Regulation of Heterotrimeric G Protein Signaling in Airway Smooth Muscle

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Heterotrimeric G proteins transduce signals from G protein-coupled receptors to regulate numerous signaling events and functions in airway smooth muscle (ASM). In this article, we detail the function and regulation of heterotrimeric G protein signaling in ASM. We further discuss recent advances in the development of experimental tools in the study of G protein signaling, and speculate how these tools might be used in therapeutic strategies that seek to mitigate bronchospasm and airway remodeling that occur in obstructive airway disease.

Keywords: G protein-coupled receptor; RGS protein; airway remodeling; inflammation; bronchospasm

The traditional view of airway smooth muscle (ASM) described a cell that either contracts or relaxes in order to regulate airway patency and thus airflow. This view implied a similarly simplistic view of the role of heterotrimeric G proteins and G protein-coupled receptors (GPCRs) in ASM: different G proteins and their upstream receptors either promoted or antagonized ASM contraction. Over the past decade, however, ASM has been revealed as a multitasking cell involved in numerous airway functions. Not surprisingly, ASM appears to use the diversity of G protein signaling to accomplish its many functions. This article will provide an overview of G protein signaling in ASM, focusing on the role of G proteins in regulating both ASM contraction and “synthetic” functions, under conditions of both normal physiology and disease.

HETEROTRIMERIC G PROTEIN CLASSIFICATION, FUNCTION

Heterotrimeric G proteins consist of α , β , and γ subunits and function as transducers of signals from GPCRs. The α subunit is the GTP binding protein, whereas the tightly associated β and γ subunits are anchored to the plasma membrane and bind the GDP-bound α subunit with high affinity to constitute the $\alpha\beta\gamma$ heterotrimer. Seventeen different G_α , 5 G_β , and 12 G_γ subunits have been identified in humans (1). The family of G proteins can be divided into four subfamilies (G_s , $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$) based on sequence homology. The G_s subfamily includes G_s and G_{olf} , and its proteins function as activators of adenylyl cyclase. The $G_{i/o}$ subfamily includes G_{i1} , G_{i2} , G_{i3} , G_{o1} , G_{o2} , G_z , G_{t1} , G_{t2} , and G_{gust} , and functions include the inhibition of adenylyl cyclase. The $G_{q/11}$ subfamily includes G_q , G_{11} , G_{14} , and $G_{15/16}$, and functions include activation of phospholipase C (PLC).

The $G_{12/13}$ subfamily includes G_{12} and G_{13} , and functions include activation of Rho guanine nucleotide exchange factors (RhoGEFs), Na^+-H^+ exchangers, and PLC- ϵ (2).

The mechanism of activation of G proteins, often discussed within the context of the classical transmembrane signaling paradigm of GPCRs, has been established through numerous cell-free and cell-based assays. An agonist-bound GPCR undergoes a conformational change that promotes its association, typically involving the third intracellular loop, with the heterotrimeric G protein (G_s in Figure 1). The C-terminus of the G α subunit is the receptor recognition domain that dictates receptor- G_α specificity. Receptor- G_α association promotes the release of GDP from G_α and binding of GTP. The active GTP-bound G_α dissociates from $G_{\beta\gamma}$ and in turn activates an effector molecule (adenylyl cyclase in Figure 1). The $G_{\beta\gamma}$ heterodimer (numerous combinations—although not all possible—of β and γ subunits exist) also has the capacity to regulate the activity of various effectors and numerous other signaling elements (discussed below). The intrinsic GTPase activity of G_α , which can be modulated by regulators of G protein signaling (RGS) proteins (discussed below), causes the hydrolysis of GTP to GDP to terminate effector activation and promote reconstitution of the membrane-bound $G_{\alpha\beta\gamma}$ trimer.

Although the most recognized functions of the G protein subfamilies reflect the actions of their α subunits, it is now appreciated that the $\beta\gamma$ subunits mediate numerous functions and greatly expand the capabilities of G proteins (reviewed in Reference 3).

