Crosstalk between $G_i$ and $G_q/G_s$ pathways in airway smooth muscle regulates bronchial contractility and relaxation

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Receptor-mediated airway smooth muscle (ASM) contraction via $G_{q3}$ and relaxation via $G_{i3}$ underlie the bronchospastic features of asthma and its treatment. Asthma models show increased ASM $G_{i3}$ expression, considered the basis for the proasthmatic phenotypes of enhanced bronchial hyperreactivity to contraction mediated by $M_i$-muscarinic receptors and diminished relaxation mediated by $\beta_2$-adrenergic receptors ($\beta_2$ARs). A causal effect between $G_i$ expression and phenotype has not been established, nor have mechanisms whereby $G_i$ modulates $G_q/G_s$ signaling. To delineate isolated effects of altered $G_i$, transgenic mice were generated overexpressing $G_{i3}$ or a $G_{i2}$ peptide inhibitor in ASM. Unexpectedly, $G_{i2}$ overexpression decreased contractility to methacholine, while $G_{i2}$ inhibition enhanced contraction. These opposite phenotypes resulted from different crosstalk loci within the $G_q$ signaling network: decreased phospholipase C and increased PKCζ, respectively. $G_{i2}$ overexpression decreased $\beta_2$AR-mediated airway relaxation, while $G_{i2}$ inhibition increased this response, consistent with physiologically relevant coupling of this receptor to both $G_i$ and $G_s$. IL-13 transgenic mice (a model of asthma), which developed increased ASM $G_{i3}$, displayed marked increases in airway hyperresponsiveness when $G_{i3}$ function was inhibited. Increased $G_{i3}$ in asthma is therefore a double-edged sword: a compensatory event mitigating against bronchial hyperreactivity, but a mechanism that evokes $\beta$-agonist resistance. By selective intervention within these multipronged signaling modules, advantageous $G_i/G_q$ activities could provide new asthma therapies.

Introduction

Airway smooth muscle (ASM) contraction and relaxation are primarily regulated by G protein–coupled receptors, the former mediated by receptors signaling to $G_s$, and the latter by those that couple to $G_i$ (1, 2). Many inflammatory cascades in asthma evoke bronchoconstriction by promoting local increases of $G_s$ receptor agonists such as acetylcholine, cysteinyl leukotrienes, prostaglandins, and histamine, which activate their cognate receptors expressed on ASM. There appear to be fewer $G_i$-coupled receptors that act via endogenous agonists to counteract bronchoconstriction, but the $\beta_2$-adrenergic receptor ($\beta_2$AR) of ASM is the target of pharmacologically administered $\beta$-agonists and is typically highly effective in relaxing constricted airways. The molecular events and critical transduction elements for these 2 classes of receptors are well recognized. Agonist binding to receptors such as the $M_i$-muscarinic receptor promote dissociation of heterotrimeric $G_s$ into $G_s$ and $G_{ir}$ subunits, with the $\alpha$ subunit activating phospholipase C (PLC; which promotes inositol-3-phosphate and diacylglycerol production) and the latter activating PKC. Receptors such as the $\beta_2$AR act via $G_{i3}$ to stimulate the effector adenyl cyclase, resulting in cAMP production and activation of PKA. Substantial interest has revolved around how these pathways might be modified in asthma, because there may be nodal points which are critical for the pathogenesis of bronchospasm, or may be particularly amenable for pharmacologic intervention. A number of studies have shown, somewhat surprisingly, that a third major class of G proteins, $G_i$ (inhibitory guanine nucleotide binding protein $\alpha$ subunits 2 and 3; $G_{i2}$ and $G_{i3}$, respectively), is increased in ASM in animal models of asthma. Early studies by Grunstein and colleagues (3) showed that rabbit tracheal smooth muscle that was passively sensitized with serum from atopic asthmatics had enhanced ex vivo contraction to acetylcholine and decreased relaxation to the $\beta$-agonist isoproterenol. These events were temporally related to increased $G_s$ protein expression, and these characteristics were attenuated by pertussis toxin (PTX), which inactivates $G_{i3}$.

