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 $G_{\alpha q}$, and relaxation via $G_{\alpha s}$, underlie the bronchospastic features of asthma and its treatment. Asthma models show increased ASM $G_{\alpha i}$ expression, considered the basis for the proasthmatic phenotypes of enhanced bronchial hyperreactivity to contraction mediated by M_3-muscarinic receptors and diminished relaxation mediated by β_2-adrenergic receptors (β_2ARs). 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 $G_{\alpha i2}$ or a $G_{\alpha i2}$ peptide inhibitor in ASM. Unexpectedly, $G_{\alpha i2}$ overexpression decreased contractility to methacholine, while $G_{\alpha i2}$ inhibition enhanced contraction. These opposite phenotypes resulted from different crosstalk loci within the G_q signaling network: decreased phospholipase C and increased PKCα, respectively. $G_{\alpha i2}$ overexpression decreased β_2AR-mediated airway relaxation, while $G_{\alpha i2}$ inhibition increased this response, consistent with physiologically relevant coupling of this receptor to both G_s and G_i. IL-13 transgenic mice (a model of asthma), which developed increased ASM $G_{\alpha i}$, displayed marked increases in airway hyperresponsiveness when $G_{\alpha i}$ function was inhibited. Increased $G_{\alpha i}$ in asthma is therefore a double-edged sword: a compensatory event mitigating against bronchial hyperreactivity, but a mechanism that evokes β-agonist resistance. By selective intervention within these multipronged signaling modules, advantageous G_s/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_q and the latter by those that couple to G_s (1, 2). Many inflammatory cascades in asthma evoke bronchoconstriction by promoting local increases of G_q receptor agonists such as acetylcholine, cysteinyl leukotrienes, prostaglandins, and histamine, which activate their cognate receptors expressed on ASM. There appear to be fewer G_s-coupled receptors that act via endogenous agonists to counteract bronchoconstriction, but the β_2-adrenergic receptor (β_2AR) of ASM is the target of pharmacologically administered β-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_3-muscarinic receptor promote disassociation of heterotrimeric G_q into G_α and $G_{\beta\gamma}$ subunits, with the α subunit activating phospholipase C (PLC; which promotes inositol-3 phosphate and diacylglycerol production) and the latter activating PKC. Receptors such as the β_2AR act via $G_{\alpha s}$ to stimulate the effector adenylyl 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 α subunits 2 and 3; $G_{\alpha i2}$ and $G_{\alpha i3}$, 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 β-agonist isoproterenol. These events were temporally related to increased $G_{\alpha i}$ protein expression, and these characteristics were attenuated by pertussis toxin (PTX), which inactivates $G_{\alpha i}$.

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 $G_{\alpha i}$ expression. It remains unclear how the 2 proasthmatic phenotypes of hyperresponsiveness to bronchoconstriction and resistance to bronchodilatation, which are mediated by G_q- 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 β_2AR, once phosphorylated by PKA, has the capacity to couple to G_i (7). Given that $G_{\alpha i}$ inhibits adenylyl 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; $G_{\alpha i2}$-IP, $G_{\alpha i2}$ inhibitory peptide expression; $G_{\alpha i2}$-OE, $G_{\alpha i2}$ overexpression; GRK2, G protein–coupled receptor kinase 2; NTG, nontransgenic littermate; PLC, phospholipase C; PTX, pertussis toxin; SMP8, smooth muscle actin promoter 8.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **117**:1391–1398 (2007). doi:10.1172/JCI30489.

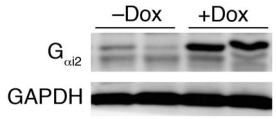

Figure 1
Induction of IL-13 in mouse airways increases $G_{\alpha i2}$ expression. Three-week-old bitransgenic mice generated to have doxycycline-inducible expression of IL-13 targeted to the airways by the CC10 promoter were fed normal food (–Dox) or doxycycline-impregnated food (+Dox) for 3 weeks. Tracheae were homogenized, and membrane preparations were subjected to Western blots performed with $G_{\alpha i2}$ and GAPDH antibodies. Induction of IL-13 increased $G_{\alpha i2}$ expression by approximately 8-fold.