$G_{q/11}$ SIGNALING AND FUNCTION IN ASM

Signaling via $G_{q/11}$ in ASM is of particular interest due to its prominent role in promoting ASM contraction. GPCRs that promote ASM contraction appear to couple primarily to G_q . Agonists most efficacious in causing ASM contraction *in vivo* or *ex vivo*, including acetylcholine, LTD4, endothelin, and histamine, tend to act on GPCRs typically associated with G_q activation (m3 muscarinic acetylcholine receptor [mAChR], cysteinyl leukotriene type 1 receptor [CysLT1R], ET[A/B] receptor, and H1 histamine receptor [H1HR], respectively). Expression of numerous other G_q -coupled receptors in ASM has been identified, although their efficacy in increasing airway resistance *in vivo* or ASM contraction *ex vivo* can be species dependent (4, 5). In mice in which the $G_{\alpha q}$ gene is ablated, methacholine-stimulated increases in lung resistance as well as tension development in tracheal rings *ex vivo* is significantly diminished (6). Whether the residual changes in lung resistance or tension development reflect physiologic coupling to other $G_{q/11}$ family members, or with G_{11} , is not known. As with G_q knockout, ablation of the m3mAChR gene causes a profound reduction in both *in vivo* and *ex vivo* ASM contraction stimulated by mAChR agonists (7).

G_q activation stimulates the activation of phospholipase C β , which hydrolyzes phosphoinositol 4,5-bisphosphate (PIP₂) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). Both $G_{\alpha q}$ and $G_{\beta\gamma}$ subunits can activate PLC. Eleven different

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Figure 1. Model of heterotrimeric G protein signaling. See text for details.

isoforms of PLC exist and exhibit distinct patterns of regulation, although PLC β 1-3 are the major isoforms activated by G_q (and G_i -derived $\beta\gamma$ subunits). The net effect of increased IP $_3$ and DAG levels is to increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) through release from internal stores and influx from membrane-bound channels (5), and to activate protein kinase C (PKC). Both actions contribute to ASM contraction. The rise in intracellular calcium promotes calcium binding to calmodulin, and calcium-calmodulin complexes with and activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chains and enables actin to activate the myosin ATPase activity required for cross-bridge cycling and contraction. Activated PKC is capable of phosphorylating a number of substrates involved in regulating contraction, including calponin and myosin light chain phosphatase (MLCP). PKC-mediated phosphorylation of calponin results in a loss of calponin's ability to inhibit myosin ATPase, whereas PKC-phosphorylated MLCP has reduced ability to dephosphorylate myosin light chain (8, 9). Pharmacologic inhibition of PKC appears to antagonize G_q -mediated contractile signaling in murine ASM *ex vivo* by affecting elements downstream of the activated G_q -coupled receptor (10), whereas up-regulation of PKC α is associated with a hypercontractile phenotype *in vivo* in mice expressing a peptide inhibitor of $G_{\alpha i2}$ (11).

Recent studies have demonstrated an important role of G_q signaling in regulating ASM growth. Understanding the mechanisms regulating ASM growth has become an area of intense investigation since the realization that chronic asthma is associated with increases in ASM mass, as evidenced by immunohistochemical analysis of pathology sections and airway biopsies (reviewed in Reference 12). By reducing airway lumen size and altering the mechanics of airway constriction in response to contractile agonists, increased ASM mass increases both fixed airway resistance and airway reactivity (13).

Whether increased ASM mass is a precipitating event in the development of asthma or a lagging (yet pathogenic) consequence of airway inflammation and asthma development is unclear. A recent study suggests that airway inflammation is sufficient to increase ASM mass *in vivo* in the guinea pig (14). That exaggerated presentation of mitogens to ASM occurs in the inflamed, asthmatic airway is suggested by analyses of bronchoalveolar lavage fluid, which demonstrate increased levels of both growth factors and numerous GPCR agonists as part of the inflammatory milieu (15-17).