Other studies have shown that cytokine exposure (4, 5) and rhinovirus infection (6) give this same physiologic phenotype, which is accompanied by a several-fold increase in ASM $G_{i3}$ expression. It remains unclear how the 2 proasthmatic phenotypes of hyperresponsiveness to bronchoconstriction and resistance to bronchodilatation, which are mediated by $G_i$, and $G_s$-coupled receptors, respectively, could be influenced by the cellular expression levels of $G_i$. Recent studies in recombinant cells have shown, however, that $\beta_2$AR, once phosphorylated by PKA, has the capacity to couple to $G_i$ (7). Given that $G_{i3}$ inhibits adenyl cyclase, this dual coupling may serve to attenuate the cAMP response. However, the magnitude of the physiologic effect in the airway of this coupling “switch” is not known, nor is it clear whether modest changes in $G_i$ could amplify or modify in some other way this bifurcated cou-

Nonstandard abbreviations used: AR, adrenergic receptor; ASM, airway smooth muscle; $G_{i3}$-IP, $G_{i3}$ inhibitory peptide expression; $G_{i3}$-OE, $G_{i3}$ overexpression; GRK2, G protein–coupled receptor kinase 2; NTG, nontransgenic littermate; PLC, phospholipase C; PTX, pertussis toxin; SMP8, smooth muscle actin promoter 8.

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Figure 1
Induction of IL-13 in mouse airways increases Gαi2 expression. Three-week-old bitransgenic mice generated to have doxycycline-inducible expression of IL-13 targeted to the airways by the CC10 promoter were fed normal food (–Dox) or doxycycline-impregnated food (+Dox) for 3 weeks. Tracheae were homogenized, and membrane preparations were subjected to Western blots performed with Gαi2 and GAPDH antibodies. Induction of IL-13 increased Gαi2 expression by approximately 8-fold.

Results

Gαi2 is increased in a genetic model of asthma, and PTX alters airway responsiveness. To assess whether the findings by Grunstein and colleagues of increased Gαi2 in rabbit models of asthma (3) are true for another model, we examined expression of Gαi2 in tracheae from the inducible IL-13–overexpressing mouse (9). In these mice IL-13 is targeted to the airway by the Clara cell secretory protein (CCSP) promoter, with induction by 3 weeks of doxycycline administration; they develop inflammation, bronchial hyperresponsiveness to methacholine, fibrosis, and airspace enlargement (9, 10). Figure 1 shows that tracheal expression of Gαi2 assessed by Western blot increased approximately 8-fold upon IL-13 induction in these transgenic mice. These data pointed toward increased Gαi as a common feature of asthmatic-like airways from passive sensitization with asthma serum (3), cytokines (4, 5), and rhinovirus exposure (6) as well as increased IL-13 production. However, these prior studies do not indicate whether isolated changes in Gα levels influence airway contraction or relaxation responses, nor do they implicate the increase in Gαi in these models as a mechanism that underlies altered airway signaling or address whether the increase represents a compensatory event. We thus proceeded with screening studies using in vivo PTX, which ablates Gαi coupling by ADP-ribosylation of the α subunit. Wild-type mice were treated with i.p. PTX or with vehicle, and 18 hours later airway resistance was measured in the intact, ventilated mouse using previously described methods (8, 11). Unexpectedly, PTX treatment increased the contractile response to methacholine (mediated by the Gαi–coupled M1–muscarinic receptor) as shown by both an increased maximal response and a left-shifted dose-response curve (Figure 2A). To assess βAR-mediated relaxation, mice were pretreated with aerosolized isoproterenol prior to methacholine challenge. With PTX treatment, βAR signaling appeared to be enhanced, in that there was little contraction to methacholine under these conditions (Figure 2B). While these findings potentially implicate Gαi function as an important element in airway contraction and relaxation, the use of systemic PTX is not specific for ASM and does not provide information as to the effects of increased Gαi expression, as is found in the aforementioned asthma models. These limitations led to the generation of transgenic mice Gαi2–OE (incorporating the full-length human Gαi2 cDNA) and Gαi2–IP (a “minigene” peptide consisting of the C-terminal portion of Gαi2) targeted to ASM.

