Integrin $\alpha_9\beta_1$ in airway smooth muscle suppresses exaggerated airway narrowing

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Exaggerated contraction of airway smooth muscle is the major cause of symptoms in asthma, but the mechanisms that prevent exaggerated contraction are incompletely understood. Here, we showed that integrin $\alpha_9\beta_1$ on airway smooth muscle localizes the polyamine catabolizing enzyme spermidine/spermine $N^2$-acyetyltransferase (SSAT) in close proximity to the lipid kinase PIP5K1$\gamma$. As PIP5K1$\gamma$ is the major source of PIP2 in airway smooth muscle and its activity is regulated by higher-order polyamines, this interaction inhibited IP3-dependent airway smooth muscle contraction. Mice lacking integrin $\alpha_9\beta_1$ in smooth muscle had increased airway responsiveness in vivo, and loss or inhibition of integrin $\alpha_9\beta_1$ increased in vitro airway narrowing and airway smooth muscle contraction in murine and human airways. Contraction was enhanced in control airways by the higher-order polyamine spermine or by cell-permeable PIP2, but these interventions had no effect on airways lacking integrin $\alpha_9\beta_1$ or treated with integrin $\alpha_9\beta_1$–blocking antibodies. Enhancement of SSAT activity or knockdown of PIP5K1$\gamma$ inhibited airway contraction, but only in the presence of functional integrin $\alpha_9\beta_1$. Therefore, integrin $\alpha_9\beta_1$ appears to serve as a brake on airway smooth muscle contraction by recruiting SSAT, which facilitates local catabolism of polyamines and thereby inhibits PIP5K1$\gamma$. Targeting key components of this pathway could thus lead to new treatment strategies for asthma.

Introduction
Asthma is characterized by exaggerated narrowing of the conducting airways of the lung in response to normally trivial bronchoconstrictor stimuli. Airway narrowing in asthma is largely dependent on contraction of airway smooth muscle. However, the pathways that regulate airway smooth muscle contraction are incompletely understood.

Integrins are a family of transmembrane heterodimers that participate in the translation of spatially fixed extracellular signals into a wide variety of changes in cell behavior (1, 2). Members of this family share a number of common functions (e.g., cell attachment, spreading, and survival) and use a number of common signaling intermediates (e.g., the focal adhesion kinase and src family kinases) (2, 3). However, the diverse and largely nonoverlapping phenotypes of lines of mice expressing KO alleles of individual integrin subunit genes underscore the biological importance of integrin specificity (4–7). Several integrins have been shown to modulate allergic genes underscore the biological importance of integrin specificity (4–7).

Results
Loss of integrin $\alpha_9$ in smooth muscle causes spontaneous airway hyperresponsiveness in vivo. We generated mice with a knockin of $\beta$-gal (LacZ) into the endogenous Itgag9 locus to study integrin $\alpha_9$ expression in the mouse lung. We counterstained with antibody against E-cadherin and $\alpha$-SMA to identify smooth muscle and distinguish airway from vascular smooth muscle (adjacent to and distant from E-cadherin staining, respectively). This confirmed that integrin $\alpha_9\beta_1$ was highly expressed in airway smooth muscle, but not vascular smooth muscle (Figure 1A). We generated mice with specific KO of integrin $\alpha_9$ in smooth muscle (referred to herein as $\alpha_9$smKO mice) by crossing Itgag9$^{9/9}_\beta$ mice (18) with mice expressing the reverse tetracycline transactivator under the control of the $\alpha$-SMA promoter ($\alpha$-sm-rTTA) and (tetO)7-cre (19). Because integrin $\alpha_9\beta_1$ was not expressed in vascular smooth muscle in the lungs, it was effectively deleted specifically in airway smooth muscle. Immunohistochemistry confirmed decreased expression of integrin $\alpha_9\beta_1$ in airway smooth muscle (Figure 1B). The high efficiency of recombination in airway smooth muscle was further confirmed by Western blot of primary cultured airway smooth muscle cells (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI60387DS1). We evaluated the tissue specificity of recombination in this line by crossing $\alpha_9$smKO...
mice with a fluorescent reporter line (20). The mT/mG double-fluorescent Cre reporter line expresses membrane-targeted tandem dimer Tomato (mTomato) prior to Cre-mediated excision and membrane-targeted GFP (mGFP) after excision. Based on the GFP expression in the lung, α9smKO mice demonstrated highly efficient recombination in smooth muscle, which was further confirmed by anti–α-SMA staining (Supplemental Figure 1B).

Airway responsiveness to acetylcholine was measured in anesthetized and paralyzed mechanically ventilated mice. α9smKO mice had significantly increased airway responsiveness (Figure 2A). α9smKO and control mice had comparable lung and airway histology, with no abnormalities in airway epithelium, and there was no evidence of inflammation in α9smKO lungs (data not shown). We assessed static distensibility by measuring static lung compliance and found no difference between α9smKO and control mice (Supplemental Figure 2). There were also no apparent differences in the volume of airway smooth muscle tissue (determined by anti–α-SMA staining) between α9smKO and control mice (data not shown). These findings suggest that the observed differences in airway responsiveness are most likely caused by differences in the function, rather than the structure, of airway smooth muscle. We previously found that heterozygous Itga9+/− mice are fully viable and have no detectable morphologic differences from Itga9+/+ mice (17). However, when we examined airway responsiveness in these animals, they exhibited an intermediate phenotype, with a small but significant increase in airway responsiveness (Figure 2B). This result suggests that even a modest reduction in the level of α9β1 expression can affect airway responsiveness and that the hyper-responsiveness in α9smKO mice is not likely to be caused by overexpression of Cre recombinase or rTTA protein.

