-control | $E_{\text{max}}$-iso | $p$ value |
|---------|---------------|-----------|-----------|-------------------|--------------|-----------|--------------------------|-------------------|-----------|
| Bradykinin | 22.5 ± 3.6 | 24.7 ± 4.1 | 0.7 | 0.2 | 0.5 | 0.67 | 39.5 ± 2.2 | 47.1 ± 4.1 | 0.03 |
| Thrombin | 30.9 ± 2.9 | 34.4 ± 3.5 | 0.4 | 0.02 | 0.028 | 43.5 ± 2.3 | 52.2 ± 3.1 | 0.027 |
| Histamine | 30 ± 5 | 32.9 ± 5.8 | 0.72 | .047 | 0.138 | 0.7 | 43.2 ± 3 | 51.5 ± 3.8 | 0.12 |

$^{a}$ Relative fluorescence units (RFU) $\times 10^3$.

$^{b}$ nM.

$^{c}$ unit/ml.
We observed a similar phenotype in mast cells depleted of RGS13 (28). We hypothesized that β₂-agonists could lead to changes in expression of RGS proteins, rather than components that propagate the G_q signaling pathway, given that the abundance and activity of RGSs is often more dynamic depending on cell type and microenvironment (29–30). We focused on RGS proteins of the R4/B subfamily, which includes RGS1–5, 8, 10, 13, 16, 18, and 21, because their function in regulating GPCR signaling in peripheral tissues is the most well-defined (31). We isolated total RNA from cultured ASM cells derived from healthy donors and evaluated RGS expression by RT-PCR and real-time quantitative PCR. We found expression of RGS2, 3, 4, 5, and 8 in these cells while RGS1, 10, 13, and 16 were absent or minimally detected (Fig. 3, A and B). Although the relative expression of each RGS could not be compared strictly by this method given the varying affinity of various primer probe sets, RGS2–5 were detected readily, and RGS3 appeared to be most abundant. Because RGS2 and 3 are widely expressed (32–34), we studied RGS5 further since its expression is restricted to only a few cell types in humans (33, 35). We detected RGS5 protein in HuASM by immunoblotting (Figs. 3D and 4C). RGS5 localized predominantly in the cytosolic fraction by immunofluorescent staining (Fig. 3C) or by subcellular fractionation and immunoblotting (Fig. 3D). The RGS5 antibody appeared to be specific as no signal was detected on immunoblots probed with antibody pre-incubated with recombinant RGS5 (Supplemental Fig. S1). We also identified RGS5 in bronchial smooth muscle and epithelial cells of human lung by immunohistochemical staining (Fig. 3E).

Changes in RGS Expression Evoked by β₂-Agonist Treatment of ASM—We investigated the effect of β₂-agonists on the expression of RGS5 in HuASM cells. We treated cells with iso-
proterenol followed by isolation of total RNA and quantification of gene expression by real-time RT-PCR. RGS5 expression decreased following isoproterenol exposure in a concentration-dependent manner (Fig. 4A). After 4 h of incubation with β2-agonist, RGS5 mRNA levels decreased by ~40%, and declined further to 25–40% of initial levels at 24–36 h (Fig. 4B). Isoproterenol treatment also led to reduced expression of RGS4 (supplemental Fig. S2) but not RGS2 and RGS3, in ASM cells (supplemental Fig. S3). The expression of RGS5 protein was also diminished ~50% 48 h following addition of isoproterenol (Fig. 4C). The partial β2-agonist salbutamol, which is more commonly used clinically for the treatment of asthma, also induced a significant reduction in RGS5 expression (Fig. 4D).

To determine if the changes in RGS5 expression induced by β2-agonist treatment were responsive to corticosteroids, we treated cells with salbutamol and dexamethasone. We used a dexamethasone concentration previously shown to prevent β2-agonist desensitization in PCLS without affecting growth factor-induced ASM proliferation (11, 36). RGS5 expression was reduced in salbutamol-treated cells compared with control (Fig. 4D). However, pretreatment with dexamethasone did not affect RGS5 expression in the presence or absence of salbutamol (Fig. 4E). Thus, since RGS proteins are known to negatively regulate output of Gi- and Gq-coupled GPCRs, their reduced expression in HuASM exposed to β2-agonist could account for the enhanced signaling we observed in these cells after GPCR stimulation.