Transgenic expression of Gαi2 and a Gαi2 competitive inhibitor. Transgenic mice were generated using smooth muscle actin promoter 8 (SMP8); the injected constructs consisted of the full-length cDNA for human Gαi2 or a cDNA encoding the carboxy terminus of human Gαi2 (58 amino acids), which also included a 5′ HA-tag (see Figure 2)
Methods). Mice were screened for the transgene by PCRs using genomic DNA derived from digested tails. Transgenic mice had a normal body weight and habitus, and their viability was the same as nontransgenic littermate (NTG) mice. To ascertain whether genomic DNA derived from digested tails. Transgenic mice had a signal). Although we were not able to reproducibly detect protein loaded to obtain a reasonable visual comparison with the NTG expressors (Figure 3B; note that the Gαi2 protein in ASM lysates from Gαi2-OE cells. Note that the protein from Gαi2-OE cells was underloaded by 10-fold compared with NTG cells, as shown by the GAPDH signals. (C) Histopathologic specimens from the lungs of the NTG and 2 representative transgenic lines. Shown are hematoxylin and eosin–stained (upper panels) and trichrome-stained (lower panels) representative sections. Original magnification, ×20.

Effects of altering Gαi2 by transgenesis on airway contractile and relaxation responses. Baseline airway resistance did not differ among Gαi2-OE, Gαi2-IP, and NTG mice (0.53 ± 0.061 cm H2O/ml/s, 0.58 ± 0.036 cm H2O/ml/s, and 0.57 ± 0.041 cm H2O/ml/s, respectively; n = 6–9; data not shown). However, Gαi2-OE mice exhibited a depressed contractile response (P < 0.005) to methacholine, primarily due to a 50% decrease in the maximal response compared to the maximal response observed in NTG mice (Figure 4A). Consistent with an antithetic relationship of Gαi2 expression and function to physiologic effect (contraction), Gαi2-IP mice had a greater response to methacholine (P < 0.005), with the maximal response being 50% greater than that of NTG mice (Figure 4A).

**Figure 3**

Gαi2-OE and Gαi2-IP in ASM cells. (A) RT-PCRs from total RNA derived from primary ASM cell cultures from NTG (lanes 1, 2, 7, 8, 13, and 14), Gαi2-IP (lanes 3, 4, 9, 10, 15, and 16), and Gαi2-OE (lanes 5, 6, 11, 12, 17, and 18) mice were performed using transgene-specific primers. h-, human. (B) Overexpression of Gαi2 protein in ASM lysates from Gαi2-OE cells. Note that the protein from Gαi2-OE cells was underloaded by 10-fold compared with NTG cells, as shown by the GAPDH signals. (C) Histopathologic specimens from the lungs of the NTG and 2 representative transgenic lines. Shown are hematoxylin and eosin–stained (upper panels) and trichrome-stained (lower panels) representative sections. Original magnification, ×20.

**Figure 4**

Gαi2-OE or Gαi2-IP in mouse ASM alters responsiveness to M3-muscarinic and β2AR agonists. (A) Intact, ventilated mice were administered the indicated doses of inhaled methacholine and airway resistance measured. The Gαi2-IP mice had a greater response to methacholine than NTG mice, while Gαi2-OE mice were less responsive than NTG. *P < 0.005 versus NTG, ANOVA. (B) Mice were pretreated with vehicle or inhaled isoproterenol and then underwent the methacholine challenge. NTG mice showed the expected decrease in maximal contraction while the Gαi2-OE mice showed no statistical difference in airway resistance with isoproterenol. The Gαi2-IP mice had an enhanced response to isoproterenol. Results are from 6–8 independent experiments. *P < 0.05; †P < 0.01.
The contractile responses to methacholine observed in the \( G_{\alpha i 2-IP} \) mice were consistent with those observed with PTX treatment (Figure 2A). The effects of \( G_{\alpha i} \) expression on \( \beta_2 \)AR-mediated relaxation responses were assessed by pretreatment of mice with aerosolized isoproterenol, followed by methacholine challenge. As shown in Figure 4B, in NTG mice this caused the expected approximately 40% decrease in maximal bronchoconstriction due to methacholine. For the \( G_{\alpha i 2-IP} \) mice, a markedly enhanced \( \beta_2 \)AR function was observed, with very little bronchoconstriction by methacholine in these mice when pretreated with isoproterenol. This effect of \( G_{\alpha i 2-IP} \) amounted to an approximate 80% decrease in resistance compared with methacholine treatment alone in these mice (\( P < 0.01 \)), and indeed resistance after isoproterenol was also less than in NTG mice under the same conditions (\( P < 0.05 \)). In the \( G_{\alpha i 2-OE} \) mice, there was an apparent small decrease in contraction in the presence of isoproterenol, but this did not reach statistical significance, suggesting impaired \( \beta_2 \)AR function. Thus, like the bronchoconstrictive response (where \( G_{\alpha i 2} \) upregulation and downregulation had opposite effects), \( G_{\alpha i 2} \) inhibition enhanced relaxation via \( \beta_2 \)AR, while \( G_{\alpha i 2} \) overexpression decreased it.