pling. A mechanism by which G_i expression alters receptor-G_q signaling is not readily apparent; however, previously unrecognized crosstalk between signaling pathways in ASM presumed to be unrelated has now been documented (8). Of particular interest in regard to the aforementioned models is the difficulty in ascribing a causal effect of increased G_i to the phenotypes in light of the complex regulatory events within many diverse pathways that are undoubtedly underway in asthma. Similarly, it is unclear whether the increase in G_i is participating in the pathologic process or is a compensatory event that may be a factor mitigating against one or both of the asthmatic phenotypes. To ascertain the role of *isolated* increased or decreased G_i function on bronchoconstrictive and dilating signaling of G protein–coupled receptors in the airway, transgenic mice were generated overexpressing $G_{\alpha i2}$ ($G_{\alpha i2}$-OE mice) and expressing a $G_{\alpha i2}$ inhibitory peptide ($G_{\alpha i2}$-IP mice) in ASM. We found that increased G_i did not cause enhanced contraction, but rather decreased the contractile response, while G_i inhibition evoked enhanced contraction. However, although these phenotypes are physiologically antithetic, their mechanistic basis was crosstalk with 2 different elements of the G_q signaling pathway. In the IL-13 transgenic mouse model of asthma and airway hyperresponsiveness, where we found increased G_i in ASM, inhibition of G_i resulted in even greater hyperresponsiveness. Thus the increase in G_i appears to be a compensatory event aimed at attenuating bronchial hyperresponsiveness. We also found that β_2AR-mediated relaxation of ASM was substantially regulated by G_i expression, which we propose is due to direct coupling of this receptor to G_i.

Results

$G_{\alpha i2}$ is increased in a genetic model of asthma, and PTX alters airway responsiveness. To assess whether the findings by Grunstein and colleagues of increased $G_{\alpha i}$ in rabbit models of asthma (3) are true for another model, we examined expression of $G_{\alpha i2}$ 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 $G_{\alpha i2}$ assessed by Western blot increased approximately 8-fold upon IL-13 induction in these transgenic mice. These data pointed toward increased G_i as a common feature of asthmatic-like airways from passive sensitization with asthmatic 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 G_i levels influence airway contraction or relaxation responses, nor do they implicate the increase in G_i 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 G_i 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 G_q-coupled M_3-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, β_2AR signaling appeared to be enhanced, in that there was little contraction to methacholine under these conditions (Figure 2B). While these findings potentially implicate G_i 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 $G_{\alpha i}$ expression, as is found in the aforementioned asthma models. These limitations led to the generation of transgenic mice $G_{\alpha i2}$-OE (incorporating the full-length human $G_{\alpha i2}$ cDNA) and $G_{\alpha i2}$-IP (a "minigene" peptide consisting of the C-terminal portion of $G_{\alpha i2}$) targeted to ASM.

Transgenic expression of $G_{\alpha i2}$ and a $G_{\alpha i2}$ competitive inhibitor. Transgenic mice were generated using smooth muscle actin promoter 8 (SMP8); the injected constructs consisted of the full-length cDNA for human $G_{\alpha i2}$ or a cDNA encoding the carboxy terminus of human $G_{\alpha i2}$ (58 amino acids), which also included a 5′ HA-tag (see

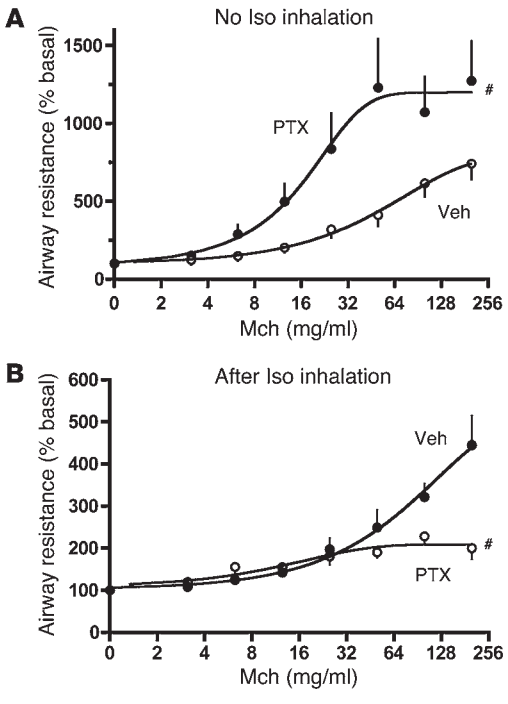

Figure 2
PTX alters airway contraction and relaxation responses. FVB/N NTG mice were treated with vehicle (veh) or i.p. PTX for 18 hours. Airway resistance and the response to inhaled methacholine (mch) was determined in intact ventilated mice (see Methods). Studies were performed without (**A**) or with (**B**) pretreatment with inhaled isoproterenol (Iso). Results are from 5 independent experiments. $^{\#}P < 0.01$ versus vehicle.

