Regulator of G-Protein Signaling–5 Inhibits Bronchial Smooth Muscle Contraction in Severe Asthma

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Severe asthma is associated with fixed airway obstruction attributable to inflammation, copious luminal mucus, and increased airway smooth muscle (ASM) mass. Paradoxically, studies demonstrated that the hypertrophic and hyperplastic ASM characteristic of severe asthma has reduced contractile capacity. We compared the G-protein–coupled receptor (GPCR)–induced Ca^{2+} mobilization and expression of GPCRs and signaling proteins related to procontractile signaling in ASM derived postmortem from subjects who died of nonrespiratory causes, with cells from subjects who died of asthma. Despite the increased or comparable expression of contraction-promoting GPCRs (bradykinin B2 or histamine H1 and protease-activated receptor 1, respectively) in asthmatic ASM cells relative to cells from healthy donors, asthmatic ASM cells exhibited reduced histamine-induced Ca^{2+} mobilization and comparable responses to bradykinin and thrombin, suggesting a postreceptor signaling defect. Accordingly, the expression of regulator of G-protein signaling–5 (RGS5), an inhibitor of ASM contraction, was increased in cultured, asthmatic ASM cells and in bronchial smooth muscle bundles of both human subjects with asthma and allergen-challenged mice, relative to those of healthy human subjects or naive mice. The overexpression of RGS5 impaired the release of Ca^{2+} to thrombin, histamine, and carbachol, and reduced the contraction of precision-cut lung slices to carbachol. These results suggest that increased RGS5 expression contributes to decreased myocyte shortening in severe and fatal asthma.

Keywords: asthma; bronchial smooth muscle; signal transduction; G-protein–coupled receptors

Chronic asthma is associated with reversible airways obstruction and extensive lung remodeling (1–6). Histological abnormalities typical of severe asthma include bronchial epithelial cell sloughing, mucus cell hyperplasia and mucus hypersecretion, subepithelial basement membrane thickening, increased myocyte number and size, the submucosal deposition of extracellular matrix components, and interstitial edema (3). A substantial body of evidence suggests that airway smooth muscle (ASM) mass is increased in the airways of patients with severe asthma (1, 6, 7).

CLINICAL RELEVANCE

We show that a negative regulator of G-protein–coupled receptor–mediated airway smooth muscle (ASM) contraction, regulator of G-protein signaling–5 (RGS5), is up-regulated in human asthmatic airways. RGS5 could contribute to a hypocontractile ASM state in severe asthma, leading to fixed airway obstruction.

Compelling studies also indicate that asthmatic ASM is characterized by hyperplasia or hypertrophy, increased synthetic and secretory function, and normal or reduced force-generating capacity (7, 8). Proliferating ASM cells phenotypically manifest a less contractile state in vitro, with reduced contractile protein expression (9). Asthmatic ASM also secretes more cytokines, chemokines, and extracellular matrix–modifying enzymes than does ASM from control samples (10–14). A fixed narrowing of the airways is hypothesized to occur in fatal asthma, which renders patients resistant to bronchodilator therapy (15). Although several studies characterized the molecular components of pathogenic ASM hyperplasia and hypertrophy in asthma (e.g., extracellular regulated kinase [ERK], phosphoinositide-3 kinase, S6 kinase, and glycogen synthase kinase 3 beta) (1, 16), abnormalities in procontractile signaling pathways in asthmatic ASM remain relatively undefined.

Soluble mediators linked to allergen-induced inflammation include histamine, leukotrienes, bradykinin, and thrombin, which evoke ASM contraction by stimulating G-protein–coupled receptor (GPCR) coupled to the activation (GTP binding) of G-protein alpha q ($G\alpha q$) (17). Activated $G\alpha q$ stimulates phospholipase $C\beta$ ($PLC\beta$), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol (3,4,5)-trisphosphate (IP3). IP3 elicits the release of Ca^{2+} from sarco/endoplasmic reticulum by the activation of IP3 receptors. The hydrolysis of GTP by $G\alpha q$ promotes pathway deactivation through the formation of inactive $G\alpha q$–GDP–$G\beta\gamma$ heterotrimers. These upstream phenomena increase the frequency of intracellular Ca^{2+} oscillations, which induce the Ca^{2+}–calmodulin–dependent protein kinase–mediated activation of myosin light chain kinase (MLCK). The phosphorylation of the myosin light chain on serine 19 by MLCK promotes actin–myosin cross-bridge formation (17).

In this study, we examined the GPCR-induced Ca^{2+} mobilization and expressed GPCR pathway components in ASM cells cultured from patients with and without asthma. We focused on the expression of regulator of G-protein signaling (RGS) proteins as potential modulators of bronchial contractility in asthma. RGS proteins have emerged as physiologically important components of cellular desensitization to GPCR stimulation by virtue of their ability to accelerate GTP hydrolysis by $G\alpha q$, and thereby blunt downstream effector activation (18). Recently, we showed that ASM cells from healthy subjects expressed RGS 2, 3, 4, and 5 (19). Here we found that the

TABLE 1. CLINICAL AND SPUTUM CHARACTERISTICS OF SUBJECTS UNDERGOING ENDOBRONCHIAL BIOPSY

	Normal	Severe Asthma
Number	8	9
Age, yr, median (range)	55 (27–61)	51 (44–57)
Male (n)	4	6
Cigarette pack years*	0 (0)	0 (0–10)
Atopy (n)	5	7
$PC_{20}FEV_1$, mg/ml, median (range)	>16	1.2 (0.5–3.3)
FEV_1 percent predicted*	105 (5)	83 (6)
FEV_1/FVC percentage*	82 (2)	72 (4)
Inhaled corticosteroids, beclamethasone diproprionate/d, median (range)	0	1,600 (1200–2,000)
Oral corticosteroids (n)	0	2
LABA (n)	0	11
Sputum cell counts		
TCC¶	2.3 (1.5)	4.0 (0.7)
Eosinophil percentage, median (range)	0.7 (0.6–0.8)	8.5 (0.5–45)
Neutrophil percentage*	67 (7)	48 (10)
Macrophage percentage*	22 (7)	27 (6)

Definition of abbreviations: LABA, long-acting β-adrenergic agonists; PC_{20}, provocation concentration producing a 20% fall in FEV_1; TCC, total cell count.
*Mean (SE).

expression of RGS5 was increased in ASM cells of patients with asthma, and that RGS5 overexpression inhibited bronchial contraction. RGS5 appears to be a physiologically relevant inhibitor of bronchial smooth muscle shortening in asthma.

MATERIALS AND METHODS

Additional methods are available in the online supplement.

Subjects

Subjects with asthma and control subjects without asthma were recruited from Leicester, United Kingdom. Subjects with asthma had a consistent history and objective evidence of asthma, as described previously (20) and as outlined in Table 1. Subjects underwent extensive clinical characterization, including video-assisted fiberoptic bronchoscopic

examination. This study was approved by the Leicestershire Ethics Committee. All patients gave written, informed consent.

Cell Culture and Tissues

Human ASM cells and bronchial tissue from healthy donors and individuals with asthma were isolated from lungs obtained postmortem from the National Disease Research Interchange (Philadelphia, PA), following previously established procedures (21). The isolation of ASM was performed in accordance with protocols approved by the Committee on Studies Involving Human Beings at the University of Pennsylvania. ASM cells were maintained in Ham's F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mM HEPES buffer. Cells in subculture during passages 2–5 were used. Clinical profiles and demographics of analyzed samples (where known) are shown in Table 2. Human embryonic kidney 293T cells (HEK293T cells) were obtained from the American Tissue Type Culture Collection (Manassas, VA) and were cultured in Eagle's minimal essential medium (Invitrogen) supplemented with 10% FBS and antibiotics, as already described.