We considered that the \( \beta_2 \)AR-mediated relaxation phenotype of \( G_{\alpha i 2-OE} \) mice could be a result of altered expression of ASM \( \beta_2 \)AR or altered function of another receptor acting via \( G_{\alpha i} \). However, \( \beta_2 \)AR expression, as determined by quantitative \( ^{125}\)I-cyanopindolol binding, did not differ among NTG, \( G_{\alpha i-OE} \), and \( G_{\alpha i-IP} \) cells (15 ± 2.1 fmol/mg, 19 ± 3.8 fmol/mg, and 20 ± 2.9 fmol/mg, respectively;
and $G_{\alpha_2}$-IP mice are oppositional, the mechanisms by which these responses are attained are different and potentially multifactorial. PKC$\alpha$ was upregulated by approximately 50% ($P < 0.01$) in $G_{\alpha_2}$-IP cells but unchanged in $G_{\alpha_2}$-OE cells (Figure 6B). Given that PKC$\alpha$ has been demonstrated to sensitize smooth muscle to contraction (12–15), this upregulation in the $G_{\alpha_2}$-IP cells is consistent with the hyperresponsive contractile phenotype. Less is known about the other PKC isoforms in regards to G protein–coupled receptor–mediated smooth muscle contraction. We noted that expression of PKC$\beta_1$ was not altered, while PKC$\epsilon$ and PKC$\delta$ were downregulated by approximately 20% in cells from both lines of mice. In terms of the $\beta_3$AR response, we found no pattern of regulation of downstream signaling elements that was consistent with the 2 phenotypes. G$\alpha_s$ was modestly decreased (about 30%) in both $G_{\alpha_2}$-OE and $G_{\alpha_2}$-IP cells, which does not explain the discordant signaling, and G protein–coupled receptor kinase 2 (GRK2) was decreased by approximately 40% in $G_{\alpha_2}$-OE cells. However, this decrease would be expected to enhance $\beta_3$AR function, instead of the decreased function we observed with $G_{\alpha_2}$-OE. Our findings are consistent with $\beta_3$AR coupling to Gs with attenuation of $\beta_3$AR stimulation of adenyl cyclase. Indeed, the primary signal that has been used to assess $\beta_3$AR-Gs coupling is p44/p42 activation (7), which was markedly enhanced (about 3-fold; $P < 0.001$) in the $G_{\alpha_2}$-OE cells (Figure 6B).

**Discussion**

The aim of these studies was to ascertain the relevance of altered $G_{\alpha_2}$ expression on airway contraction and relaxation. As introduced earlier, a several-fold increase in $G_{\alpha_2}$ has been found in...
Numerous roles of G\textsubscript{i} in regulating contraction and relaxation signaling in ASM. Increased (green arrows) or decreased (red arrows) expression or function of given parameters are indicated. Dotted lines denote pathways and critical mechanisms of action from increased G\textsubscript{i2}-IP; dashed lines denote pathways and critical mechanisms of action from decreased G\textsubscript{i2}. AC, adenylyl cyclase; M\textsubscript{2}AR, muscarinic receptor.