Integrin α9β1 inhibits airway narrowing and smooth muscle contraction in vitro. To directly determine whether the differences in pulmonary resistance are due to differences in airway narrowing, we examined ex vivo airway contraction in mouse lung slices, a protocol modified from the one reported by Sanderson’s laboratory (21). Airway narrowing was determined by the decrease of lumen area induced by methacholine, as shown in Figure 2C and Supplemental Video 1. Methacholine induced significantly more narrowing in airways from α9smKO than control mice (Figure 2D). There were no differences in the duration of contraction between α9smKO and control animals. To exclude effects caused by genetic compensation, we also induced loss of smooth muscle ex vivo by infecting lung slices prepared from Itga9fl/fl mice with adenovirus expressing Cre recombinase. In vitro loss of integrin α9 induced a very similar and significant increase in airway narrowing (Figure 2E). The high efficiency of recombination in lung slices was further confirmed by Western blot (Supplemental Figure 3). We also incubated lung slices with hamster monoclonal blocking antibody against integrin α9β1 (22); this in vitro inhibition of α9β1 ligation also increased airway narrowing compared with hamster IgG control (Figure 2F).

Loss of integrin α9 in smooth muscle also increased methacholine-induced in vitro force generation by excised tracheal rings (Figure 3A). As we observed with lung slices, integrin α9β1-blocking antibody or gene deletion ex vivo by infection with adenovirus-Cre increased force generation in response to methacholine (Figure 3C and E). To determine whether loss of integrin α9β1 ligation on airway smooth muscle specifically enhances signaling through acetylcholine receptors, we examined the effects of integrin α9β1 inhibition on contraction mediated by serotonin (5-HT), which
induces contraction by activating a separate G protein–coupled receptor (23). Lung slices and tracheal rings from α9smKO mice showed increased contractile responses to 5-HT (Supplemental Figure 4, A and B). Integrin α9β1-blocking antibody also enhanced 5-HT–induced contraction in both mouse lung slices and tracheal rings (Supplemental Figure 4, C and D). 5-HT and cholinergic agonists induce airway smooth muscle contraction by activating PLC, which converts phosphatidylinositol 4,5-bisphosphate (PIP2) to 1,4,5-trisphosphate (IP3). IP3 then binds to IP3 receptors on the sarcoplasmic reticulum (SR) to induce release of calcium into the cytosol, which triggers downstream events that lead to smooth muscle contraction. Alternatively, smooth muscle contraction can be induced by influx of extracellular calcium, for example, by activating voltage-gated calcium channels through addition of extracellular KCl. To determine whether loss of integrin α9 enhances smooth muscle contraction downstream of changes in intracellular calcium, we examined the effects of integrin α9β1 gene deletion, blocking antibody, and ex vivo deletion by adenovirus-Cre on KCl-induced contraction and found no effect (Figure 3, B, D, and F). To exclude possible nonspecific effects of blocking antibody on cells other than airway smooth muscle, we examined the effects of blocking antibody on α9smKO lung slices and found no further increase in airway narrowing (Supplemental Figure 5).

Tracheal rings contain a number of cell types, including a layer of epithelial cells. To evaluate whether the phenomenon we observed was due to indirect effects through airway epithelial cells, we removed epithelium from tracheal rings. As expected, epithelium removal enhanced the magnitude of force generation in response to methacholine. However, the difference between WT and α9smKO tracheal rings was sustained (Supplemental Figure 6), which suggests that the inhibitory effect of integrin α9β1 on smooth muscle contraction does not depend on effects on epithelial cells. Together, these results suggest that integrin α9β1 regulates airway smooth muscle contraction by modulating the pathway by which activation of G protein–coupled receptors stimulates calcium release.

Integrin α9–mediated inhibition of airway smooth muscle contraction is mediated by modulation of polyamine catabolism. We have previously shown that the integrin α9 cytoplasmic domain specifically binds to the polyamine catabolizing enzyme spermidine/spermine N1-acetyltransferase (SSAT) and that this effect is critical for integrin α9β1–dependent cell migration (24, 25). To determine whether the interaction of the integrin α9 cytoplasmic domain with SSAT is also important for integrin α9β1–mediated modulation of airway smooth muscle contraction, we evaluated the effects of stabilizing SSAT (and thus effectively enhancing SSAT activity) on airway contraction using the polyamine analog N1,N11-bis(ethyl)norspermine...
tetrahydrochloride (BE3-3-3), which prevents proteasome-mediated SSAT degradation (26). BE3-3-3 inhibited airway contraction in WT lung slices and tracheal rings, but had no effect on the enhanced contraction induced by loss or blockade of integrin α9 (Figure 4, A–D). These results support the hypothesis that integrin α9-dependent inhibition of airway smooth muscle contraction depends on the interaction of the integrin α9 cytoplasmic domain with SSAT.

We next investigated whether the metabolism of polyamines plays a role in integrin α9β1-mediated inhibition of airway narrowing. The oxidative catabolism of the higher-order polyamines, spermidine and spermine, is accomplished by the concerted action of 2 different enzymes, SSAT and polyamine oxidase (PAO). Cytosolic SSAT N1-acetylates both spermidine and spermine, which then serve as substrates for PAO (27). Because PAO strongly prefers acetylated polyamines to unmodified polyamines as its substrates, SSAT is generally considered the rate-controlling enzyme in the back conversion of the higher-order polyamines spermidine and spermine to the lower-order polyamine putrescine. We thus examined the effects of exogenous spermine (which should compensate for spermine catabolism by SSAT) or exogenous putrescine in order to determine whether integrin α9β1 specifically inhibits airway smooth muscle contraction by reducing concentrations of higher-order polyamines or by increasing the concentration of the end product putrescine. Spermine clearly increased contraction of control lung slices and tracheal rings, but had no effect on α9smKO lung slices or tracheal rings (Figure 5, A–D). Putrescine had no effect on airway contraction. Similar results were also obtained with WT lung slices and tracheal rings treated with polyamines in the presence of integrin α9–blocking antibody (Supplemental Figure 7). To exclude the possibility that we failed to see additive effects of integrin α9 loss and spermine treatment because each intervention causes maximal airway narrowing or force generation, we examined the additional effects of another enhancer of airway smooth muscle contraction, IL-13. IL-13 clearly further enhanced contraction in lung slices treated with either blocking antibody or spermine (Supplemental Figure 8, A and B), which suggests that the absence of additive effects of loss or blockade of integrin α9 and spermine are not the result of either