Statistical Analysis

Functional data were analyzed by one-way or two-way ANOVA or the Student t test, using PRISM software (GraphPad, La Jolla, CA). Statistical outliers were determined by Grubb's test. We considered $P < 0.05$ statistically significant.

RESULTS

Reduced Excitation–Contraction Coupling in Asthmatic ASM

To evaluate signaling pathways modulating myocyte contractility in severe asthma, we cultured ASM cells postmortem from patients who died of respiratory failure because of severe asthma, and compared their functional responses to those from control subjects without asthma. We measured GPCR-induced Ca^{2+} mobilization after stimulation with substances present at elevated concentrations in asthmatic airways (histamine, bradykinin, and thrombin) (22) (Figures 1A–1C). We incubated cells with a Ca^{2+}-binding fluorophore, and detected intracellular

TABLE 2. CLINICAL PROFILES AND DEMOGRAPHICS OF ANALYZED HUMAN SAMPLES

Status	Sex	Age (yr)	Race	Smoking Status	Causes of Death
	Male	37	African American	S	Intracranial hemorrhage
Control	Male	19	African American	NS	Closed head injury
	Male	50	White	NS	Intracranial hemorrhage
	Male	49	Hispanic	S	Cerebrovascular accident
	Male	25	White	S	Cardiac arrest
	Female	23	African American	S	Unknown
	Female	19	African American	NS	Unknown
	Male	21	White	S	Head trauma
	Female	49	Hispanic	NS	Unknown
	Female	47	White	NS	Cerebrovascular accident
	Male	28	African American	S	Gunshot wound
	Male	28	Asian	NS	Head trauma
	Female	22	White	S	Gunshot wound
	Female	24	White	NS	Cerebrovascular accident
	Male	31	African American	NS	Anoxia, secondary to asthma attack
	Male	13	White	NS	Anoxia, secondary to asthma attack
Asthma	Male	44	Hispanic	NS	Anoxia, secondary to asthma attack
	Female	46	White	NS	Anoxia, secondary to asthma attack
	Female	15	Hispanic	NS	Anoxia, secondary to asthma attack
	Male	59	White	S	Stroke
	Female	48	White	NS	Anoxia, secondary to asthma attack
	Male	9	White	NS	Anoxia, secondary to asthma attack
	Female	44	White	NS	Respiratory arrest
	Male	44	Hispanic	NS	Anoxia, secondary to asthma attack

Definition of abbreviations: NS, nonsmoker; S, smoker.

fluorescence over a period of several minutes after stimulation. The addition of agonist led to a rapid increase in intracellular Ca^{2+}, followed by a slower decline in concentration, consistent with IP3-mediated Ca^{2+} mobilization from intracellular stores (representative kinetic tracings are shown in Figure 1 at *left*). Although the response to bradykinin and thrombin was similar in asthmatic and nonasthmatic ASM cells (Figures 1A and 1B), histamine induced significantly less Ca^{2+} flux in asthmatic cells (Figure 1C). These results suggest that ASM cells from subjects with asthma manifest impaired excitation–contraction signaling responses to some but not all GPCR ligands.

GPCR and Signaling Protein Expression in Asthmatic ASM

Thapsigargin, which raises intracellular Ca^{2+} concentrations by blocking the sarco/endoplasmic reticular Ca^{2+} ATPase (SERCA) pump and depleting endoplasmic reticulum stores, or the Ca^{2+} ionophore (ionomycin) triggered equivalent Ca^{2+} influx in asthmatic and nonasthmatic ASM cells (Figures 1D and 1E), indicating intact Ca^{2+} homeostasis mechanisms in asthmatic cells. Alternatively, the selectively reduced responses of asthmatic ASM cells to histamine relative to control samples could have resulted from reduced receptor expression. However, we found increased bradykinin B2 receptor (B2R) expression in asthmatic ASM cells
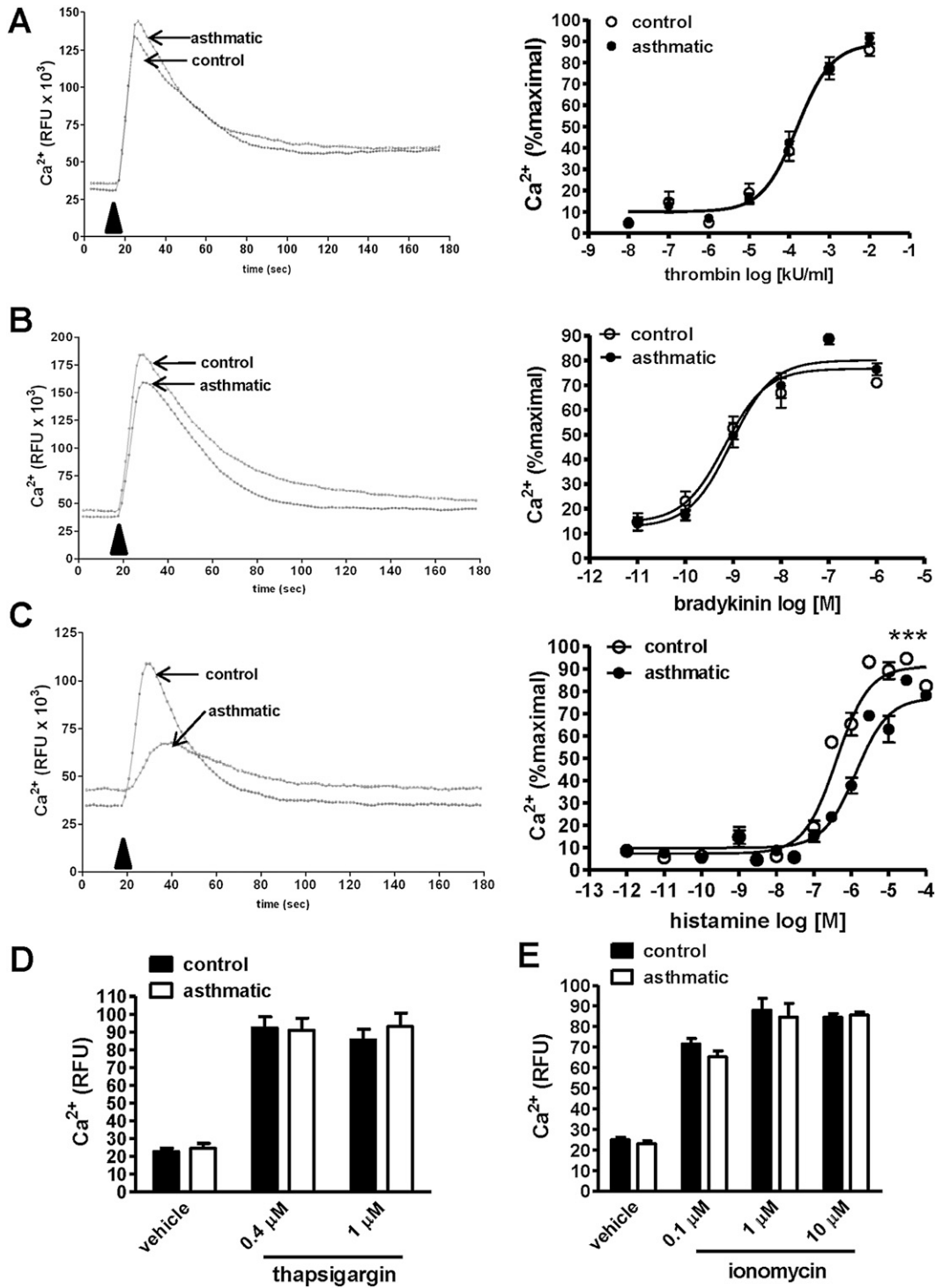
Figure 1. Decreased G-protein–coupled receptor (GPCR)–evoked Ca^{2+} mobilization in asthmatic airway smooth muscle (ASM). (*A–D*) ASM cells derived from healthy donors or subjects with asthma were labeled with Ca^{2+}-binding fluorophore, followed by stimulation with increasing concentrations of bradykinin (*A*), thrombin (*B*), or histamine (*C*), and by measurement of intracellular Ca^{2+} by fluorimetry. Representative kinetic tracings for bradykinin (100 nM), thrombin (10 U/ml), and histamine (1 μM) are shown at *left*. *Arrowheads* indicate the time of stimulus addition. Relative fluorescence units (RFU) were normalized to the cell number and percent maximal response to each concentration at *right*. Graphs represent the mean ± SEM of seven independent experiments performed in quadruplicate, using cells derived from 3–5 individual donors in each group. ***$P <$ 0.0001, least-squares fit, $logEC_{50}$ and E_{max}. (*D* and *E*) Cytosolic Ca^{2+} was measured upon stimulation with the indicated concentrations of thapsigargin (*D*) or ionomycin (*E*), as already described. The data in *D* and *E* represent the mean ± SEM of two experiments performed in quadruplicate, using cells from separate donors.