The effects of altering G\textsubscript{i} expression and function on methacholine-promoted bronchoconstriction are not readily explained by alterations in muscarinic receptor–G protein interactions (i.e., at the receptor–G protein interface). The M\textsubscript{1}-muscarinic receptor couples to G\textsubscript{i}, and there is no evidence of promiscuous coupling of this receptor to G\textsubscript{s}. Nor can the phenotype be attributed to altered M\textsubscript{2}-muscarinic receptor function. Although the M\textsubscript{3} subtype is clearly the major muscarinic receptor that mediates airway contraction to muscarinic agonists, we recognize that the M\textsubscript{2} subtype also contributes (16). This subtype, which is G\textsubscript{i}-coupled, appears to promote contraction via decreasing CAMP and eliciting nonselective cation conductance (17). However, our results with the G\textsubscript{i2}-OE mice cannot be explained by enhanced M\textsubscript{2}-receptor function, because in these mice we found a decreased bronchoconstrictive response to methacholine, and when M\textsubscript{2}-G\textsubscript{i} coupling would be expected to be diminished with the G\textsubscript{i2}-OE minigene mice, we observed enhanced contraction. Rather, the mechanisms for the phenotypes appear to be due to crosstalk between G\textsubscript{i} and downstream mediators of contraction. While the phenotypes of the G\textsubscript{i2}-OE and G\textsubscript{i2}-IP mice are opposites, the mechanism for these phenotypes does not appear to be caused by regulation of a single transduction element. Instead, 2 elements that are closely linked in receptor-G\textsubscript{i} signaling, PLC\textsubscript{3} and PKC\textalpha, are each independently regulated. Increased G\textsubscript{i2} resulted in downregulation of PLC\textsubscript{3}, the effector for G\textsubscript{i}-coupled receptor activation. In contrast, PLC\textsubscript{3} expression was unchanged in G\textsubscript{i2}-IP ASM, but PKC\textalpha, whose activation is a consequence of PLC activation, was upregulated. In a positive feedback loop, PKC\textalpha sensitizes smooth muscle to receptor-mediated contraction via G\textsubscript{q} receptors. This effect has been particularly well documented in vascular smooth muscle, where PKC increases the myofilament force sensitivity to intracellular Ca\textsuperscript{2+} concentration (12–15). Interestingly, increased G\textsubscript{i2} has been reported in many animal models of cardiac hypertrophy and heart failure (18–20) as well as in human heart failure (21). Taken together with the various animal models of asthma, an increase in G\textsubscript{i2} may be a generalized response to cellular hypertrophy, which in asthma contributes to the increased ASM mass (22). Although G\textsubscript{i2} is not increased in hearts overexpressing \beta\textsubscript{2}AR, coupling of \beta\textsubscript{2}AR to G\textsubscript{i} appears to provide protection against \beta\textsubscript{2}AR-mediated cardiomyopathy (23).

The \beta\textsubscript{2}AR-mediated relaxation phenotype is consistent with the recent recognition that this receptor, a classic G\textsubscript{i}-coupled receptor, can also couple to G\textsubscript{s} (7). What has remained unknown is the physiologic relevance of \beta\textsubscript{2}AR-G\textsubscript{i} coupling and whether changes in G\textsubscript{s} expression can modulate a physiologic effect (as opposed to G\textsubscript{i}, being in such excess that its expression is not rate limiting). It has not been entirely clear whether \beta\textsubscript{2}AR-G\textsubscript{i} coupling serves to substantially modulate G\textsubscript{i2}-mediated bronchodilation or whether it is more relevant to non–cAMP-dependent signaling, such as p44/p42 MAP kinase activation. We show here that inhibition of \beta\textsubscript{2}AR-G\textsubscript{i} coupling via the minigene markedly enhanced \beta\textsubscript{2}AR-mediated ASM relaxation. This is consistent with the concept that even with normal levels of G\textsubscript{i}, \beta\textsubscript{2}AR actively couples to G\textsubscript{s}, which has important physiologic consequences. The results with the G\textsubscript{i2}-OE mice revealed that increased G\textsubscript{i} expression was associated with increased \beta\textsubscript{2}AR-G\textsubscript{s} signaling, which competed with stimulation of \beta\textsubscript{2}AR-mediated relaxation via G\textsubscript{i}. This indicates that increasing expression levels by pathologic processes, or by pharmacologic means, will have an impact on the airway relaxation response. Several downstream signaling elements potentially related to \beta\textsubscript{2}AR signaling were regulated by overexpression of G\textsubscript{i2} or expression of the G\textsubscript{i2}-IP minigene. However, these changes were inversely related to the observed gain, or loss, of \beta\textsubscript{2}AR function. For example, GRK2 (which phosphorylates and...
desensitizes β2AR) was downregulated in Gαi2-OE smooth muscle. However, if anything this would cause an increase in β2AR function, rather than the decreased function that was observed in these mice. Similarly, p44/p42 MAP kinase phosphorylates GRK2 and impairs its function (24). The former kinase was markedly activated in the Gαi2-OE smooth muscle cells, but β2AR function was impaired, as opposed to enhanced, in these mice. Gαi2 was decreased approximately 30% in both Gαi2-OE and Gαi2-IP cells, and thus this change is unlikely to represent a major factor given the opposite β2AR phenotypes in the 2 mice. Similarly, PKCs and PKCθ were downregulated in both mice.