**Figure 3**
Increased tension generation in tracheal rings from α9smKO mice. (A and B) Force generation in response to methacholine (A) and KCl (B) by tracheal rings from WT control or α9smKO mice. (C and D) Force generation in response to methacholine (C) and KCl (D) by tracheal rings from WT mice pretreated in vitro with blocking antibody or control antibody. (E and F) Tracheal rings from Itga9fl/fl mice were infected with adenovirus expressing Cre recombinase or GFP (as control) for 3 days before assessment of contractile response to methacholine (E) and KCl (F). Results (mean ± SEM) are representative of 2 independent experiments (n = 10). *P < 0.01.

**Figure 4**
The effect of airway smooth muscle integrin α9β1 depends on SSAT. Airway narrowing in lung slices (A and C) and tension in tracheal rings (B and D) from control and α9smKO mice, either (A and B) untreated or (C and D) treated with control or blocking antibody, in the presence or absence of 50 μM BE3-3-3 for 24 hours. Results (mean ± SEM) are representative of 2 independent experiments (n = 10). *P < 0.01.
agent inducing maximal contraction. Together, these results are consistent with the hypothesis that integrin α9β1 normally inhibits airway smooth muscle contraction by facilitating local decreases in the concentration of higher-order polyamines.

Integrin α9β1 modulates smooth muscle calcium oscillation frequency and myosin light chain phosphorylation. Our finding that loss of integrin α9β1 enhanced airway smooth muscle contraction in response to cholinergic agonists and 5-HT, but not depolarization by KCl, suggests that this integrin is modulating a pathway downstream of G protein–coupled receptors, but upstream of Ca2+ release. Previous studies have shown that G protein–coupled receptor agonists induce cyclic release and reuptake of calcium, leading to cytosolic Ca2+ oscillations, and that the magnitude of the resultant smooth muscle contraction is best correlated with the frequency of these Ca2+ oscillations (28). To evaluate whether integrin α9β1 modulates airway smooth muscle contraction through effects on Ca2+ release, we loaded lung slices with Oregon Green 488 BABTA-1-AM and evaluated Ca2+ oscillations at video rate (Supplemental Video 2). Methacholine-induced Ca2+ oscillations occurred at markedly higher frequency in slices from α9smKO mice (Figure 6A). The frequency of Ca2+ oscillations was increased by spermine treatment and decreased by BE3-3-3 treatment, but only in lung slices from control mice (Figure 6B), completely consistent with our observations for airway contraction. These findings suggest that ligated integrin α9β1 inhibits airway smooth muscle contraction by acting downstream of G protein–coupled receptors, but upstream of Ca2+ release from the SR.

Contraction of airway smooth muscle is dependent on activation of actin and myosin cross-bridges and phosphorylation of myosin light chain (MLC) by MLC kinase. The degree to which MLC is phosphorylated can determine the velocity of smooth muscle shortening and maximum contraction (29). We found that methacholine-induced MLC phosphorylation was increased in smooth muscle of α9smKO animals. As above, MLC phosphorylation was inhibited by BE3-3-3 and enhanced by spermine in smooth muscle dissected from control airway tracheal rings, but unaffected by either treatment in smooth muscle from α9smKO mice (Figure 6C).

Integrin α9β1, SSAT, and polyamines have similar roles in human airways. To determine whether the pathway we identified in murine airways is also relevant to airway narrowing in humans, we examined the effects of integrin α9β1 inhibition and of BE3-3-3 and spermine treatment on narrowing of airways in human lung slices and on force generation in human bronchial rings. First, we confirmed that integrin α9β1 was highly expressed in human airway smooth muscle cells by costaining of integrin and α-SMA (Supplemental Figure 9). As we found in murine airways, human airway contraction was increased by integrin α9β1–blocking antibody in both bronchial rings and lung slices (Figure 7, A and E). Human airway contraction was also inhibited by BE3-3-3 and enhanced by spermine (Figure 7, C and F). There were no effects of blocking antibody on KCl-induced contraction (Figure 7, B and D). Overall, these data support an important role of the pathway we describe in human airway smooth muscle and suggest that SSAT could be a potential target for the treatment of asthma.

PIP2 and PIP5K1γ play critical roles in inhibition of smooth muscle contraction by integrin α9β1. One of the best-characterized effects of spermine and spermidine that is not shared by the breakdown product putrescine is dramatic enhancement of the activity of the lipid kinase PIP5K1γ. PIP5K1γ adds a second phosphate to phosphatidylinositol 4-phosphate (PIP4P) to generate PIP2 (30) and is the major lipid kinase responsible for generation of PIP2 in airway smooth muscle (31). PIP2 is the essential precursor for IP3, which, as described above, is generated by PLC in response to G protein–coupled receptor activation and directly mediates cyclic Ca2+.
release from the SR. Importantly, PIP5K1γ is the only lipid kinase with a focal adhesion targeting domain, which has been previously shown to directly bind the focal adhesion protein talin, which in turn binds directly to the cytoplasmic domains of integrin β subunits, including β1 (32, 33). We therefore hypothesized that integrin αβ1 might inhibit airway smooth muscle contraction by localizing SSAT and locally catabolizing spermine and spermidine, thus decreasing PIP5K1γ activity, reducing local PIP2 production, and resulting in less IP3 production. If this hypothesis is correct, we should be able to overcome the inhibitory effects of ligated integrin αβ1 by adding excess PIP2. We therefore incubated lung slices with either a cell-permeable form of PIP2 (diC16-PIP2) or its carrier vehicle (carrier 3) and evaluated airway narrowing in response to methacholine. diC16-PIP2 significantly increased airway narrowing in control slices, to the same level seen in slices from untreated αβ1smKO mice, but had no additional effect in lung slices from αβ1smKO mice or from mice treated with integrin αβ1-blocking antibody (Figure 8, A and B).