Both thrombin (capable of activating G_q through protease-activated receptors [PARs]) (18, 19) and lysophosphatidic acid (LPA) (EDG receptors) (20) are strong stimulators of cultured ASM DNA synthesis and cell proliferation. These effects appear

to be mediated by sufficient activation of the p42/p44 MAPK (via PKC-mediated phosphorylation of Raf-1) and p70S6K pathways, obligate pathways for ASM proliferation (18, 21, 22), which induce promitogenic transcription factor activation, cyclin D1 induction, and up-regulate the translational machinery necessary for cell cycle progression (23). Moreover, numerous GPCR agonists including thrombin and LPA, as well as agonists that are minimally mitogenic by themselves (e.g., leukotriene D4, endothelin, histamine, thromboxane, and sphingosine-1-phosphate) have also been shown to potentiate the mitogenic effects of receptor tyrosine kinase signaling (20, 22, 24–26). Two recent studies have shed light on mechanisms contributing to this effect. Billington and coworkers (27) demonstrated that thrombin can promote late-phase (>4 h) PI3K (and downstream p70S6K) activity, and that activity appears limiting in the mitogenic effect of epidermal growth factor (EGF). With growth factors providing strong, sustained p42/p44 activity, and the GPCR augmenting PI3K/p70S6K activity, combined RTK and GPCR stimulation results in a greater than additive effect on ASM proliferation. Interestingly, GPCR-mediated activation of PI3K involves $G_{\beta\gamma}$ subunits, as sequestration of $G_{\beta\gamma}$ subunits could partially inhibit the cooperative effects of EGF and thrombin on PI3K/p70S6K activity and ASM growth. Moreover, G_q appears to be the principal source of these $G_{\beta\gamma}$ subunits, as the (G_q -specific) RGS N-terminal domain of GRK2, but not (G_i -inactivating) pertussis toxin, could similarly suppress the cooperative effect of EGF and several GPCR agonists (thrombin, histamine, and thromboxane) on ASM mitogenic signaling and growth (27, 28).

The role of G_q in the cooperative mitogenic signaling by RTKs and GPCRs is further supported by studies from the Groningen group that demonstrate that the synergistic effect of methacholine on PDGF-stimulated growth of guinea pig ASM in culture is mediated by the G_q -coupled m3 mAChR and not the G_i -coupled m2 mAChR (29). The physiologic relevance of this finding is supported by a subsequent study demonstrating that the long-acting anticholinergic tiotropium bromide (which is preferentially selective for the m3 mAChR) inhibited the increase in ASM mass caused by repeated allergen challenge in the guinea pig (14).

G_q -dependent activation of PKC and p42/p44 also promotes phosphorylation and activation of phospholipase A2 (PLA2), which contributes to rapid prostaglandin synthesis in ASM cells stimulated with bradykinin (acting on B2 bradykinin receptors) (30). G_q activation in ASM by LPA, endothelin, or carbachol can also promote actin polymerization through a Rho-dependent mechanism (31, 32), suggesting that effectors other than PLC can be activated by G_q in ASM.

G_i SIGNALING AND FUNCTION IN ASM

ASM expresses several different GPCRs capable of stimulating G_i (24, 33–40), although the manner and context in which many of these receptors function are unclear. Members of the $G_{\alpha i}$ family expressed in ASM include $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ (41). A role for G_i in mediating specific cellular signaling or function can be discerned experimentally through the use of pertussis toxin, which catalyzes the ADP-ribosylation of the $G_{\alpha i/o}$ family subunits, thereby locking them in the inactive, GDP-bound state. $G_{\alpha i}$ activation is typically associated with inhibition of $G_{\alpha s}$ -stimulated adenylyl cyclase activity and thus reduced cAMP generation. The most prominent and best understood functions of G_i in ASM have been deduced from analyses of the m2 muscarinic acetylcholine receptor (m2mAChR). The mAChR agonists acetylcholine (42) and carbachol (43) reduce β -agonist-stimulated cAMP accumulation in a pertussis toxin-

sensitive manner, implicating G_i activation by the m2mAChR. In mice lacking the m2mAChR, forskolin-promoted inhibition of contraction stimulated by a muscarinic receptor agonist is enhanced, this functional gain attributed to relief of G_i -dependent antagonism of adenylyl cyclase activity that promotes cAMP-dependent PKA activation. Excepting studies showing similar but weaker G_i -dependent effects of A1/A3 adenosine (36), somatostatin (40), and LPA (EDG) receptors (44), little evidence exists of other G_i -coupled receptors antagonizing cAMP accumulation or its relaxant effect on ASM.