The current work shows a complex, multifactorial interplay between G, expression and contraction and relaxation signaling via G protein-coupled receptors in the airway (Figure 8). From the standpoint of the pathogenesis of bronchospasm, the increase in Gαi2 observed in asthmatic-like airways (3–6) appears to serve a protective effect: the increase acts to attenuate bronchial hyperreactivity and exacerbate asthma. On the other hand, the increase in Gαi2 impaired β-agonist bronchodilatation. This appears to be due to an enhancement of β2AR-Gαi2 coupling, which opposes β2AR-Gαi2 functional stimulation of adenyl cyclase. Furthermore, decreasing Gαi2 function acts to increase β2AR-mediated bronchodilatation, as would be expected based on this mechanism. Therefore, in the absence of β-agonist treatment the increase in Gαi2 in asthma acts to attenuate bronchospasm, but also limits the effectiveness of β-agonists in relieving bronchoconstriction. This multipronged set of regulatory events may indicate that pharmacologic or genetic methods to modulate Gαi2 could have limited clinical utility. However, there may be opportunities to exploit these relationships by manipulating other downstream effects of altered Gαi2 expression. For example, if decreasing Gαi2 expression and function could be accomplished while also inhibiting PKCα, then both the relaxation and the constrictive pathways would be affected in a manner that would be expected to be clinically beneficial for hyperresponsiveness and β-agonist efficacy (Figure 8).

Methods

Transgenic mice. These studies were approved by the Institutional Animal Care and Use Committees of the University of Cincinnati College of Medicine and the University of Maryland School of Medicine. Gαi2-OE and Gαi2-IP in mice were targeted to ASM using a construct based on the mouse SMP8 and the SV40 polyadenylation region as previously described (25). Briefly, for the Gαi2-OE construct, the full-length human Gαi2 cDNA was subcloned into the neo/Xbal sites between the SMP8 promoter and the SV40 polyadenylation site of the construct. For Gαi2-IP, PCR techniques were used to create a cDNA encoding the influenza HA tag (YPYDVPDYA) in-frame with the last 58 amino acids of human Gαi2 which was cloned into the same sites as above in the SMP8 construct. For transgenic generation, the final constructs were excised with NotI, purified, and microinjected into fertilized zygotes from superovulated FVB/N mice. Surviving zygotes were implanted into pseudopregnant females. Offspring were screened for the presence of transgenes using PCRs of genomic DNA from tail clips, with the 3′ primer being in the SV40 polyadenylation region. Hemizygous mice from generations 4–6 that were 8–10 weeks of age were used for all studies. FVB/N littermates that were negative by transgene screening were used as the NTG controls. Transgene mRNA expression from cultured ASM cell total RNA was confirmed by RT-PCR using an oligo-dT primer for reverse transcription. For the PCR, primers were designed that were specific for the human, versus mouse, Gαi2 (5′-GGCGGTGTCTACGCAACACAT-3′ and 5′-CTCGCTCTTGGGCATGTACTAC-3′). For Gαi2-IP, the 5′ primer was on the HA-tag sequence (5′-TACCCATACGGCTCAGACTAC-3′ and 5′-CACCAGTCTTCTGAGTTACTTGC-3′). The dosycycline-inducible IL-13 bitransgenic mice were generated as previously described (9). IL-13 induction was initiated in 3-week-old mice by addition of doxycycline (625 mg/kg) in their Chow for 3 weeks (9).