We first confirmed that PIP5K1γ was highly expressed in tracheal smooth muscle tissue (Supplemental Figure 10). We then confirmed that PIP5K1γ associates with integrin αβ1 in airway smooth muscle by IP and immunofluorescent staining. IP of integrin αβ1 from lysates of WT murine tracheal smooth muscle tissue also precipitated PIP5K1γ (Figure 8C). Integrin αβ1 was also found in the complex precipitated by anti-SSAT (Figure 8D). We also found that PIP5K1γ and integrin αβ1 colocalized in focal adhesions of cultured murine airway smooth muscle, whereas a mutant form of PIP5K1γ lacking the talin-binding (integrin-targeting) domain did not colocalize with integrin αβ1 (Figure 8E). This colocalization was not dependent on PIP5K1γ kinase activity, since kinase-dead PIP5K1γ and integrin αβ1 colocalized. To more directly examine the functional significance of PIP5K1γ in these responses, we constructed adenoviruses expressing mcherry-tagged full-length murine PIP5K1γ, a kinase-dead version, or a version expressing a splice variant lacking the talin-binding domain and examined the effects on methacholine-induced contraction of WT lung slices. Full-length PIP5K1γ enhanced airway narrowing, but the kinase-dead version and the version lacking the talin-binding domain did not (Figure 9A). Interestingly, full-length PIP5K1γ did not further enhance airway narrowing in lungs slices treated with integrin αβ1-blocking antibody, whereas the kinase-dead mutant showed a dominant-negative inhibitory effect (Figure 9B). This suggests that in the absence of ligated integrin αβ1, there was sufficient PIP5K1γ present at baseline to saturate the requirement for local PIP2. We also generated adenovirus expressing shRNA for PIP5K1γ. Knockdown efficiency was found to be greater than 90% in mouse fibroblasts (Supplemental Figure 11). Knockdown of PIP5K1γ decreased airway contraction in lung slices in the presence or absence of integrin αβ1-blocking antibody (Figure 9C), which confirmed the important role this enzyme plays in the contractile response to methacholine. Although PIP5K1γ shRNA did not reduce contraction of slices treated with blocking antibody to the level seen with shRNA in WT slices, this partial response is likely explained by incomplete knockdown in lung slices (Figure 9D).

To further test our hypothesis, we measured PIP5K1γ activity by immunoprecipitating the enzyme from lysates of tracheal smooth muscle cells from control and αβ1smKO mice in the presence of absence of BE3-3-3 or spermine treatment. PIP5K1γ kinase activity was higher in αβ1smKO than in control smooth muscle (Figure 10A). Activity was increased by spermine and decreased by BE3-3-3 in WT smooth muscle, but neither treatment had any effect in muscle from αβ1smKO mice (Figure 10B). Overall, these data support the hypothesis that integrin αβ1 and SSAT prevent airway narrowing at least in part through local modulation of PIP5K1γ activity and PIP2 production.

**Discussion**

Exaggerated responses to agonists of G protein–coupled receptors expressed on airway smooth muscle represent a central feature of human asthma. Although the core pathway linking receptor ligation to airway smooth muscle contraction has been well established, much remains unknown about how this core pathway is modulated and why this pathway is hyperresponsive in asthma. Interactions between integrins and the surrounding ECM have
been shown to regulate airway smooth muscle proliferation (34, 35) and to connect smooth muscle cells to the matrix, allowing contraction to be translated into the force generation required for airway narrowing (14). Here, we describe a role for ligation of integrin α9β1, in preventing exaggerated contraction of airway smooth muscle, which we believe to be novel. Integrin-mediated prevention of smooth muscle contraction depends on the previously described association of the integrin α9 cytoplasmic domain with SSAT and is caused by catabolism of higher-order polyamines (spermine and spermidine). A reduction in local concentrations of polyamines appears to decrease the contractile response to G protein–coupled receptor agonists by reducing the activity of the integrin-associated lipid kinase PIP5K1γ, which reduces the concentration of available PIP2 and thus inhibits IP3-mediated calcium release from the smooth muscle SR (Figure 11). These conclusions are supported by our findings that exogenous PIP2 enhanced contraction only in control airway smooth muscle and that overexpression of WT PIP5K1γ, but not a kinase-dead version or a version that cannot localize to integrins, also enhanced contraction only in smooth muscle containing functional, ligated integrin α9β1.

Integrin α9β1 is highly expressed in airway smooth muscle. This integrin has been shown to interact with a wide variety of ligands, including the endothelial counter receptor VCAM-1 (36), tenascin C (37), osteopontin (38, 39), cellular fibronectin (40), several members of the disintegrin and metalloprotease (ADAM) family (41–43), coagulation factor XIII (44), tissue transglutaminase (44), VEGFA (45, 46), VEGFC and VEGFD (47), and von Willebrand factor (44). Our current findings suggest that integrin α9β1 ligation is required for inhibition of airway smooth muscle contraction, since blocking antibody that inhibits interactions with ligands was as effective as KO or knockdown of the integrin in enhancing airway smooth muscle contraction. However, given the large number of potential ligands, we have not attempted here to identify the relevant ligand on or near airway smooth muscle.