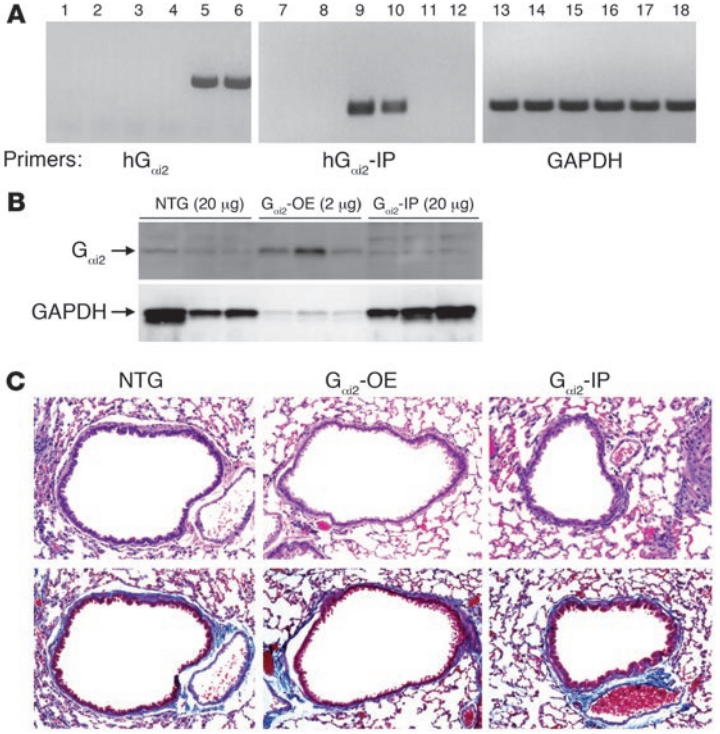

Figure 3
$G_{\alpha i2}$-OE and $G_{\alpha i2}$-IP in ASM cells. (**A**) RT-PCRs from total RNA derived from primary ASM cell cultures from NTG (lanes 1, 2, 7, 8, 13, and 14), $G_{\alpha i2}$-IP (lanes 3, 4, 9, 10, 15, and 16), and $G_{\alpha i2}$-OE (lanes 5, 6, 11, 12, 17, and 18) mice were performed using transgene-specific primers. h-, human. (**B**) Overexpression of $G_{\alpha i2}$ protein in ASM lysates from $G_{\alpha i2}$-OE cells. Note that the protein from $G_{\alpha i2}$-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.

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 the transgenes were expressed in the cell type of interest, we prepared RNA from cultures of primary ASM cells that were established from tracheal explants of heterozygous F2 and F3 mice. We detected mRNA for the $G_{\alpha i2}$ transgene by RT-PCR using human-specific $G_{\alpha i2}$ primers and for the $G_{\alpha i2}$-IP transgene using primers based on sequence from the 5′ HA-tag and human $G_{\alpha i2}$. As shown in Figure 3A, the human $G_{\alpha i2}$ transcript was identified only in ASM cells derived from $G_{\alpha i2}$-OE mice. Immunoblots using $G_{\alpha i2}$ antisera further showed overexpression of $G_{\alpha i2}$ protein by approximately 10-fold in the ASM cells derived from the full-gene overexpressors (Figure 3B; note that the $G_{\alpha i2}$-OE protein was underloaded to obtain a reasonable visual comparison with the NTG signal). Although we were not able to reproducibly detect protein expression of the $G_{\alpha i2}$-IP minigene, RT-PCR revealed the chimeric HA–$G_{\alpha i}$-IP transcript in ASM cells only in the $G_{\alpha i}$-IP–derived cell lines, not the NTG or $G_{\alpha i2}$-OE lines. Formalin-fixed sections of the lungs revealed no microscopic evidence of increased ASM mass, basement membrane thickening, inflammation, fibrosis, or parenchymal distortion in either transgenic line. Figure 3C shows representative sections that include both airway and parenchyma. Mean systolic blood pressure measured noninvasively by tail cuff readings over a period of 5 days did not differ among NTG, $G_{\alpha i2}$-OE, and $G_{\alpha i2}$-IP mice (125 ± 1 mmHg, 125 ± 3 mmHg, and 126 ± 5 mmHg, respectively; n = 10; data not shown).

Effects of altering $G_{\alpha i}$ by transgenesis on airway contractile and relaxation responses. Baseline airway resistance did not differ among $G_{\alpha i2}$-OE, $G_{\alpha i2}$-IP, and NTG mice (0.53 ± 0.061 cm H_2O/ml/s, 0.58 ± 0.036 cm H_2O/ml/s, and 0.57 ± 0.041 cm H_2O/ml/s, respectively; n = 6–9; data not shown). However, $G_{\alpha i2}$-OE mice exhibited a depressed constrictive 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 G_i expression and function to physiologic effect (contraction), $G_{\alpha i2}$-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).