Airway physiology. Invasive assessment of respiratory mechanics was performed using an intact, intubated, anesthetized mouse model similar to that previously reported (8, 11). Briefly, mice were anesthetized with approximately 60 mg/kg pentobarbital, after which the trachea was cannulated with an 18-gauge metal needle. Mice were then mechanically ventilated using a computer-controlled rodent ventilator (flexiVent version 4.01; SCIREQ) to deliver a tidal volume of 10 ml/kg (approximately 250 μl/breath) at a rate of 150 breaths per minute, with positive end-expiratory pressure of 2.5 cm H2O. Dynamic lung resistance was determined by fitting a linear first-order single-compartment model of airway mechanics to measurements of airway pressure, volume, and air flow made during application of single sinusoidal perturbation with an amplitude of 150 μl at 2.5 Hz for approximately 1.2 seconds using software provided by the manufacturer (flexiVent version 4.01; SCIREQ). Then mean of 2 measurements of resistance made prior to administration of methacholine was established as baseline. Increasing concentrations of methacholine were subsequently delivered to the airway by transiently diverting the inspiratory limb of the ventilator through the reservoir of an ultrasonic nebulizer for 30 seconds. Resistance was measured at 30-second intervals for 5 minutes after each dose, and the maximum resistance value following each dose was used to establish the dose-response curves. In studies to assess the relaxation effects of inhaled β-agonist, isoproterenol (1.0 mg/ml) was delivered by aerosol, and then the constrictive response to varying doses of methacholine was determined as described above. In some studies, mice were pretreated with PTX (100 μg/kg body weight i.p.) injected 18 hours before being studied. Data were fit by nonlinear curve fitting (Prism version 4.0; GraphPad). Since full dose-response sigmoid-like curves cannot be attained in vivo, ANOVA was used to compare the response data across all doses rather than a simpler model that relies on extrapolated values to obtain and compare the maximal responses.

Tracheal ring studies. Studies of mouse tracheal ring contractility were performed as reported previously in detail (26). Tracheae were excised and cut into rings of 5 mm in length. They were mounted on stainless steel wires connected to isometric force transducers and maintained at 37°C in a physiologic saline solution (118 mM NaCl, 11 mM glucose, 4.73 mM KCl, 1.2 mM MgCl2, 0.026 mM EDTA, 1.2 mM KH2PO4, 2.5 mM CaCl2, 25 mM NaHCO3, pH 7.40) with bubbled 95% O2 and 5% CO2. Rings were stretched to a tension of 5 mN, which we have previously determined to be an optimal passive tension for maximizing active force (25). Following a 30-minute equilibration period, rings were contracted with 60 mM KCl, and the maximal response over the next 5 minutes was recorded. With KC1 remaining in the bath, the relaxation response to isoproterenol was determined by perfusing rings with the indicated concentrations of isoproterenol (Figure 5), and the isometric force was continuously measured over the ensuing 5 minutes. In some studies, rings were incubated with 0.1 μM pilocarpine for 30 minutes at 37°C prior to, and during, KC1 and isoproterenol treatments.

Airway smooth muscle culture. Primary cultures of murine ASM cells were established from tracheal explants of NTG, Gαi2-OE, and Gαi2-IP mice as previously reported (25). The trachea between the larynx and main stem bronchi was removed and placed on a dish containing Hanks’ balanced...
Western blots. Primary ASM cells in monolayers were washed 3 times with PBS and lysed in a solubilization buffer containing 1% IGEPAL, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate-buffered saline. The protease inhibitors benzamidine, soybean trypsin inhibitor, and aprotinin (all at 5 μg/ml) were included in this and all subsequent steps. Lysates were passed 3 times through a 21-gauge needle and then rotated at 4°C for 1 hour in microfuge tubes. The tubes were then centrifuged to pellet unsolubilized material and the supernatants aliquoted and frozen at -70°C. Proteins from whole trachea were prepared in a similar manner, but were first homogenized with a polytron. Equal amounts of protein (typically 15 μg) were loaded in all lanes. Western blots were performed using standard enhanced chemiluminescence techniques as previously described in detail (8, 27), with signals from the membranes acquired with a Fuji LAS-3000 charged-coupled acquisition system. Quantification of the immunoreactive bands was performed with software from the manufacturer (ImageGauge version 4.22; Fuji). Membranes were stripped and reprobed for GAPDH; these signals were used to control for minor variations in protein loading or transfer.

Statistics. All curve fitting was by nonlinear regression techniques using Prism software (version 4.0; GraphPad). Data are presented as mean ± SEM. Comparisons were by 2-tailed paired or unpaired Student’s t tests unless otherwise indicated, with significance considered at P < 0.05.

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