compared with control samples, whereas the expression of histamine H1 receptor (H1R) and thrombin receptors protease-activated receptor 1 (PAR1) was equivalent (Figures 2A–2C). These results suggest that the reduced histamine-induced Ca^{2+} mobilization in these cells resulted from a postreceptor defect.

Because the abundance of effectors mediating Ca^{2+} flux immediately downstream of GPCRs (Gαq and phospholipase C beta 1 [PLCβ1]) was similar in asthmatic and nonasthmatic ASM cells (Figure 2D), we further analyzed gene expression related to GPCR signaling by a quantitative PCR array (a full gene list is provided in Table E1 in the online supplement). Notably, the expression of cell-cycle genes (cyclin D1, E1, and E2), prosurvival factors (Akt1), cytokines (IL-1β and vascular endothelial growth factor [VEGF]), and matrix metalloproteinases (MMP9; an ∼ 30-fold increase) was increased in ASM cells from subjects with asthma relative to control subjects, consistent with the previously described proliferative/synthetic phenotype of asthmatic ASM. Although the expression of several GPCRs and signaling molecules was increased in asthmatic ASM cells compared with control samples (e.g., the adenosine A2a receptor [A2aR], 12-fold; the sphingosine-1–phosphate-1 receptor, 10-fold; and β-arrestin 1/2, 7-fold), we did not detect corresponding significant changes in the expression of these proteins, with the exception of A2a receptors (Figures 3A–3D).

RGS5 Is Up-Regulated in Asthmatic ASM

Because our studies thus far failed to reveal changes in procontractile signaling components that could underlie the abnormal histamine response of asthmatic ASM cells, we considered other modulators of GPCR signal transduction pathways. The expression of RGS proteins, which are potent negative regulators of
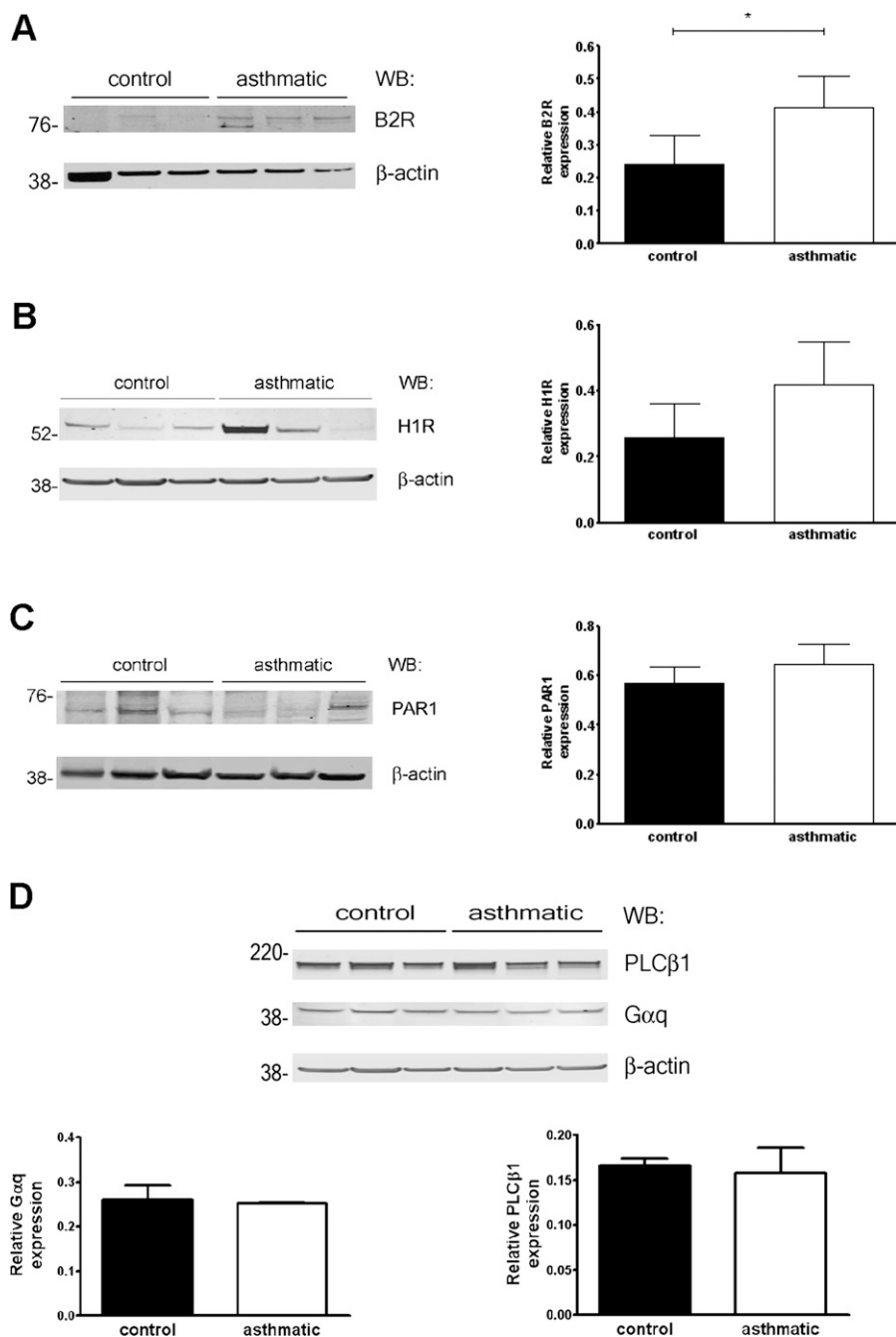
Figure 2. GPCR and signaling protein expression in ASM. (*A–C*) Expression of bradykinin B2 (*A*), histamine H1 (*B*), or PAR1 (*C*) receptors was determined by immunoblotting. *P = 0.04, Mann-Whitney U test. (*D*) Relative expression of signaling proteins in control and asthmatic ASM cells was determined by densitometry (normalized to β-actin, $n = 3$–7 donors/group). WB, Western blot.
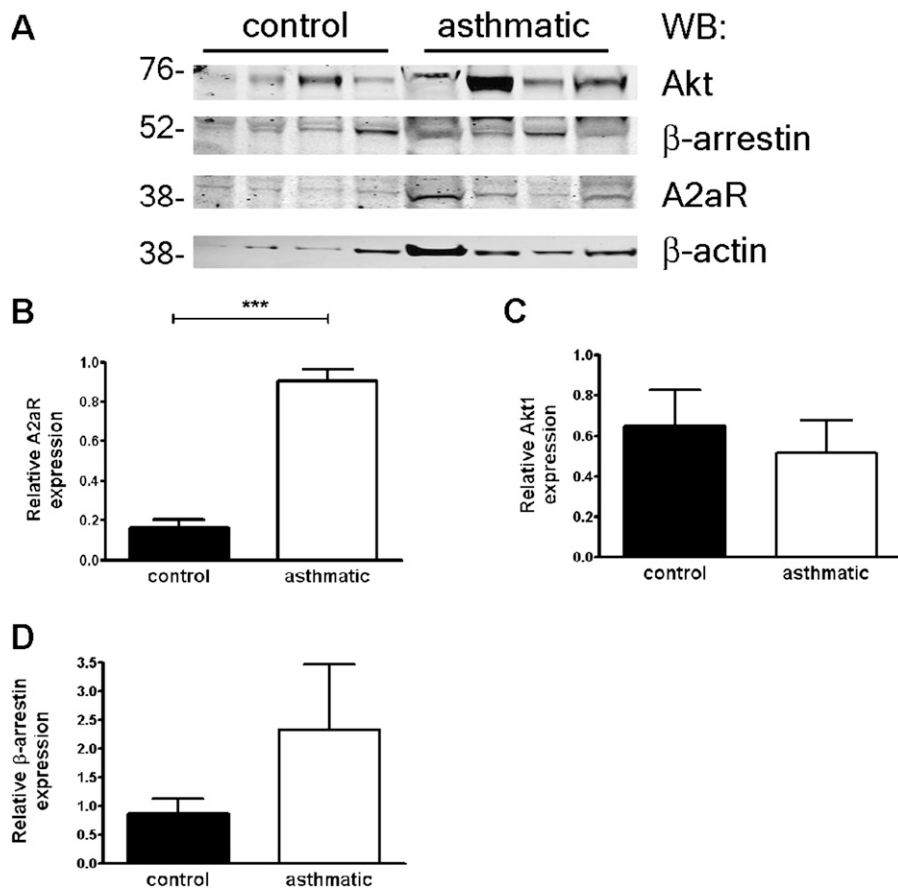
Figure 3. Protein expression in asthmatic and nonasthmatic ASM cells. (*A–D*) Relative expression of proteins (adenosine A2a [A2a] receptors, Akt1, and β-arrestin) in control and asthmatic ASM cells was determined by densitometry (mean ± SEM normalized to β-actin, $n = 3–7$ donors/group). ***$P = 0.0001$, unpaired t test.