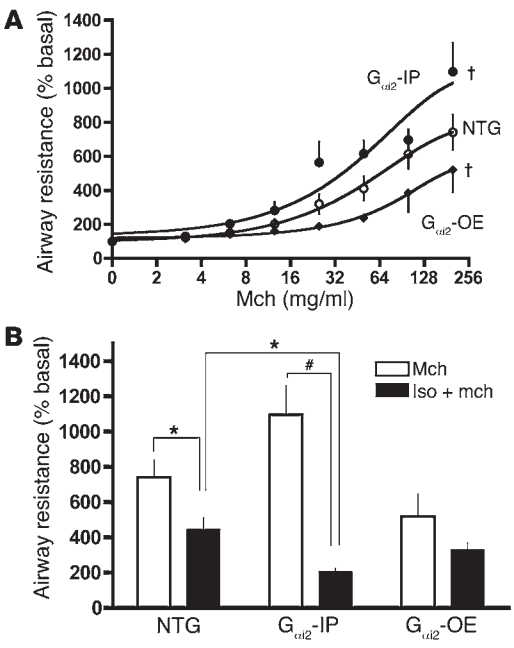

Figure 4
$G_{\alpha i2}$-OE or $G_{\alpha i2}$-IP in mouse ASM alters responsiveness to M_3-muscarinic and β_2AR agonists. (**A**) Intact, ventilated mice were administered the indicated doses of inhaled methacholine and airway resistance measured. The $G_{\alpha i2}$-IP mice had a greater response to methacholine than NTG mice, while $G_{\alpha i2}$-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 $G_{\alpha i2}$-OE mice showed no statistical difference in airway resistance with isoproterenol. The $G_{\alpha i2}$-IP mice had an enhanced response to isoproterenol. Results are from 6–8 independent experiments. *$P < 0.05$; #$P < 0.01$.

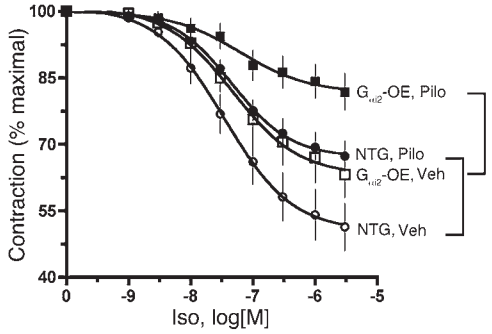

Figure 5
M_2-muscarinic receptor activation does not alter the G_i-effect on β_2AR-mediated smooth muscle relaxation. Shown are experiments where isolated tracheal rings were pretreated with 0.1 μM pilocarpine (pilo) or vehicle, contracted with 60 mM KCl, and then exposed to increasing concentrations of isoproterenol. The brackets clarify the effects of pilocarpine compared with vehicle, which were not significantly different, in the NTG and $G_{\alpha i2}$-OE groups (n = 4 experiments).

The contractile responses to methacholine observed in the $G_{\alpha i2}$-IP mice were consistent with those observed with PTX treatment (Figure 2A). The effects of G_i expression on β_2AR-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 $G_{\alpha i2}$-IP mice, a markedly enhanced β_2AR function was observed, with very little bronchoconstriction by methacholine in these mice when pretreated with isoproterenol. This effect of $G_{\alpha i2}$-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 $G_{\alpha i2}$-OE mice there was an apparent small decrease in contraction in the presence of isoproterenol, but this did not reach statistical significance, suggesting impaired β_2AR function. Thus, like the bronchoconstrictive response (where $G_{\alpha i2}$ upregulation and downregulation had opposite effects), $G_{\alpha i2}$ inhibition enhanced relaxation via β_2AR, while $G_{\alpha i2}$ overexpression decreased it.

We considered that the β_2AR-mediated relaxation phenotype of $G_{\alpha i2}$-OE mice could be a result of altered expression of ASM β_2AR or altered function of another receptor acting via G_i. However, β_2AR 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;