We have previously shown that another effect of integrin α9β1, accelerated cell migration, depends on the interaction between the integrin α9 cytoplasmic domain and SSAT (24, 25). SSAT is the rate-limiting step in catabolism of higher-order polyamines (48), which converts spermine to spermidine and then spermidine to putrescine. Because SSAT is rapidly ubiquitinated and degraded in cells, for in vitro experiments it was necessary to use the polyamine analog BE3-3-3, which binds to SSAT and prevents its degradation. By preventing SSAT degradation, this drug effectively enhances SSAT activity, and it has thus proven a useful tool to probe SSAT-mediated effects in cells. In this study, we found that airway narrowing in lung slices and force generation by tracheal rings were decreased by BE3-3-3 treatment only in tissues in which integrin α9 was expressed and functional; BE3-3-3 had no effect on tissues of α9smKO mice or animals treated with integrin α9β1–blocking antibody. Furthermore, exogenous spermine also enhanced airway narrowing and force generation only in tissues in which integrin α9 was expressed and functional on airway smooth muscle. Together, these results strongly suggest that integrin α9β1 normally inhibits airway smooth muscle contraction through interaction with SSAT and local breakdown of higher-order polyamines.

The 2 best-characterized cytosolic effects of spermidine and spermine that are not shared by putrescine are inward rectification of inwardly rectifying potassium (Kir) channels (49, 50) and dramatic enhancement of PIP5K1γ activity (31, 51). We have previously shown that integrin α9β1–mediated enhancement of cell migration is caused by altered rectification of a specific Kir channel, Kir4.2 (25), and have found that Kir4.2 is also the major Kir channel expressed in airway smooth muscle (unpublished observations). However, we found that inhibiting Kir channels by barium chloride had no effect on force generation by tracheal rings or lung slices (our unpublished observations). PIP5K1γ is the major source of PIP2 in airway smooth muscle cells (31). Conversion of PIP2 to IP3 is a critical step in the pathway by which G protein–coupled receptor agonists induced calcium efflux from the SR, an important step regulating smooth muscle contraction. We found that increased cellular PIP2 concentration or increased PIP5K1γ kinase activity.
activity reversed the inhibitory effects of integrin α9β1. Furthermore, the absence of this integrin enhanced the frequency of calcium oscillations in airway smooth muscle, and this effect was inhibited by BE3-3-3 and spermine only in smooth muscle that expressed integrin α9. These data support the hypothesis that integrin α9β1 and SSAT prevent airway narrowing at least in part through modulation of PIP5K1γ activity and PIP2 generation, and that these effects are mediated by enhanced calcium release from intracellular stores. The absence of any effects of integrin α9 loss on the contractile response to KCl, which bypasses all these steps by inducing influx of extracellular calcium through voltage-gated calcium channels, provided further support for this interpretation.

PIP2 also plays an important role in linking actin to integrin-mediated adhesion sites, a key step in agonist-induced force generation by airway smooth muscle (52, 53). However, as noted above, loss of integrin α9 on airway smooth muscle specifically

Figure 8
Interaction between PIP5K1γ and integrin α9β1 in airway smooth muscle cells. (A and B) Airway narrowing was measured on lung slices from control and α9smKO mice, either (A) untreated or (B) treated with control or blocking antibody, that were also treated with either cell-permeable diC16-PIP2 and carrier 3 (the lipid carrier) or carrier 3 alone. *P < 0.01. (C) Co-IP of integrin α9β1 and PIP5K1γ. Lysate from mouse tracheal tissue was subjected to IP by anti-integrin α9 or rabbit IgG control followed by protein G–sepharose. Precipitated proteins (or cell lysates; Lys) were detected with anti-integrin α9 and anti-PIP5K1γ. (D) Co-IP of integrin α9β1 and SSAT. Mouse tracheal tissues were treated with BE3-3-3 (50 μM) for 24 hours before lysis. Lysates were subjected to IP by anti-SSAT or rabbit IgG control and detected with anti–integrin α9 and SSAT. (E) Mouse airway smooth muscle cells were cotransfected by integrin α9–EGFP (α9eGFP) and PIP5K1γ-mcherry fusion cDNA (m661, full-length PIP5K1γ; m661KD, kinase-dead mutation; m635, truncated PIP5K1γ lacking the 26 amino acids that interact with talin). Scale bar: 10 μm.
inhibited responses to G protein–coupled receptor ligation, with no effect on contractile responses to smooth muscle depolarization by KCl. If integrin $\alpha_9\beta_1$ was principally modulating smooth muscle contraction by enhancing interactions between actin and other anchoring integrins, we would have also expected effects on KCl-induced contraction. However, we cannot exclude some contribution of PIP2's effects on integrin anchoring as an amplifier of the inhibitory response we describe herein.

It is tempting to speculate that the pathway we describe could have relevance to the exaggerated airway narrowing that characterizes human asthma. For example, our results would predict that genetic or acquired defects in integrin $\alpha_9\beta_1$, its relevant ligands, or SSAT could result in functionally important airway hyperresponsiveness in humans. Furthermore, these results suggest that stabilization of SSAT or inhibition of PIP5K1$\gamma$ could represent novel therapies for prevention of airway smooth muscle contraction in asthma.

**Methods**

**Reagents.** Polyclonal antiserum was raised in rabbits against the mouse integrin $\alpha_9$ cytoplasmic domain peptide NRKENEDGWDWVQKNQ and affinity purified. Hamster monoclonal antibody SS2AC against murine integrin $\alpha_9$ was previously described (54). Human integrin $\alpha_9\beta_1$–blocking antibody Y9A2 was generated in our laboratory (55). Both blocking antibodies bind the extracellular domains of the integrin, as determined by flow cytometry, and block ligand binding, as determined by cell adhesion assays (36, 54, 56). BE3-3-3 was from TOCRIS. Adenoviruses expressing GFP or Cre recombinase were gifts from L. Wu (UCLA, Los Angeles, California, USA). diC16-PIP2 and carrier vehicle were from Echelon Biosciences Inc. Mouse monoclonal antibody against PIP5K1$\gamma$ was from BD Biosciences. Mouse monoclonal antibody against $\alpha$-SMA was from Sigma-Aldrich. An integrin $\alpha_9\beta_1$–specific mutant form of the third fibronectin type III repeat in tenascin-C(TNfn3RAA) was previously described (57).