GPCRs, is often dynamically regulated in diseased tissue or in response to environmental stimuli (18). We hypothesized that RGS expression could be altered in asthmatic ASM, and specifically RGS5, which we previously identified as a physiological, relevant modulator of GPCR signaling in healthy ASM cells (19). Similar to control cells, ASM from asthmatic cells predominantly expressed RGS 2–5 (Figure 4A). Because unique primer pairs detected individual RGS gene expressions, relative amounts of each could not be compared by this method. We evaluated the expression of specific RGSs individually by real-time PCR. Although quantities of RGS 2, 3, and 4 were similar in ASM cells from healthy donors and from donors with asthma (Figures 4B–4D), RGS5 mRNA was significantly increased in asthmatic ASM relative to control ASM (Figure 4E). To determine whether RGS5 protein expression was increased in cells from subjects with asthma relative to subjects without asthma, we prepared lysates from cultured ASM cells and analyzed RGS5 quantitatively by immunoblotting. Concentrations of RGS5 were significantly increased in ASM cells from several patients with asthma, compared with control subjects (Figures 5A and 5B).

Increased RGS5 Expression in the Asthmatic Lung

To ascertain whether the changes in amounts of RGS5 in ASM cells from subjects with asthma correlated with the expression of RGS5 *in vivo*, we measured RGS5 mRNA in bronchial tissue from subjects with asthma and subjects without asthma by quantitative PCR. RGS5 was significantly increased in asthmatic lung tissue relative to control lung tissue (Figure 6A). To determine which cells express RGS5 protein *in vivo*, we evaluated endobronchial biopsies from subjects with asthma and age-matched healthy control subjects by immunohistochemistry. We obtained samples from patients with severe asthma according to

criteria established by the Global Initiative for Asthma (Table 2). RGS5 was expressed predominantly in ASM bundles *in situ*, and bronchial smooth muscle from subjects with asthma demonstrated significantly increased numbers of RGS5$^+$ ASM cells compared with healthy subjects (Figure 6B). Furthermore, RGS5 mRNA concentrations were 6-fold higher in whole-lung tissue from allergen-sensitized and allergen-challenged mice compared with naive mice (Figure 6C), and RGS5 was detected in lung ASM of challenged mice (Figure 6D). Lung epithelium also stained with the RGS5 antibody in wild-type (WT) mice. However, similar epithelial staining was present in lung sections from in $Rgs5^{-/-}$ mice (Figure E1 in the online supplement). Thus, the expression of RGS5 in murine lungs is restricted to ASM and is up-regulated *in vivo* in the setting of asthmatic/allergic pulmonary inflammation in both mice and humans.