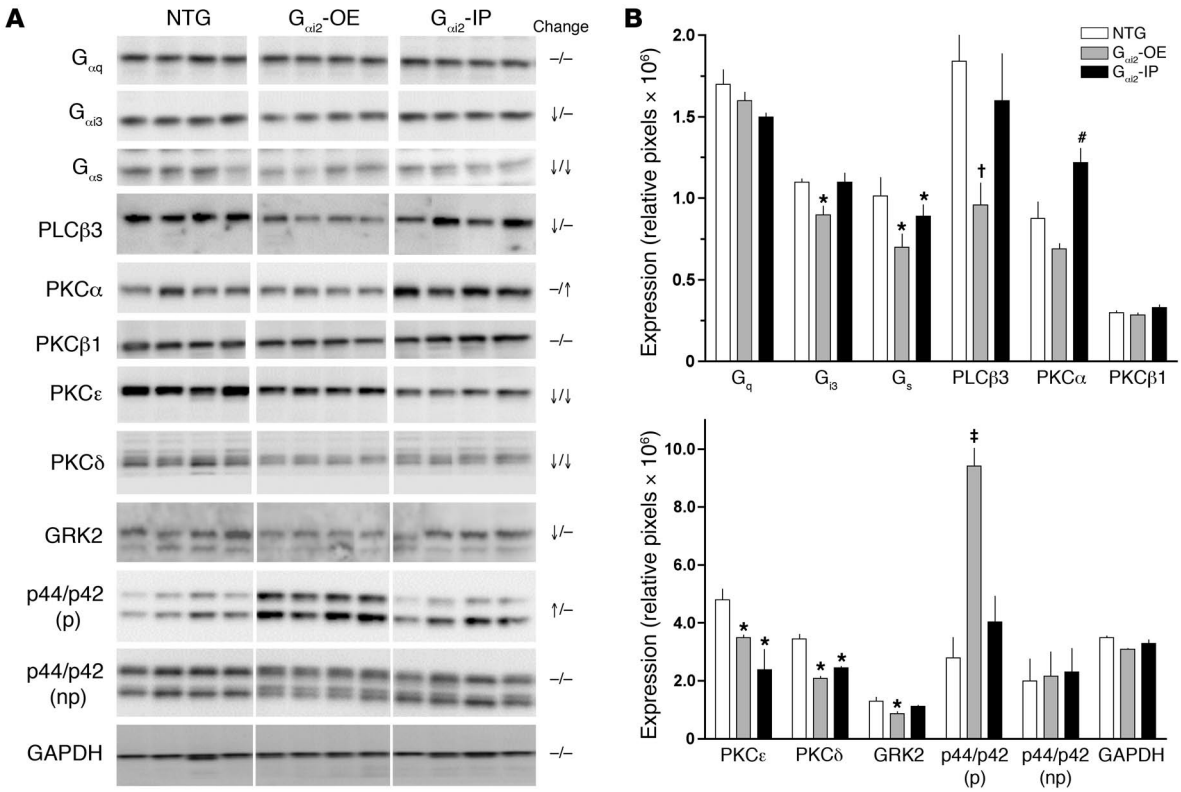

Figure 6
Downstream modulation of signal transduction elements by altered $G_{\alpha i}$ in ASM cells. (**A**) Western blots from 4 independent ASM cell lysates for each line. Symbols at right of blots denote significant increase (up arrows), significant decrease (down arrows), or no change (dashes) in protein expression from $G_{\alpha i2}$-OE and $G_{\alpha i2}$-IP derived cells (left and right symbols, respectively) compared with NTG cells. (**B**) Quantification of Western blot data, presented as mean ± SEM. *$P < 0.05$; #$P < 0.01$; †$P < 0.005$; ‡$P < 0.001$ versus NTG.

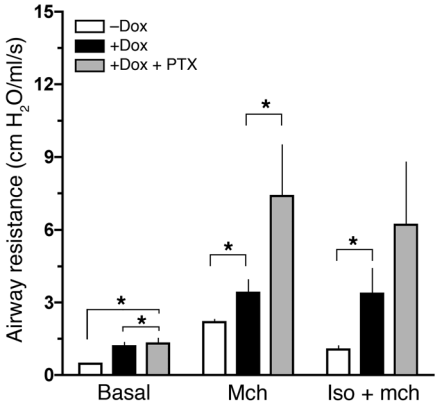

Figure 7
The increase in $G_{\alpha i}$ in IL-13 mice limits airway hyperresponsiveness. Shown are experiments where the IL-13 doxycycline-inducible mice were studied in the absence or presence of induction with doxycycline. The latter were treated with i.p. PTX or vehicle. Airway resistance was measured using the ventilated mouse model, at a single concentration of aerosolized methacholine (10 mg/ml). After resistance returned to baseline, mice were treated with the same concentration of methacholine with isoproterenol. PTX enhanced methacholine-promoted airway constriction (compare black versus shaded bars under methacholine). These results are consistent with the increased $G_{\alpha i}$ found in these mice after IL-13 induction acting to attenuate hyperresponsiveness. Results are from independent experiments with 2–3 mice in each group. *$P < 0.05$.

n = 4; data not shown). We thus considered the potential for enhanced function of a classic receptor-G_i–coupled pathway (such as the M_2-muscarinic pathway) that might antagonize G_s-coupled relaxation at adenylyl cyclase. To address this potential mechanism, excised tracheal rings were studied ex vivo to measure relaxation responses during KCl contraction. Rings were treated with vehicle or the M_2-muscarinic receptor agonist pilocarpine (0.1 μM) and then contracted continuously with 60 mM KCl. Relaxation was then ascertained in response to increasing concentrations of isoproterenol. As expected, with NTG rings, pilocarpine modestly blunted isoproterenol-mediated relaxation from 51% ± 5.0% to 62% ± 5.0% of maximal contraction (a 20% decrease; Figure 5). However, there was no accentuation of this effect in the $G_{\alpha i2}$-OE rings, with pilocarpine reducing isoproterenol-mediated relaxation by an amount comparable to that observed with NTG rings: from 67% ± 3.3% to 81% ± 4.0% (a 22% decrease; Figure 5).