**Mouse lines.** $\text{Itga9}^{0/0}$ (18), Gt(ROSA)26Sor tm4(ACTB-tdTomato,-EGFP)Luo/J (20), and (tetO)$^7$-Cre (19) mouse lines have been described previously. $\alpha$-sm-tTA mice were provided by M. Shipley (Washington University School of Medicine, St. Louis, Missouri, USA). Mice were maintained on a C57BL/6 background and housed at the UCSF animal care facility.

**Integrin $\alpha_9$ reporter mouse generation.** A knockin ES cell clone was obtained from Knock-Out Mouse Project (www.komp.org; project no. VG12163). LacZ cDNA was inserted downstream of the endogenous $\text{Itga9}^\text{promoter}$. 

**Figure 9** Dependence of effect of airway smooth muscle integrin $\alpha_9\beta_1$ on PIP5K1$\gamma$. (A and B) Airway narrowing in lung slices treated with control antibody (A) or blocking antibody (B) and infected by adenovirus expressing full-length, truncated, or kinase-dead PIP5K1$\gamma$. (C) Airway narrowing in lung slices treated with control antibody or blocking antibody and infected by adenovirus expressing full-length, truncated, or kinase-dead PIP5K1$\gamma$. Mouse lung slices were incubated with shRNA-expressing adenovirus for 0, 1, 2, or 3 days. Data (mean ± SEM) represent 2 independent experiments with similar results. *$P < 0.01$.

**Figure 10** PIP5K1$\gamma$ activities are regulated by integrin $\alpha_9\beta_1$ and polyamines. (A) Mouse tracheal smooth muscle tissues (5 mice per group) were lysed, PIP5K1$\gamma$ kinase was subjected to IP by anti-PIP5K1$\gamma$ antibody, and kinase activity was calculated as the transfer of $[^\gamma\text{32P}]$ to PI4P from $[^\gamma\text{32P}]$ ATP. Developed blots for all samples in each group are shown. (B) Mouse tracheal smooth muscle tissues were incubated with or without spermine (100 $\mu$M) or BE3-3-3 (50 $\mu$M) for 24 hours. PIP5K1$\gamma$ activity is expressed as a percentage of control. Data are mean ± SEM ($n = 5$). *$P < 0.01$. 

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μα muscle cells were established from tracheal explants of rat anti-mouse E-cadherin (R&D Systems) and Cy3-conjugated anti-mouse
12 hours. After staining, sections were washed with PBS, then incubated with X-gal (1 mg/ml; Invitrogen) at 37°C for
tions were cut and incubated with X-gal (1 mg/ml; Invitrogen) at 37°C for 10 minutes. Methacholine was added after the first recording. The lumen area
Multiple airways in 1 lung slice were recorded in time lapse (1 frame/min) for
microscope was equipped with a temperature chamber maintained at 37°C. The whole lung slice was imaged (area
were placed in a 15-ml organ bath by 2 stainless-steel wires (0.2 mm in diameter) passed through the lumen. One wire was fixed to the organ
for active cilia beating in the airways.
measurement of intracellular Ca2+ oscillation. The protocol was modified from that of Bai et al. (21). Briefly, lung slices were labeled by Oregon green 488 BAPTA-AM (20 μM in HBSS containing 0.1% Pluronic F-127 and 100 μM sulfobromophthalein). For 45 minutes at 30°C and de-esterified for 30 minutes at 30°C in HBSS containing 100 μM sulfobromophthalein. Lung slices were placed in coverglass chambers (Lab-Tek) and immobilized by Slice Anchor. Fluorescence imaging was performed with a Nikon spinning-disk confocal microscope at 20 frames/s using a x10 Nikon objective. Changes in fluorescence intensity from selected regions of interest (5 × 5 pixels) were analyzed by ImageJ software.
Tracheal ring contraction. Tracheal ring segments approximately 3 mm in length were suspended in a 15-ml organ bath by 2 stainless-steel wires (0.2 mm diameter) passed through the lumen. One wire was fixed to the organ

Figure 11
Integrin α6β1, inhibits airway smooth muscle contraction by interacting with SSAT and regulating PIP5K1α kinase activity. CaM, calmodulin; DAG, diacylglycerol; MLCK, MLC kinase; Pu, putrescine.

Measurement of airway response to acetylcholine. WT and αsmKO mice were anesthetized with ketamine/sylazine, attached to a rodent ventilator and pulmonary mechanics analyzer (FlexiVent; SIRAQ Inc.), and ventilated at a tidal volume of 9 ml/kg, a frequency of 150 breaths/minute, and 2 cmH2O positive end-expiratory pressure. Mice were paralyzed with pancuronium (0.1 mg/kg intraperitoneally). A 27-gauge needle was placed in the tail vein, and measurements of airway mechanics were made continuously using the forced oscillation technique. Mice were given increasing doses of acetylcholine (0.03, 0.1, 0.3, 1, and 3 μg/g body weight) administered through the tail vein to generate a concentration-response (58).

Measurement of lung static compliance. WT and αsmKO mice were anesthetized with ketamine (100 mg/kg), sylazine (10 mg/kg), and acepromazine (2–3 mg/kg). A tracheostomy was performed, and a tubing adaptor (20 gauge) was used to cannulate the trachea. The mice were then attached to a rodent ventilator and pulmonary mechanics analyzer (FlexiVent; SIRAQ Inc.) and ventilated at a tidal volume of 9 ml/kg, a frequency of 150 breaths/minute, and 2 cmH2O positive end-expiratory pressure. The animals were then paralyzed with pancuronium (0.1 mg/kg intraperitoneally) and allowed to adjust to the ventilator. Static compliance was calculated from quasistatic pressure/volume curves that were generated by stepwise inflation and deflation of the lungs.