RGS5 Knockdown Enhances Agonist-induced Intracellular Calcium Flux and Protein Phosphorylation—To evaluate the consequences of reduced RGS5 levels on pro-contractile signaling pathways in HuASM, we used RNA interference to eliminate RGS5 expression. We transfected cells with a scrambled siRNA or two distinct RGS5-specific siRNAs and measured RGS5 mRNA abundance by real-time PCR. Compared with cells expressing the control siRNA, cells transfected with either RGS5 siRNA had 80% less RGS5 mRNA (Fig. 5A). Similar to isoproterenol treatment, transfection of RGS5 siRNA led to substantially reduced RGS5 protein expression compared with control (Fig. 5B). We evaluated the rise in intracellular calcium concentration after stimulation with GPCR agonists in control
and RGS5-depleted cells. Compared with control cells, cells expressing RGS5 siRNA had a significantly higher cytosolic Ca\(^{2+}\) concentration in response to thrombin, bradykinin, and histamine (Fig. 5, C–F and Table 2). In contrast, the Ca\(^{2+}\) response to endothelin-1 (which also activates a Gq-linked GPCR) or the Ca\(^{2+}\) ionophore ionomycin was similar in control

**TABLE 2**

| Agonist   | Basal-scrambled siRNA | Basal-RGS5 siRNA | p value | EC\(_{50}\)-scrambled siRNA | EC\(_{50}\)-RGS5 siRNA | p value | E\(_{\text{max}}\)-scrambled siRNA | E\(_{\text{max}}\)-RGS5 siRNA | p value |
|-----------|----------------------|------------------|---------|-----------------------------|-----------------------|---------|-------------------------------|----------------------------|---------|
| Bradykinin\(^b\) | 6.2 ± 2.9\(^a\) | 6 ± 2.4 | 0.98 | 0.34 | 0.38 | 0.95 | 25.7 ± 2.2 | 37.6 ± 2.4 | 0.0006 |
| Thrombin\(^c\)  | 5.1 ± 2.2 | 5.8 ± 2.7 | 0.85 | 0.02 | 0.017 | 0.85 | 27.8 ± 1.5 | 35.8 ± 1.9 | 0.004 |

\(^a\) Relative fluorescence units (RFU) \(\times 10^3\).

\(^b\) nM.

\(^c\) unit/ml.
and RGS5 knockdown cells (Fig. 5, G and H). These studies show that reduced RGS5 expression selectively enhances Ca$^{2+}$ signaling induced by activation of thrombin, histamine, and bradykinin receptors but has no effect on endothelin-1 or receptor-independent responses. Thus, RGS5 negatively regulates Gq-linked signaling pathways in HuASM.

To evaluate the effect of RGS5 siRNA on the excitation-contraction pathway downstream of Ca$^{2+}$, we examined protein phosphorylation. We treated cells with bradykinin for up to 15 min and assessed MLC phosphorylation by immunoblotting cell lysates. Compared with cells expressing the scrambled siRNA, cells with extinguished RGS5 expression had more basal and bradykinin-evoked MLC phosphorylation (Fig. 6A). The increased basal protein phosphorylation in RGS5-depleted cells is concordant with previous studies (28, 37). Consistent with the function of RGS5 as an upstream negative regulator at the level of G protein activation, phosphorylation of other GPCR-activated kinases (Erk and Akt) was also increased in cells expressing RGS5 siRNA compared with control (Fig. 6, B and C).