$G_{\alpha i2}$ expression regulates multiple downstream signaling elements. Potential crosstalk between $G_{\alpha i2}$ expression and function and relevant downstream mediators of contraction and relaxation was assessed by Western blot analyses from primary ASM cells derived from NTG, $G_{\alpha i2}$-IP, and $G_{\alpha i2}$-OE mice (Figure 6). Of particular interest — because of the counterintuitive effects on contraction — was expression of elements involved in airway constriction: $G_{\alpha q}$, PLC, and PKC isoforms. $G_{\alpha q}$ levels were not altered in either transgenic. However, its effector, PLCβ3, was downregulated by more than 50% ($P < 0.005$) in the $G_{\alpha i2}$-OE cells but was not different in the $G_{\alpha i2}$-IP cells compared with NTG cells (Figure 6B). These results are consistent with our physiologic studies showing that the $G_{\alpha i2}$-OE mice were hyporesponsive to methacholine. However, this does not explain the hyperresponsiveness observed in $G_{\alpha i2}$-IP mice, which indicates that while the phenotypes of the $G_{\alpha i2}$-OE and $G_{\alpha i2}$-IP mice are oppositional, the mechanisms by which these responses are attained are different and potentially multifactorial. PKCα was upregulated by approximately 50% ($P < 0.01$) in $G_{\alpha i2}$-IP cells but unchanged in $G_{\alpha i2}$-OE cells (Figure 6B). Given that PKCα has been demonstrated to sensitize smooth muscle to contraction (12–15), this upregulation in the $G_{\alpha i2}$-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 PKCβ1 was not altered, while PKCε and PKCδ were downregulated by approximately 20% in cells from both lines of mice. In terms of the β_2AR 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 $G_{\alpha i2}$-OE and $G_{\alpha i2}$-IP cells, which does not explain the discordant signaling, and G protein–coupled receptor kinase 2 (GRK2) was decreased by approximately 40% in $G_{\alpha i2}$-OE cells. However, this decrease would be expected to enhance β_2AR function, instead of the decreased function we observed with $G_{\alpha i2}$-OE. Our findings are consistent with β_2AR coupling to G_i with attenuation of β_2AR stimulation of adenylyl cyclase. Indeed, the primary signal that has been used to assess β_2AR-G_i coupling is p44/p42 activation (7), which was markedly enhanced (about 3-fold; $P < 0.001$) in the $G_{\alpha i2}$-OE cells (Figure 6B).

Increased $G_{\alpha i2}$ in a mouse asthma model is a beneficial compensatory event attenuating airway hyperresponsiveness. The physiologic consequences of transgenic overexpression of $G_{\alpha i2}$ revealed a decrease, rather than the expected increase, in airway constriction to methacholine. In addition, the molecular events that we defined as a consequence of increased $G_{\alpha i2}$ were consistent with this phenotype. This suggested that the increase in $G_{\alpha i}$ that has been observed in several animal models of asthma is a compensatory event, acting to attenuate bronchial hyperresponsiveness. To test this, we used the inducible IL-13 mice, which displayed a significant increase in $G_{\alpha i2}$ upon IL-13 induction (Figure 1). These mice are known to be hyperresponsive, which is a key aspect of their phenotype (10), but the question remains as to whether inactivating $G_{\alpha i}$ would further increase airway contractility in these mice, thus revealing the partial protective effect against hyperresponsiveness provided by increased $G_{\alpha i2}$. The IL-13 mice were given normal chow or chow with doxycycline for 3 weeks, and then some mice were treated with PTX i.p. and studied 18 hours later. As shown in Figure 7, IL-13 induction increased methacholine-promoted airway constriction. Treatment with PTX further enhanced contraction, amounting to a 2-fold increase compared with doxycycline-only mice. These data are consistent with the aforementioned concept that the increase in $G_{\alpha i}$ that occurs in this animal model of asthma (and others as well) acts in a compensatory manner to limit airway hyperresponsiveness. The β-agonist isoproterenol afforded protection against bronchoconstriction in the noninduced IL-13 mice. Interestingly, β-agonist provided no protection in the IL-13–induced mouse, where extensive airway constriction due to methacholine was observed, consistent with depressed βAR function. However, there was a nonsignificant trend toward less constriction in the presence of isoproterenol with PTX treatment, which supports improved βAR function.

Discussion

The aim of these studies was to ascertain the relevance of altered $G_{\alpha i2}$ expression on airway contraction and relaxation. As introduced earlier, a several-fold increase in $G_{\alpha i2}$ has been found in

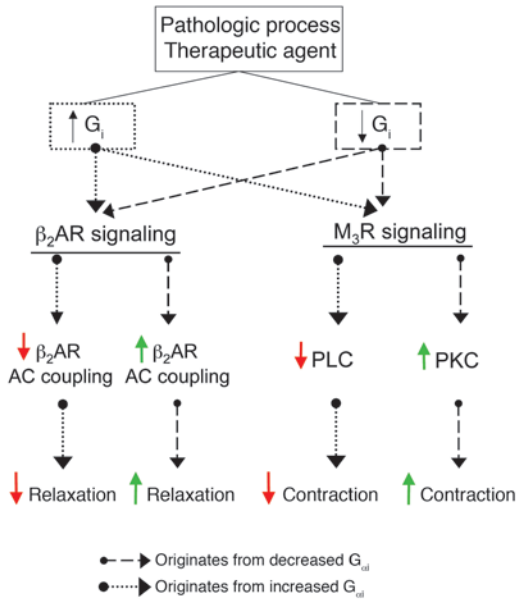

Figure 8
Multiple roles of G_i in regulating contraction and relaxation signaling in ASM. Increased (green arrows) or decreased (red arrows) expression or function of given parameters are indicated. Dotted lines denote pathways and critical mechanisms of action from increased $G_{\alpha i}$; dashed lines denote pathways and critical mechanisms of action from decreased $G_{\alpha i}$. AC, adenylyl cyclase; M_3R, M_3-muscarinic receptor.