RGS5 Overexpression Inhibits Ca^{2+} Release in ASM and Bronchial Contractions

To assess the functional significance of elevated RGS5 expression in the bronchi of subjects with asthma for smooth muscle Ca^{2+} signaling, we expressed RGS5 in ASM cells from healthy donors by lentiviral transduction. The overexpression of RGS5 in ASM cells (Figure 7A) led to a greater than 50% reduction in Ca^{2+} mobilization in response to bradykinin, thrombin, and histamine (Figure 7B). Because we could not detect Ca^{2+} flux in ASM stimulated with carbachol, most likely attributable to the previously described down-regulation of M3 muscarinic receptors in ASM cultures (23) (Figure 7C), we expressed RGS5 in HEK293T cells, which have endogenous M3 receptors. Compared with cells expressing empty vector, RGS5-overexpressing cells showed significantly reduced Ca^{2+} after stimulation with carbachol (Figure 7D).
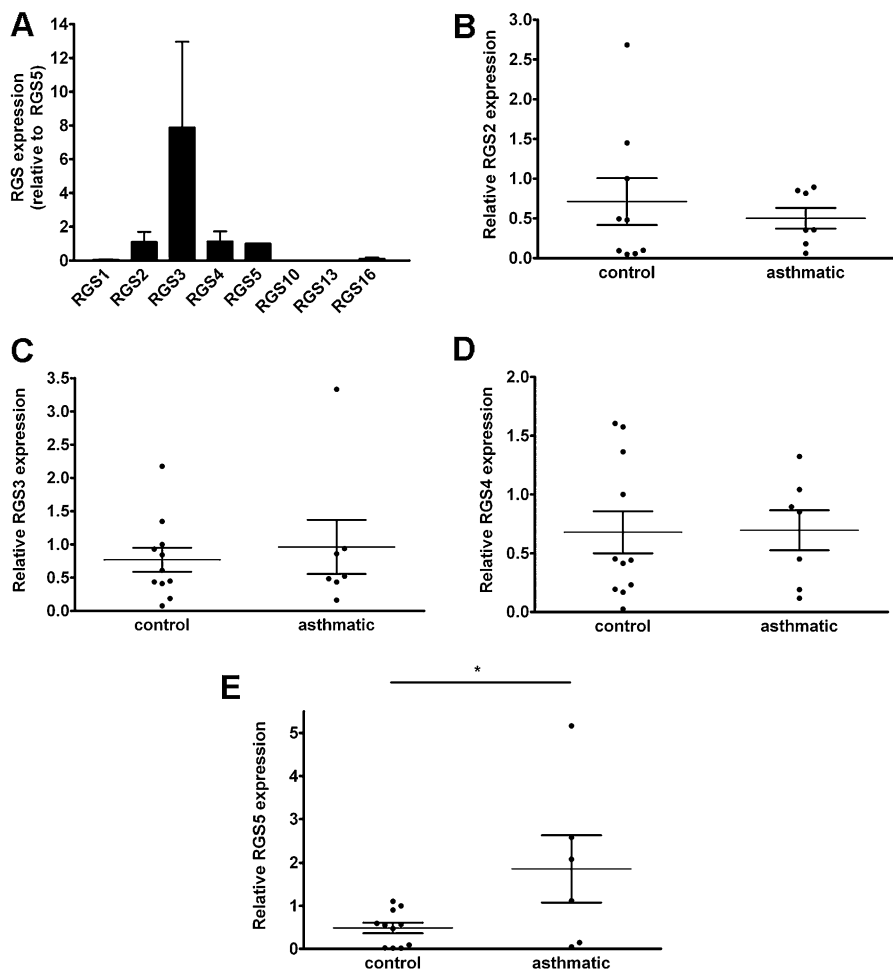
Figure 4. Regulator of G-protein signaling (RGS) expression in asthmatic ASM. (*A*) Expression of RGS mRNAs was determined in ASM from subjects with asthma by real-time PCR (mean ± SEM of seven donors relative to RGS5, set as 1). (*B–E*) Expression of individual RGS mRNAs in asthmatic and nonasthmatic ASM cells was determined by real-time PCR (mean ± SEM relative to a single control donor, set as 1). *$P =$ 0.03, unpaired t test.

To determine how RGS5 overexpression affected GPCR-evoked contractility, we transduced precision-cut lung slices (PCLS) with lentivirus encoding RGS5, and measured airway luminal narrowing by microscopy. Because our previous work indicated that these lung slices do not contract well to agonists other than carbachol (data not shown) (24), we measured airway contraction in response to carbachol. RGS5 mRNA concentrations were significantly increased in lung slices transduced with RGS5-encoding lentivirus compared with control virus (LacZ) (Figure 7E). The overexpression of RGS5 significantly reduced the maximal contraction of PCLS elicited by carbachol (Figure 7F). In concordance with our previous study using PCLS from RGS5-deficient mice (19), the overexpression of RGS5 did not affect the potency of carbachol (log[EC_{50}], 0.68 ± 0.1 μM versus 0.73 ± 0.14 μM), but reduced the maximal contraction (E_{max}, 94% ± 3.3% versus 80.2% ± 3.4%; $P = 0.007$). Thus, RGS5 inhibits the contraction of human bronchi in response to muscarinic receptor activation.

DISCUSSION

Although allergic inflammation has a central function in the establishment and maintenance of airway hyperresponsiveness (AHR) in asthma, only 50% of patients with asthma are atopic (25). Moreover, the degree of inflammation present in asthmatic lungs may not correlate with the degree of AHR (26). Such findings suggest that intrinsic abnormalities in lung structural cells, including ASM, merit distinct consideration in severe or fatal disease. Unfortunately, only a few studies have addressed

the excitation–contraction coupling and contractile function of ASM cells because of the difficulties in obtaining ASM cells in the setting of fatal asthma (death from asthma is relatively rare), and because of the lack of adequate techniques to address the
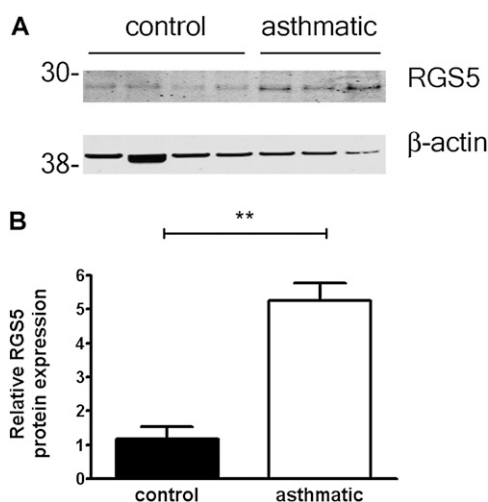
Figure 5. RGS5 protein is up-regulated in asthmatic ASM cells. (*A*) Expression of RGS5 in asthmatic and nonasthmatic ASM cells was evaluated by immunoblotting. (*B*) Relative expression of RGS5 in control and asthmatic ASM cells was determined by densitometry (mean ± SEM of four control donors and three donors with asthma, normalized to β-actin). **$P = 0.003$, unpaired t test.
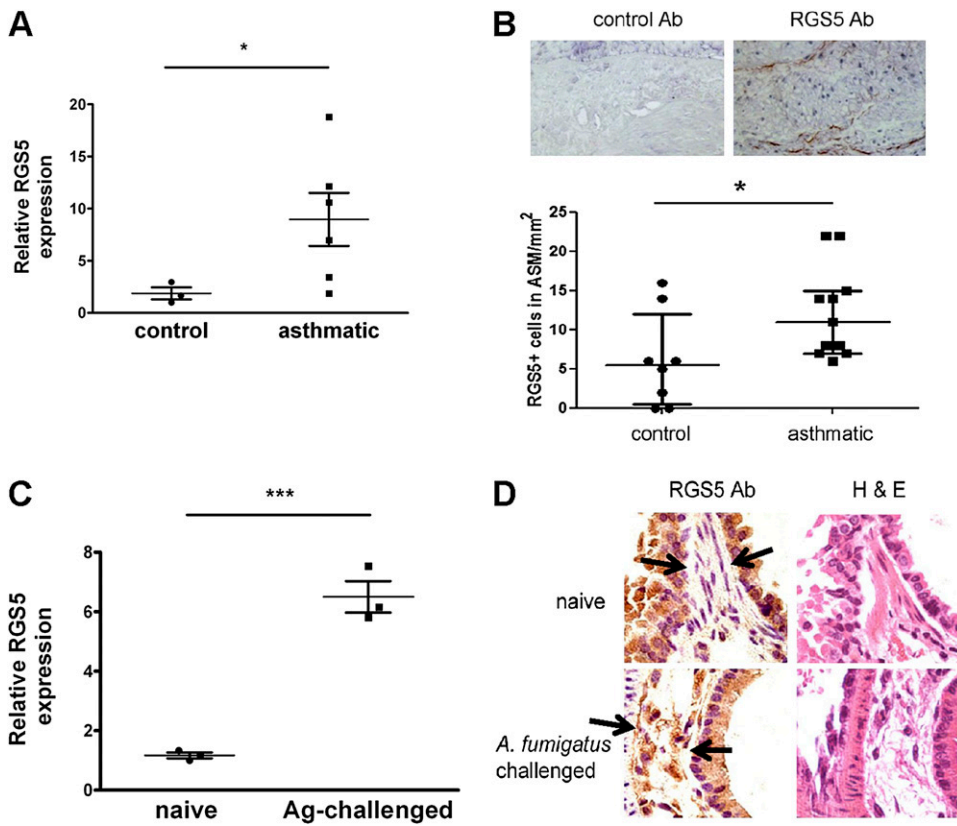
Figure 6. Increased RGS5 expression in asthmatic bronchi. (A) RGS5 mRNA expression in bronchial tissue from donors with and without asthma (Table 2) was evaluated by real-time PCR (mean ± SEM of 3–6 donors). *$P = 0.04$, Mann-Whitney U test. (B) Representative photomicrographs of a bronchial biopsy from a donor with asthma (magnification, ×200), stained with either isotype control antibody (Ab; left) or RGS5 antibody (right), illustrating RGS5+ cells within the ASM bundle (dot-plot of the number of RGS5+ cells/mm² ASM). Horizontal bars represent the medians and interquartile ranges. *$P = 0.028$, Mann-Whitney U test. (C) RGS5 mRNA expression in bronchial tissue from PBS-challenged or Af-challenged mice was evaluated by real-time PCR (mean ± SEM of three mice/group). Ag, allergen. ***$P = 0.0006$, unpaired t test. (D) Paraffin-embedded sections of lungs from naive and allergen (Aspergillus fumigatus)-challenged mice were evaluated by immunohistochemistry, using an RGS5 antibody. Images (magnification, ×40) are derived from a single mouse, representative of three mice/group, and RGS5 staining is brown. H&E, hematoxylin and eosin. Arrows show the sites of RGS5 expression.