**RGS5 Overexpression Inhibits Intracellular Calcium Flux and MLC Phosphorylation**—To examine the effects of increased RGS5 levels on procontractile signaling in HuASM, we measured agonist-evoked Ca$^{2+}$ flux in cells infected with lentiviruses encoding either a control protein (β-galactosidase) or RGS5. Overexpression of RGS5 in HuASM blunted bradykinin-induced increases in cytosolic Ca$^{2+}$ and MLC phosphorylation compared with control cells (Fig. 7, A and B). To address the role of RGS5 in the enhanced excitation-contraction coupling induced by isoproterenol, we expressed β-gal or RGS5 in HuASM cells and treated them with medium alone or isoproterenol for 48 h prior to measurement of bradykinin-evoked Ca$^{2+}$ flux. Cells overexpressing RGS5 had reduced intracellular Ca$^{2+}$ concentrations after bradykinin stimulation compared with control in the presence or absence of isoproterenol pretreatment (Fig. 7C). Thus, RGS5-overexpressing cells are resistant to isoproterenol-induced hypersensitivity. Collectively, these studies indicate that RGS5 inhibits Gq-linked procontractile pathways in HuASM. Reduced RGS5 expression likely contributes to the augmented signaling observed in cells chronically exposed to isoproterenol.

**Increased Bronchial Contractility of Rgs5$^{-/-}$ Mice**—To investigate how RGS5 regulates AHR, we compared carbachol-evoked contraction of PCLS from WT and Rgs5$^{-/-}$ mice. We detected Rgs5 transcripts in whole lung from WT but not KO mice (Fig. 8A). Bronchial slices from RGS5-deficient mice contracted more than WT airways to equivalent concentrations of carbachol (Fig. 8B). Similar to the Ca$^{2+}$ responses of primary human ASM cells expressing RGS5-specific siRNA, RGS5 deficiency in mice was associated with increased maximal bronchial contraction ($E_{\text{max}}$: WT = 70.8 ± 5.7%; KO = 93.5 ± 2.9% (p = 0.001)) while the $E_{50}$ of carbachol was unaffected (WT =

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**FIGURE 6. Enhanced agonist-evoked protein phosphorylation in RGS5-deficient ASM.** A–C, cells were transfected with scrambled or RGS5-specific siRNAs as in Fig. 5. 72 h after transfection, serum-starved cells were treated with bradykinin (10 nM) for the indicated times followed by collection of cell lysates and immunoblotting with the indicated antibodies. Blots are from a representative experiment, and bar graphs show the mean ± S.E. of 3–5 independent experiments (black bars, scrambled siRNA; white bars, RGS5 siRNA; *, p < 0.05, t test).
These studies indicate that RGS5 regulates bronchial smooth muscle contraction.

DISCUSSION

This is the first study to define a function for RGS proteins in bronchial smooth muscle. We demonstrated expression of several RGS proteins in primary HuASM cells and showed that exposure to β2-adrenergic agonists lead to reduced expression of both RGS4 and RGS5. Depletion of RGS5 in these cells either by prolonged exposure to isoproterenol or by RNA interference sensitized excitation-contraction pathways, as evidenced by enhanced intracellular Ca2+ flux and MLC phosphorylation. Thus, β2-agonists may inadvertently "prime" signaling routes in HuASM by depleting them of negative regulatory components such as RGS proteins, whose activity controls contraction of naïve cells under physiological conditions.

Evidence suggests that chronic exposure to β-agonists alters ASM contraction and gene expression (21, 38–39). Liggett and colleagues found significant changes in expression of >500 genes in mice overexpressing β-adrenergic receptors. As but one example, ASM cells from these mice had reduced expression of phospholamban, a protein that inhibits activity of the sarcoplasmic reticulum Ca2+ ATPase2 (SERCA2), which promotes reuptake of cytosolic Ca2+ into the ER (38). Mice deficient in phospholamban had reduced airway contraction to methacholine. Decreased phospholamban abundance was surprising given the enhanced contraction of tracheas from these mice (22), indicating that β-agonists may influence the transcriptional program and contractile properties of ASM in complex ways.
evoked by several agonists, it did not lead to substantial changes in G protein (Ga, Gβγ, Gap) or effector (PLCβ) expression (Fig. 2 and data not shown). These differences might be explained simply by the different agonists used.