a number of models of asthma, and it has been proposed that both bronchial hyperresponsiveness to contraction and impaired β_2AR-mediated relaxation can be attributed to this increase (3–6). Undoubtedly, there are expression changes in many signaling elements in these asthma models, and thus a causal relationship between $G_{\alpha i}$ expression and the above phenotypes cannot be established, nor can the specific effects of altered $G_{\alpha i}$ expression, in isolation, be ascertained. In the current work we show that increased $G_{\alpha i2}$ resulted in *decreased* contractile responsiveness, which contradicts the aforementioned proposed paradigm. Furthermore, as a complementary and independent approach, we examined the effect of lowering $G_{\alpha i}$ function via a minigene. This resulted in *increased* contraction, which is the opposite phenotype to $G_{\alpha i2}$-OE, and thus supports the notion that up- or downregulation of G_i levels has opposing effects on contraction. The phenotype of the PTX-treated mice is additional confirmation of the $G_{\alpha i2}$-IP results, and also indicates that the effect of the minigene was not because of its interaction with receptor-G_q signaling, since PTX ADP-ribosylates $G_{\alpha i}$. Thus, the increase in $G_{\alpha i}$ observed in animal models of asthma and/or bronchial hyperresponsiveness is clearly not the cause of contractile hyperresponsiveness: it actually decreased the methacholine response. Indeed, it appears that an increase in $G_{\alpha i}$ is a beneficial compensatory event, acting to limit the extent of hyperresponsiveness. To confirm this notion, the IL-13–inducible mice, which have airway hyperresponsiveness and increased $G_{\alpha i2}$, were treated with PTX and displayed further enhancement of bronchoconstriction due to methacholine.

The effects of altering G_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_3-muscarinic receptor couples to G_q, and there is no evidence of promiscuous coupling of this receptor to G_i. Nor can the phenotype be attributed to altered M_2-muscarinic receptor function. Although the M_3 subtype is clearly the major muscarinic receptor that mediates airway contraction to muscarinic agonists, we recognize that the M_2 subtype also contributes (16). This subtype, which is G_i coupled, appears to promote contraction via decreasing cAMP and eliciting nonselective cation conductance (17). However, our results with the $G_{\alpha i2}$-OE mice cannot be explained by enhanced M_2-receptor function, because in these mice we found a *decreased* bronchoconstrictive response to methacholine, and when M_2-G_i coupling would be expected to be diminished with the $G_{\alpha i2}$-IP minigene mice, we observed *enhanced* contraction. Rather, the mechanisms for the phenotypes appear to be due to crosstalk between G_i and downstream mediators of contraction. While the phenotypes of the $G_{\alpha i2}$-OE and $G_{\alpha i2}$-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_q signaling, PLCβ3 and PKCα, are each independently regulated. Increased $G_{\alpha i}$ resulted in downregulation of PLCβ3, the effector for G_q-coupled receptor activation. In contrast, PLCβ3 expression was unchanged in $G_{\alpha i2}$-IP ASM, but PKCα, whose activation is a consequence of PLC activation, was upregulated. In a positive feedback loop, PKCα sensitizes smooth muscle to receptor-mediated contraction via G_q receptors. This effect has been particularly well documented in vascular smooth muscle, where PKC increases the myofilament force sensitivity to intracellular Ca^{2+} concentration (12–15). Interestingly, increased $G_{\alpha i}$ 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 $G_{\alpha i}$ may be a generalized response to cellular hypertrophy, which in asthma contributes to the increased ASM mass (22). Although $G_{\alpha i2}$ is not increased in hearts overexpressing β_2AR, coupling of β_2AR to G_i appears to provide protection against β_2AR-mediated cardiomyopathy (23).