contractile function of ASM cells in vitro. Here we studied upstream, receptor-related events leading to cellular contraction (Ca^{2+} flux) and the expression of components of procontractile GPCR pathways in ASM cells from patients with and without asthma, in an attempt to uncover new or unique therapeutic targets.

Our experiments revealed differences in GPCR-evoked Ca^{2+} mobilization in asthmatic and nonasthmatic ASM cells. We found reduced peak Ca^{2+} concentrations after stimulation with histamine, but not bradykinin or thrombin, in asthmatic ASM cells compared with nonasthmatic ASM cells. Our results differ from those of Mahn and colleagues (27), who found reduced responses of asthmatic ASM to bradykinin. They observed that the recovery of Ca^{2+} concentrations to baseline was delayed in asthmatic cells, which they attributed to depleted sarcoplasmic reticulum Ca^{2+} stores resulting from decreased SERCA concentrations. In contrast, we detected equivalent responses to the SERCA inhibitor thapsigargin and similar kinetics of Ca^{2+} recovery after the GPCR stimulation of healthy and asthmatic ASM cells. These results indicate that Ca^{2+} stores were comparable in our samples. Our study and that of Mahn and colleagues contain the key difference that Mahn and colleagues (27) obtained bronchial specimens from subjects with mild to moderate asthma, whereas we studied ASM cells from bronchi obtained postmortem from patients who died of asthma.

We found that the total expression of receptor-stimulated signaling components such as Gαq and PLCβ1 was equivalent in our ASM samples, whereas amounts of bradykinin B2 and adenosine A2a receptor were significantly increased in asthmatic cells relative to control cells. We are unaware of any extensive comparison of GPCR expression in asthmatic and nonasthmatic ASM cells reported by others, and B2 receptor concentrations were not assessed in the study of Mahn and colleagues (27). Cytokines, including IFN-γ and TGF-β, which

are both increased in asthmatic airways (5), up-regulate ASM GPCR expression in cultured ASM cells (cysteinyl leukotriene D1 and B2, respectively) (28, 29). Asthmatic ASM cells also express higher concentrations of C-C chemokine receptor 3, which may mediate migration and cytokine secretion (30). Although the amounts of Gαq were increased in bronchial tissue from allergen-sensitized and challenged mice in a previous study, we observed no changes in Gαq expression in asthmatic human ASM cells compared with control cells. These findings are similar to those of McGraw and colleagues (31), who demonstrated unchanged Gαq concentrations in Gαi2 inhibitory peptide transgenic mice, which demonstrate increased AHR relative to wild-type mice in the absence of allergic inflammation. Currently, the physiological significance of altered G-protein expression in these models has not been clearly established.

Because no obvious reductions in receptor, G-protein, or effector expression could account for the impaired histamine-evoked Ca^{2+} mobilization in asthmatic ASM cells, we analyzed the expression of other genes that could potentially regulate excitation–contraction pathways. Consistent with the proliferative phenotype of asthmatic cells observed by others (8), the expression of cyclins and antiapoptosis factors (Akt, Elk1/4, and Bcl2) was substantially increased compared with that in control cells. In addition, asthmatic cells displayed a synthetic/secretory phenotype, as evidenced by the up-regulation of cytokines and matrix-modifying enzymes such as MMP9, VEGF, C-C chemokine ligand 2, and connective tissue growth factor, among others, as shown in previous studies (5, 32). We also detected increased expression of adenosine A2a and sphingosine-1–phosphate receptors in asthmatic ASM cells, which were reported by others to affect bronchial smooth muscle tone (33–35), suggesting physiological relevance to asthma.