In another series of reports, Grunstein and co-workers showed that chronic exposure of ASM to salmeterol induced transcriptional up-regulation of phosphodiesterase 4D5, which promotes cAMP degradation, through a Goi-Erk-MKP1 dependent mechanism (20, 41–42). Although procontractile signaling elements such as IP3 and Ca2+ flux in ASM cells exposed to salmeterol were not examined in these studies, rabbit tracheas treated with salmeterol contracted more to acetylcholine, which was reversed by either glucocorticoid or phosphodiesterase inhibitor pretreatment. Although it has been shown recently that cAMP may promote Ca2+ signaling by directly sensitizing IP3 receptors to IP3 independently of PKA (43), it is unclear from these studies whether PDE4 up-regulation altered the procontractile signaling pathway in ASM cells directly.

In contrast to these studies, we provide evidence that exposure to β agonists directly affects propagation of Goi-mediated procontractile signaling in cultured ASM cells by decreasing expression of key negative regulators of the pathway. The reduced expression of RGS4 and RGS5 in isoproterenol-treated ASM is concordant with recent studies of Penn and co-workers (39), who performed microarray studies on HuASM cells expressing GFP or cells expressing a heat-stable inhibitor of PKA kinase activity, PKI, and cultured in the presence of epidermal growth factor and IL-1β. Cells expressing PKI had 14-fold more RGS4 and 4-fold more RGS5 than control cells, suggesting negative regulation of RGS4/5 transcription by PKA. However, while we found that β agonist-induced changes in RGS4/5 expression in HuASM were unaffected by steroid pretreatment, in the previous study glucocorticoid pretreatment of the cells before stimulation with growth factors induced a marked down-regulation of RGS4 and RGS5 (39). In addition, stimulation of cardiomyocytes with high doses of isoproterenol did not affect RGS4 or 5 expression in a recent report (44).

Although these studies collectively suggest involvement of the cAMP-PKA axis in transcriptional regulation of RGS4/5 in ASM, other cell-type specific factors could be involved. Thus, further dissection of pathways involved in regulation of RGS expression is needed.

Loss of RGS GAP activity enhances downstream output in response to GPCR stimulation (28), which we observed in RGS5-depleted ASM cells. However, endothelin-1-induced Ca2+ responses were unaffected by the loss of RGS5, suggesting that it does not regulate signaling through ET–B receptors in ASM. Receptor-selective regulation of GPCR signaling by RGS proteins has been observed, which may be a result of direction interactions with GPCRs or with linkers or activity-modifying proteins such as spinophilin (45). Although such interactions have not been extensively characterized for RGS5, our results suggest that it specifically regulates a subset of GPCR responses in ASM.

RGS4 and RGS5 are closely related isoforms, which display similar Ga GAP activity (46–47). RGS4 has been shown to inhibit bradykinin- and thrombin-induced signaling in other cell types (48–49). Although we did not formally
assess the role of RGS4 in regulating ASM signaling in this study using siRNA, we hypothesize that the reduced expression of both RGS4 and RGS5 induced by isoproterenol leads to a cumulative loss in GAP activity. This would be predicted to enhance GPCR signaling responses, which have been demonstrated in cells from knock-in mice expressing a mutant Goαi2 resistant to all RGS GAP activity (50). The unique, non-redundant function of RGS5 in the regulation of AHR is confirmed by the increased bronchial contraction of PCLS from RGS5-deficient mice compared with WT. It is also possible that GPCR/Go-independent signaling mechanisms could contribute to increased excitation-contraction coupling in ASM cells exposed to isoproterenol.

Studies of RGS4/5 knock-out mice in models of allergen-induced pulmonary inflammation are needed to fully assess their role in regulating AHR, and these are underway. However, interpretation of such experiments is likely to be challenging given expression of RGS4 and -5 in multiple cell types in lung including bronchial smooth muscle and epithelial cells (our study and (51)), vascular smooth muscle (52), and leukocytes (53). Leukocyte recruitment and activation induced by allergen exposure has an integral function in generating inflammation in the asthmatic lung. Given the capacity of RGS proteins to regulate cell migration (54), RGS4/5 deficiency may also affect the inflammatory component of asthma. Nonetheless, our studies indicate that the down-regulation of both RGS4 and RGS5 and resultant hyper-responsiveness of ASM after chronic β2 agonist exposure should be considered when evaluating the appropriateness of these agents for the treatment of asthma.

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