Mouse lung slices. Murine lung slices were prepared as described by Bai and Sanderson (21) from 6- to 10-week-old mice on the C57BL/6 background. After the chest cavity was opened, the lungs were inflated with 1.0 ml warm (37°C) 2% low-melting agarose through a tracheal catheter, and 0.2 ml air was injected to flush the agarose out of the airways. After gelling of the agarose in the whole lung by cooling to 4°C, a single lobe was removed and cut into serial sections of 140 μm thickness with a vibratome (VT1000S; Leica) at 4°C. Slices were maintained in DMEM supplemented with antibiotics at 37°C and 10% CO2 for up to 3 days.

Human lung slices. Human lung slices were prepared by the previously described protocol (59) modified in our lab. Human lobectomy samples (obtained from explanted lungs during lung transplantation) were warmed in a 37°C water bath, then inflated with a warm agarose solution (4% in PBS, 37°C) via the mainstem bronchus. To solidify the agarose, the tissue was cooled with ice for 30 minutes. 400-μm slices were prepared with a vibratome (as above) from tissue sections containing visible airways. The viability of the slices was confirmed by active cilia beating in the airways.

Measurement of airway contraction. Lung slices were maintained overnight in DMEM with or without polyamines, BE3-3-3, and/or antibodies. 2 lung slices were placed in a 6-well plate with 3.0 ml DMEM and held by a Slice Anchor (SHD-22L/10; Warner Instruments). The whole lung slice was imaged (area scan) with a Leica DM6000 inverted microscope using a x5 objective. The microscope was equipped with a temperature chamber maintained at 37°C. Multiple airways in 1 lung slice were recorded in time lapse (1 frame/min) for 10 minutes. Methacholine was added after the first recording. The lumen area of the airway was measured by ImagePro software (Media Cybernetics Inc.).

Measurement of airway smooth muscle culture. Primary cultures of murine airway smooth muscle cells were established from tracheal explants of αsmKO mice and WT controls. The trachea was removed and placed on a dish containing HBSS supplemented with antibiotic solution. Tracheal segments were dissected into 2- to 3-mm squares and placed in a 6-well plate. After adherence, 10 minutes. Methacholine was added after the first recording. The lumen area of the airway was measured by ImagePro software (Media Cybernetics Inc.).

Measurement of airway smooth muscle culture. Primary cultures of murine airway smooth muscle cells were established from tracheal explants of αsmKO mice and WT controls. The trachea was removed and placed on a dish containing HBSS supplemented with antibiotic solution. Tracheal segments were dissected into 2- to 3-mm squares and placed in a 6-well plate. After adherence, DMEM with 20% FCS was added to cover the explants. Explanted tracheas were subsequently removed when there was local confluence of the outgrowth of smooth muscle cells. The purity of airway smooth muscle cells was confirmed by anti-α-SMA staining.

ES cell blastocyst injection was performed in the transgenic mouse core of the Gladstone Research Institute, germline transmission was confirmed, and heterozygous reporter mice were used to obtain lung sections.

Immunostaining. Fresh mouse lungs were inflated by intratracheal injection with cold PBS containing 15% sucrose mixed with ornithine-carbamyltransferase (OCT), then embedded in OCT. Mouse trachea was embedded in OCT directly. Cryostat sections (5 μm) were cut onto glass slides and stored at −80°C after 1 hour air drying. For immunofluorescent staining, tissues were fixed in cold acetone for 5 minutes at −20°C. Nonspecific binding was blocked by incubation with 5% goat serum in PBS for 30 minutes at room temperature, followed by overnight incubation with rabbit anti–integrin α6 at 4°C. Samples were visualized with Alexa Fluor 488 goat anti–rabbit antibody (Invitrogen).

Lungs from LacZ knockin mice were inflated by 4% paraformaldehyde in PBS for 1 hour at 4°C. Fixed tissues were transferred to PBS containing 20% sucrose overnight (4°C), after which they were embedded in OCT. 10-μm sections were cut and incubated with X-gal (1 mg/ml; Invitrogen) at 37°C for 12 hours. After staining, sections were washed with PBS, then incubated with rat anti-mouse E-cadherin (R&D Systems) and Cy3-conjugated anti-mouse α-SMA (Sigma-Aldrich) followed by Alexa Fluor 488 goat anti-rabbit (Invitrogen).

Airway smooth muscle culture. Primary cultures of murine airway smooth muscle cells were established from tracheal explants of αsmKO mice and WT controls. The trachea was removed and placed on a dish containing HBSS supplemented with antibiotic solution. Tracheal segments were dissected into 2- to 3-mm squares and placed in a 6-well plate. After adherence, DMEM with 20% FCS was added to cover the explants. Explanted tracheas were subsequently removed when there was local confluence of the outgrowth of smooth muscle cells. The purity of airway smooth muscle cells was confirmed by anti-α-SMA staining.

Measurements of intracellular Ca2+ oscillation. The protocol was modified from that of Bai et al. (21). Briefly, lung slices were labeled by Oregon green 488 BAPTA-AM (20 μM in HBSS containing 0.1% Pluronic F-127 and 100 μM sulfobromophthalein) for 45 minutes at 30°C and de-esterified for 30 minutes at 30°C in HBSS containing 100 μM sulfobromophthalein. Lung slices were placed in coverglass chambers (Lab-Tek) and immobilized by Slice Anchor. Fluorescence imaging was performed with a Nikon spinning-disk confocal microscope at 20 frames/s using a x10 Nikon objective. Changes in fluorescence intensity from selected regions of interest (5 × 5 pixels) were analyzed by ImageJ software.
bath, whereas the other was connected to a force-displacement transducer (FT03; Grass Instrument Co.) for the measurement of isometric force. A resting tension of 0.5 g was applied, and rings were equilibrated for 45 minutes. The buffer solution, containing modified Krebs-Henseleit solution (117.5 mM NaCl, 5.6 mM KCl, 2.5 mM CaCl₂, 1.18 mM MgSO₄, 25 mM NaHCO₃, 1.28 mM NaH₂PO₄, and 5.55 mM glucose), was maintained at 37°C and oxygenated with 95% O₂ and 5% CO₂. In some experiments, rings were incubated with antibodies or adenosine in a 37°C incubator with 5% CO₂ for 24 hours prior to study. Rings were first contracted by 60 mM KCl, and only those that could generate more than 2.5 mN tension were used for experiments. Rings were washed and re-equilibrated, and contractile response was then evaluated with increasing concentrations of methacholine (10⁻⁴ to 10⁻³ M) or KCl (3.75 to 60 mM). To evaluate whether integrin α₅β₁ modulates airway contraction effects on epithelial cells, we completely removed epithelium in some rings by gentle rubbing with roughened PE-10 tubing. Epithelial removal was confirmed for each individual ring by microscopic observation of H&E-stained sections.