To sum up most important finding of this study, the expression of RGS5 mRNA and protein was significantly up-regulated in
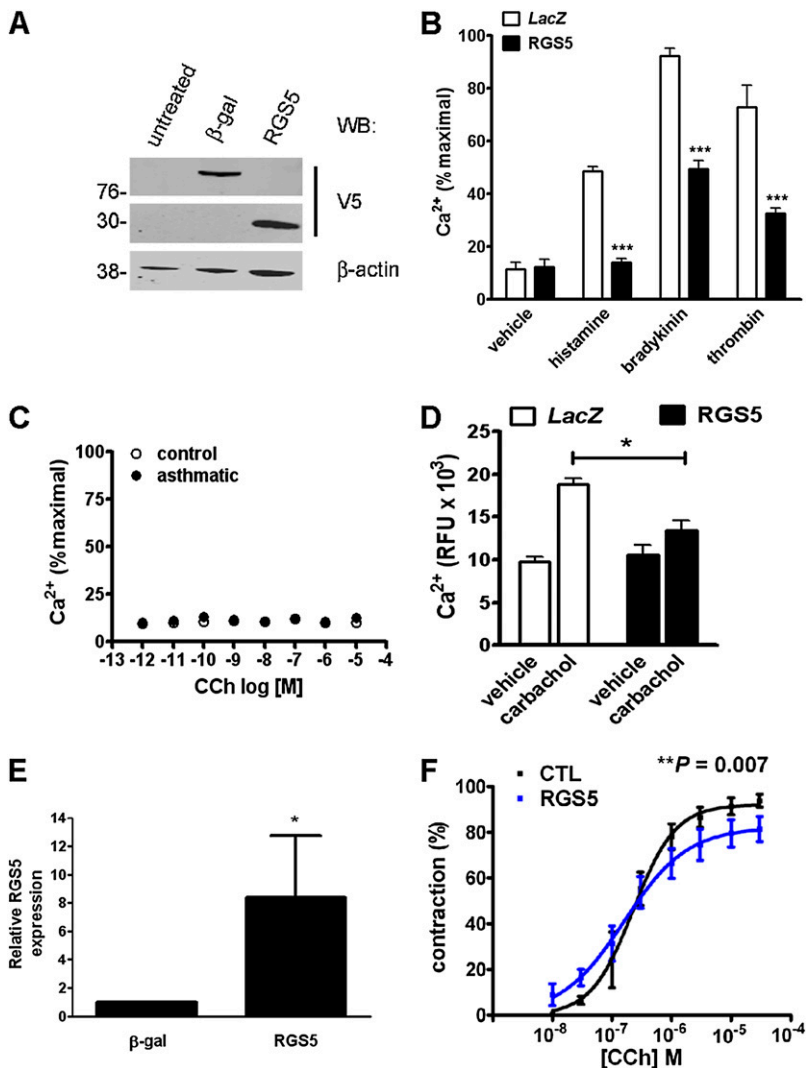
Figure 7. RGS5 inhibits GPCR-induced Ca^{2+} mobilization and airway contraction. (*A* and *B*) ASM derived from healthy donors was left untreated or was transduced with lentivirus encoding β-galactosidase or RGS5. (*A*) The expression of recombinant proteins was determined by immunoblotting cell lysates with anti-V5 antibody. β-gal, β-galactosidase. (*B*) Control or lentivirus-transduced cells were stimulated with histamine (10 μM), bradykinin (100 nM), or thrombin (1 U/ml), followed by measurement of intracellular Ca^{2+} concentrations, as in Figure 1. Data represent the mean ± SEM of two independent experiments performed in quadruplicate, using cells derived from two healthy donors. ***$P < 0.001$, one-way ANOVA. (*C*) Human ASM cells were stimulated with various concentrations of carbachol (CCh), followed by measurement of intracellular Ca^{2+} concentrations by fluorimetry. Graphs represent the mean ± SEM of two independent experiments performed in quadruplicate, using cells derived from two individual donors. (*D*) HEK293T cells were transfected with plasmids encoding RGS5 or β-galactosidase (control), followed by the measurement of Ca^{2+} flux after stimulation with carbachol (100 μM) or vehicle alone. Data represent the mean ± SEM of two independent experiments, measured in quadruplicate. ***$P < 0.01$, two-way ANOVA. (*E*) Precision-cut human lung slices (PCLS) were prepared as described in MATERIALS AND METHODS. The expression of RGS5 in slices was determined by quantitative PCR. *$P = 0.01$, Mann-Whitney U test. (*F*) PCLS were contracted to various concentrations of carbachol, followed by measurement of airway luminal diameter by microscopy. The data represent mean ± SEM of 3–4 airways in each group, as performed in two independent experiments. CTL, control.

asthmatic ASM compared with nonasthmatic cells and lung tissue. Asthmatic ASM cells displayed reduced histamine responses relative to nonasthmatic ASM cells, but had comparable to responses to bradykinin and thrombin. These results suggest that RGS5 is a regulator of histamine-mediated contractile signaling in asthmatic ASM, but may not couple to bradykinin or PAR1 receptors in these cells. Our previous work demonstrated that short interfering (si)RNA-mediated RGS5 knockdown in healthy ASM cells augmented thrombin responses (19). In a separate study, RGS5 specifically inhibited angiotensin II type 1A receptor–mediated but not muscarinic M3 receptor–mediated ERK activation in vascular smooth muscle cells. In contrast, Anger and colleagues found that RGS5 impaired carbachol-mediated IP3 formation and ERK activation in COS-7 cells expressing M3 receptors (36). Collectively, these results suggest the receptor-selective, and perhaps cell type–specific, regulation of signaling by RGS5 (37).

We also demonstrated that the overexpression of RGS5 inhibited PCLS contraction to carbachol. A current limitation of our PCLS transfection involved the nonselective overexpression/knockdown in the slices, and future studies using lentivirus driven by a smooth muscle–specific promoter will allow us to achieve selective expression in ASM. However, agonist-induced bronchoconstriction is visualized directly, which is mediated by ASM contraction. Although the contribution of factors secreted by

epithelial cells or other cells cannot be formally excluded, the sections are aggressively washed immediately before the addition of an agonist, which induces a reduction in airway diameter within minutes. We showed that RGS5 overexpression blunts the Ca^{2+} signaling evoked by procontractile ligands, including carbachol (Figures 7B and 7D). In addition, because we observed previously that PCLS from $Rgs5^{-/-}$ mice contracted significantly more to carbachol than did airways from WT mice (19), these studies indicate that RGS5 is a physiologically relevant modulator of airway contraction.

We also determined previously that the prolonged exposure of ASM to β-adrenergic agonists down-regulates RGS5 expression (19). Thus, we may be underestimating the extent of RGS5 up-regulation in ASM from patients with severe asthma who died of asthma, given that such patients were probably exposed to sustained high doses of β-agonist. What mechanisms might underlie the increased RGS5 quantities in asthmatic ASM? RGS5 is up-regulated in arterial smooth muscle in models of skin wound–healing and tumor angiogenesis (38), suggesting that extracellular matrix–modifying enzymes and growth factors linked to airway remodeling in asthma also modulate RGS5 expression. Inflammatory cytokines (Th2-related, IL-1, and TNF-α) also directly influence GPCR responsiveness (39). IL-13 increases the Ca^{2+} responsiveness of ASM to histamine, bradykinin, and acetylcholine

(Ach), and the contraction of tracheas to Ach (40). Although our preliminary work indicates that IL-13 does not affect RGS5 expression in ASM (data not shown), numerous cytokines and chemokines modify RGS protein transcription in other cell types (18).

Finally, whether the up-regulation of RGS5 in asthmatic ASM is beneficial or maladaptive remains unclear. Recently, Li and colleagues demonstrated that transgenic mice expressing a cardiac-specific *Rgs5* transgene were resistant to hypertrophic cardiomyopathy and fibrosis, whereas $Rgs5^{-/-}$ mice were more sensitive to pressure overload–induced cardiomyopathy than were WT mice (41). The up-regulation of RGS5 could be a compensatory event that protects ASM from chronic hyperstimulation in asthma. Future studies of bronchial contraction in mice with varying amounts of RGS5 expression in models of allergic and nonallergic pulmonary inflammation will be needed to address this and other issues more fully.