The β_2AR-mediated relaxation phenotype is consistent with the recent recognition that this receptor, a classic G_s-coupled receptor, can also couple to G_i (7). What has remained unknown is the physiologic relevance of β_2AR-G_i coupling and whether changes in G_i expression can modulate a physiologic effect (as opposed to G_i being in such excess that its expression is not rate limiting). It has not been entirely clear whether β_2AR-G_i coupling serves to substantially modulate G_s-mediated bronchodilatation or whether it is more relevant to non–cAMP-dependent signaling, such as p44/p42 MAP kinase activation. We show here that inhibition of β_2AR-G_i coupling via the minigene markedly enhanced β_2AR-mediated ASM relaxation. This is consistent with the concept that even with normal levels of G_i, β_2AR actively couples to G_i, which has important physiologic consequences. The results with the $G_{\alpha i2}$-OE mice revealed that increased G_i expression was associated with increased β_2AR-G_i signaling, which competed with stimulation of β_2AR-mediated relaxation via G_s. 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 β_2AR signaling were regulated by overexpression of $G_{\alpha i2}$ or expression of the $G_{\alpha i2}$-IP minigene. However, these changes were inversely related to the observed gain, or loss, of β_2AR function. For example, GRK2 (which phosphorylates and

desensitizes β_2AR) was downregulated in $G_{\alpha i2}$-OE smooth muscle. However, if anything this would cause an increase in β_2AR 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 $G_{\alpha i2}$-OE smooth muscle cells, but β_2AR function was impaired, as opposed to enhanced, in these mice. $G_{\alpha s}$ was decreased approximately 30% in both $G_{\alpha i2}$-OE and $G_{\alpha i2}$-IP cells, and thus this change is unlikely to represent a major factor given the opposite β_2AR phenotypes in the 2 mice. Similarly, PKCε and PKCδ were downregulated in both mice.

The current work shows a complex, multifactorial interplay between G_i 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 $G_{\alpha i}$ observed in asthmatic-like airways (3–6) appears to serve a protective effect: the increase acts to attenuate bronchial hyperresponsiveness from G_q-mediated receptor signaling. Given that virtually all bronchospastic mediators act via G_q-coupled receptors (e.g., M_3-muscarinic, LT1-cysteinyl leukotriene, and H1-histamine), it would appear that not only is the increase in G_i beneficial in asthma, attempts to adjust these levels at or below normal will increase bronchial hyperreactivity and exacerbate asthma. On the other hand, the increase in $G_{\alpha i}$ impaired β-agonist bronchodilatation. This appears to be due to an enhancement of β_2AR-$G_{\alpha i}$ coupling, which opposes β_2AR-G_s functional stimulation of adenylyl cyclase. Furthermore, decreasing G_i function acts to increase β_2AR-mediated bronchodilation, as would be expected based on this mechanism. Therefore, in the absence of β-agonist treatment the increase in $G_{\alpha i}$ 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 G_i could have limited clinical utility. However, there may be opportunities to exploit these relationships by manipulating other downstream effects of altered G_i expression. For example, if decreasing G_i 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. $G_{\alpha i2}$-OE and $G_{\alpha i2}$-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 $G_{\alpha i2}$-OE construct, the full-length human $G_{\alpha i2}$ cDNA was subcloned into the *NcoI/XhoI* sites between the SMP8 promoter and the SV40 polyadenylation site of the construct. For $G_{\alpha i2}$-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 $G_{\alpha i2}$, 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, $G_{\alpha i2}$ (5′-GGCGGTTGTCTACAGCAACACCAT-3′ and 5′-CTTGCTCTGTGGGCATGTACTCACT-3′). For $G_{\alpha i2}$-IP, the 5′ primer was based on the HA-tag sequence (5′-TACCCATACGACGTCCCAGACTAC-3′ and 5′-CCACAGTCCTTCAGGTTGTTCTTG-3′). The doxycycline-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 H_2O. 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 $MgCl_2$, 0.026 mM EDTA, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM NaH_2CO_3, pH 7.40) with bubbled 95% O_2 and 5% CO_2. 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 KCl 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, KCl and isoproterenol treatments.

Airway smooth muscle culture. Primary cultures of murine ASM cells were established from tracheal explants of NTG, $G_{\alpha i2}$-OE, and $G_{\alpha i2}$-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

saline solution supplemented with a 2× concentration of antibiotic-antimycotic solution (Invitrogen). After additional surrounding tissue was removed, the tracheal segment was cut longitudinally and dissected into 2- to 3-mm squares. Segments from a single trachea were then placed intima-side-down in a sterile 60-mm dish. After adherence, Dulbecco's modified Eagle's medium supplemented with 20% FCS and 2× antibiotic-antimycotic was added to cover the explants. Explanted trachea were subsequently removed when there was local confluency. Once the initial seed dish became confluent, cells were harvested by trypsinization and passed into 75-cm^2 flasks. As previously described (25), over 90% of these cells were smooth muscle cells, as determined by immunohistochemistry performed with an antibody raised against smooth muscle α-actin. Cells were studied at passages 5–8.

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 tracheae 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 $P < 0.05$.

Acknowledgments

The authors thank Judy Cain, Cheryl Theiss, and Mary Rose Schwarb for technical assistance and Esther Moses for manuscript preparation. The authors were supported by NIH grants HL045967, HL071609, and HL065899 (to S.B. Liggett), HL072068 (to D.W. McGraw), and AI45898 and HL076383 (to M.E. Rothenberg).

Received for publication September 27, 2006, and accepted in revised form February 6, 2007.

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