Isolated human bronchi. Bronchi with diameters between 3 and 8 mm were dissected free of lung and connective tissue and cut into 3- to 4-mm-thick rings. The tissues were then incubated overnight at 37°C in DMEM with antibiotics, polyamines, or BE3-3-3 and used for contraction in 13-ml organ baths as described above.

Adenovirus production. Clones for chimerically-tagged full-length murine PIP5Kιγ, kinase-dead mutant, and truncated mutant lacking the 26 amino acids that interact with talin were provided by R.A. Anderson (University of Wisconsin–Madison, Madison, Wisconsin, USA). cDNAs were subcloned into pshuttle-CMV vector. Adenoviruses were then produced and amplified using the AdEasy XL Adenoviral System (Agilent Technologies). For infection, 10⁹ pfu viruses were used for each lung slice.

shRNA-mediated knockdown of mouse PIP5Kιγ. shRNA was designed using pSicoOligomaker 1.5 (http://web.mit.edu/jacks-lab/protocols/pSico.html). shRNAs were cloned into the lentiviral vector pSicoR and verified by sequencing. Lentiviruses were produced in HEK 293T cells and used to infect mouse embryonic fibroblasts. Infected cells were enriched by fluorescence-activated cell sorting and selected for high GFP expression. The efficiency of shRNA-mediated knockdown was evaluated by Western blot. shRNA 2045 (targeting 5'-GAAGACCTGCAGAAGATAA-3') knocked down PIP5Kιγ protein more than 90% (Supplemental Figure 4). shRNA 1791 (targeting 5'-GAAGAGATGTCGATGATGAA-3') had no effect on PIP5Kιγ expression and was used as control shRNA. To knock down PIP5Kιγ in mouse lung slices, the shRNA expression cassette was cloned into the pShuttle vector, and adenovirus was generated as described above.

Fluorescent protein expression and microscopy. Mouse airway smooth muscle cells were prepared as described above. Cells within passage 4 were serum starved for 24 hours before transfection. Fluorescent protein-tagged human integrin α₅ and mouse PIP5Kιγ cDNAs were cotransfected by Nucleofector (Basic Smooth Muscle Cells [SMC] Nucleofector Kit; Lonza). Cells were plated on Tfn3R3AA-coated coverglass chambers and imaged 24 hours later on a total internal reflection fluorescence microscope (TE2000E; Nikon), using a Photometrics Cascade II EM CCD camera and Nikon NIS Elements software (all installed at the Nikon Imaging Center at UCSF).

PIPSKιγ kinase assay. Mouse tracheal smooth muscle tissues were lyzed in IP buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 5.0 mM NaF; 2 mM Na₃VO₄; 1 mM EDTA; 0.1 mM EGTA; 10% glycerol; and proteinase inhibitor cocktail), centrifuged, and incubated with protein A-Sepharose and rabbit anti-PIPSKιγ antibody (ab109192; Abcam) at 4°C overnight. Sepharose were then washed 3 times with IP buffer. PIP5Kιγ kinase activity was assayed following a previously described method for lipid kinases (60). Kinase activity was assayed at room temperature in a 40-µl final reaction volume consisting of 20 µl buffer substrate solution and 20 µl IP protein. Reaction conditions consisted of 30 mM Tris (pH 7.4), 1.2 mM MgCl₂, 0.3 µg/ml BSA, 60 µM dioctylthioptreitol, 0.06 mg/ml PIP4/DOPS (1:1; Avanti Polar Lipids), 3.5 µM ATP, and 22 µcI γ²P ATP (Perkin Elmer). To prepare lipids, 1 mg DOPS powder was suspended in 1 ml water and sonicated in a water bath sonicator for 20 seconds to create a 1-mg/ml solution of DOPS carrier lipid. 1 mg solid PI4P was then suspended in the DOPS solution and sonicated in a water bath sonicator for 20 seconds to generate 1 mg/ml PI4P/DOPS lipid vesicles at a 1:1 ratio. The kinase reaction was started by the addition of ATP and was terminated after 2 hours by spotting 4 µl of the reaction onto 0.2 µm nitrocellulose membrane. When spotting was complete, the membrane was dried under a heat lamp for 5 minutes. The membrane was washed once with 200 ml of 1% phosphoric acid in 1 M NaCl for 30 seconds, followed by 4 washes with 5 minutes, and finally for an additional 3 hours. The membrane was dried for 1 hour, exposed for 43 hours on a phosphor screen, and scanned using the Typhoon (GE Healthcare). The intensity of each spot was quantified by densitometry using Spot (60). PIP5Kιγ activity was normalized by protein concentrations in tissue lysates and expressed as a percentage of WT tracheal tissue activity.

Statistics. All error bars denote SEM. Results were analyzed with SigmaStat software using 1-way ANOVA followed by Newman-Keuls test. A P value less than 0.05 was considered significant.

Study approval. All studies in mice were approved by the institutional review board of UCSF. Written informed consent was obtained from all subjects, and the study was approved by the UCSF Committee on Human Research. Human lung tissues were obtained from explanted lung during lung transplantation.

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