References

1. Hershenson MB, Brown M, Camoretti-Mercado B, Solway J. Airway smooth muscle in asthma. *Annu Rev Pathol* 2008;3:523–555.
2. Gil FR, Lauzon AM. Smooth muscle molecular mechanics in airway hyperresponsiveness and asthma. *Can J Physiol Pharmacol* 2007;85:133–140.
3. Halayko AJ, Amrani Y. Mechanisms of inflammation-mediated airway smooth muscle plasticity and airways remodeling in asthma. *Respir Physiol Neurobiol* 2003;137:209–222.
4. An SS, Bai TR, Bates JH, Black JL, Brown RH, Brusasco V, Chitano P, Deng L, Dowell M, Eidelman DH, *et al.* Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. *Eur Respir J* 2007;29:834–860.
5. Tran T, Halayko AJ. Extracellular matrix and airway smooth muscle interactions: a target for modulating airway wall remodelling and hyperresponsiveness? *Can J Physiol Pharmacol* 2007;85:666–671.
6. Bentley JK, Hershenson MB. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. *Proc Am Thorac Soc* 2008;5:89–96.
7. Seow CY, Schellenberg RR, Pare PD. Structural and functional changes in the airway smooth muscle of asthmatic subjects. *Am J Respir Crit Care Med* 1998;158:S179–S186.
8. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, Burgess JK, Black JL. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001;164:474–477.
9. Halayko AJ, Salari H, Ma X, Stephens NL. Markers of airway smooth muscle cell phenotype. *Am J Physiol* 1996;270:L1040–L1051.
10. Black JL, Roth M. Intrinsic asthma: is it intrinsic to the smooth muscle? *Clin Exp Allergy* 2009;39:962–965.
11. John AE, Zhu YM, Brightling CE, Pang L, Knox AJ. Human airway smooth muscle cells from asthmatic individuals have CXCl8 hypersecretion due to increased NF-kappa B p65, C/EBP beta, and RNA polymerase II binding to the CXCl8 promoter. *J Immunol* 2009;183:4682–4692.
12. Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* 2001;28:233–236.
13. Chan V, Burgess JK, Ratoff JC, O'Connor BJ, Greenough A, Lee TH, Hirst SJ. Extracellular matrix regulates enhanced eotaxin expression in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* 2006;174:379–385.
14. Kaur D, Saunders R, Berger P, Siddiqui S, Woodman L, Wardlaw A, Bradding P, Brightling CE. Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. *Am J Respir Crit Care Med* 2006;174:1179–1188.
15. Tliba O, Panettieri RA Jr. Noncontractile functions of airway smooth muscle cells in asthma. *Annu Rev Physiol* 2009;71:509–535.
16. Liu W, Liang Q, Balzar S, Wenzel S, Gorska M, Alam R. Cell-specific activation profile of extracellular signal–regulated kinase 1/2, Jun

17. N-terminal kinase, and p38 mitogen–activated protein kinases in asthmatic airways. *J Allergy Clin Immunol* 2008;121:893–902.
17. Deshpande DA, Penn RB. Targeting G protein–coupled receptor signaling in asthma. *Cell Signal* 2006;18:2105–2120.
18. Bansal G, Druey KM, Xie Z. R4 RGS proteins: regulation of G-protein signaling and beyond. *Pharmacol Ther* 2007;116:473–495.
19. Yang Z, Cooper PR, Damera G, Mukhopadhyay I, Cho H, Kehrl JH, Panettieri RA Jr, Druey KM. {Beta}-agonist associated reduction in RGS5 expression promotes airway smooth muscle hyperresponsiveness. *J Biol Chem* 2011;286:11444–11455.
20. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346:1699–1705.
21. Panettieri RA, Murray RK, DePalo LR, Yadvish PA, Kotlikoff MI. A human airway smooth muscle cell line that retains physiological responsiveness. *Am J Physiol* 1989;256:C329–C335.
22. Abraham WM, Scuri M, Farmer SG. Peptide and non-peptide bradykinin receptor antagonists: role in allergic airway disease. *Eur J Pharmacol* 2006;533:215–221.
23. Gosens R, Nelemans SA, Grootte Bromhaar MM, McKay S, Zaagsma J, Meurs H. Muscarinic M3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. *Am J Respir Cell Mol Biol* 2003;28:257–262.
24. Ressmeyer AR, Larsson AK, Vollmer E, Dahlen SE, Uhlig S, Martin C. Characterisation of guinea pig precision-cut lung slices: comparison with human tissues. *Eur Respir J* 2006;28:603–611.
25. Beasley R, Pekkanen J, Pearce N. Has the role of atopy in the development of asthma been over-emphasized? *Pediatr Pulmonol* 2001;149–150.
26. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998;157:4–9.
27. Mahn K, Hirst SJ, Ying S, Holt MR, Lavender P, Ojo OO, Siew L, Simcock DE, McVicker CG, Kanabar V, *et al.* Diminished sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) expression contributes to airway remodelling in bronchial asthma. *Proc Natl Acad Sci USA* 2009;106:10775–10780.
28. Amrani Y, Moore PE, Hoffman R, Shore SA, Panettieri RA Jr. Interferon-gamma modulates cysteinyl leukotriene receptor–1 expression and function in human airway myocytes. *Am J Respir Crit Care Med* 2001;164:2098–2101.
29. Kim JH, Jain D, Tliba O, Yang B, Jester WF Jr, Panettieri RA Jr, Amrani Y, Pure E. TGF-beta potentiates airway smooth muscle responsiveness to bradykinin. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L511–L520.
30. Joubert P, Lajoie-Kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V, Chakir J, Laviolette M, Hamid Q, Lamkhioued B. CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* 2005;175:2702–2708.
31. McGraw DW, Elwing JM, Fogel KM, Wang WC, Glinka CB, Mihlbachler KA, Rothenberg ME, Liggett SB. Crosstalk between G_i and G_q/G_s pathways in airway smooth muscle regulates bronchial contractility and relaxation. *J Clin Invest* 2007;117:1391–1398.
32. Simcock DE, Kanabar V, Clarke GW, Mahn K, Karner C, O'Connor BJ, Lee TH, Hirst SJ. Induction of angiogenesis by airway smooth muscle from patients with asthma. *Am J Respir Crit Care Med* 2008;178:460–468.
33. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International union of pharmacology: XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 2001;53:527–552.
34. Rosenfeldt HM, Amrani Y, Watterson KR, Murthy KS, Panettieri RA Jr, Spiegel S. Sphingosine-1–phosphate stimulates contraction of human airway smooth muscle cells. *FASEB J* 2003;17:1789–1799.
35. Roviezzo F, Di Lorenzo A, Bucci M, Brancaleone V, Vellecco V, De Nardo M, Orlotti D, De Palma R, Rossi F, D'Agostino B, *et al.* Sphingosine-1–phosphate/sphingosine kinase pathway is involved in mouse airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 2007;36:757–762.
36. Anger T, Klintworth N, Stumpf C, Daniel WG, Mende U, Garlichs CD. RGS protein specificity towards G_q- and $G_{i/o}$-mediated ERK 1/2 and AKT activation, *in vitro*. *J Biochem Mol Biol* 2007;40:899–910.
37. Wang Q, Liu M, Mullah B, Siderovski DP, Neubig RR. Receptor-selective effects of endogenous RGS3 and RGS5 to regulate

mitogen-activated protein kinase activation in rat vascular smooth muscle cells. *J Biol Chem* 2002;277:24949–24958.

38. Gu S, Cifelli C, Wang S, Heximer SP. RGS proteins: identifying new gaps in the understanding of blood pressure regulation and cardiovascular function. *Clin Sci (Lond)* 2009;116:391–399.

39. Amrani Y, Panettieri RA Jr. Modulation of calcium homeostasis as a mechanism for altering smooth muscle responsiveness in asthma. *Curr Opin Allergy Clin Immunol* 2002;2:39–45.

40. Tliba O, Deshpande D, Chen H, Van Besien C, Kannan M, Panettieri RA Jr, Amrani Y. IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. *Br J Pharmacol* 2003; 140:1159–1162.

41. Li H, He C, Feng J, Zhang Y, Tang Q, Bian Z, Bai X, Zhou H, Jiang H, Heximer SP, *et al*. Regulator of G protein signaling 5 protects against cardiac hypertrophy and fibrosis during biomechanical stress of pressure overload. *Proc Natl Acad Sci USA* 2010;107:13818–13823.