Several studies have suggested that G_i activation can contribute to increased ASM contraction, presumably via direct PLC activation. $G_{\beta\gamma}$ subunits are capable of stimulating PLC β 1-3 isoforms and thereby stimulating Ca^{2+} flux, and studies have shown the ability of pertussis toxin to partially reduce carbachol- (45), LPA- (44), A1 adenosine- (46), and chemokine- (33) stimulated phosphoinositide hydrolysis or Ca^{2+} flux in ASM. The pertussis toxin-mediated reduction of mAChR agonist signaling suggests that both G_q and G_i , stimulated by the m3mAChR and m2mAChR, respectively, can cooperate to regulate $[Ca^{2+}]_i$ and promote contraction. Pertussis toxin pretreatment also reduces acetylcholine-stimulated contraction of porcine ASM strips *ex vivo* (47), and tracheal rings from mice lacking the m2mAChR have a lesser tension generation in response to carbachol than do rings from wild-type mice (48). In contrast to these findings, a recent study by McGraw and colleagues (11) suggests that $G_{\alpha i}$ can function to mitigate ASM contraction *ex vivo* and *in vivo*. Mice treated with pertussis toxin, or expressing an inhibitory peptide of $G_{\alpha i2}$, exhibit increased MCh-induced airway resistance, whereas $G_{\alpha i2}$ overexpression attenuated MCh-induced resistance. $G_{\alpha i2}$ overexpression was associated with a reduction in the PLC β 3, and $G_{\alpha i2}$ inhibition was associated with increased PKC α expression (discussed above), suggesting that the effects on contractile signaling were not direct effects of $G_{\alpha i}$ signaling *per se* but the consequence of complex regulation of downstream effectors affecting $G_{\alpha i}$ - $G_{\alpha q}$ crosstalk.

A role for G_i -coupled receptors in modulating growth in ASM is suggested by studies that demonstrate that pertussis toxin partially inhibits ASM DNA synthesis stimulated by numerous GPCR ligands, including carbachol (activating the m2 mAChR), LPA, sphingosine-1-phosphate, endothelin, and thrombin (12, 24, 49–54). However, these effects may be species specific, as inhibition of human ASM proliferation by pertussis toxin in cultures stimulated with any GPCR agonist is typically not observed (27, 28). The mechanism mediating G_i -stimulated growth of ASM is unclear, although actions of both α and $\beta\gamma$ subunits may be involved. $G_{\beta\gamma}$ has the potential to stimulate p42/p44 MAPK via activation of PLC and PKC (as described above), and can also mediate p42/p44 activation through Src-dependent transactivation of the EGF receptor (55). However, none of these mechanisms has been established in ASM. Transactivation of the EGF receptor does not appear to be a mechanism, as it is not induced by thrombin, carbachol, or LPA in human ASM cultures (22, 51). Moreover, increased p42/p44 MAPK signaling does not appear to mediate the synergistic effect of several GPCR agonists on EGF-stimulated ASM growth (22, 27, 28, 51).

Other functional consequences of G_i activation in ASM include effects on cytoskeleton organization, sensitization of adenylyl cyclase, and chemotaxis. G_i is capable of activating Rho through activation of Rho guanine nucleotide exchange factors (GEFs), and in ASM this can mediate both actin polymerization and possibly contractile sensitization (32, 56, 57). Adenylyl cyclase sensitization, characterized by greater increases in cAMP accumulation by adenylyl cyclase activity in response to forskolin,

occurs with chronic treatment of human ASM cultures with carbachol (as well as numerous other agonists of G_i -coupled receptors) in a PKC-insensitive, pertussis toxin-sensitive manner (58). A similar sensitization of adenylyl cyclase occurs in neuronal cells chronically exposed to opioids (contributing to tolerance to morphine), suggesting a compensatory mechanism to counteract persistent G_i -coupled receptor activation (59). Finally, a recent study by Govindaraju and coworkers (33) demonstrates that IL-8 stimulates the chemokine receptors CXCR1 and CXCR2 to promote both ASM cell contraction and migration. CXCR1 and CXCR2 have been widely characterized as pertussis toxin-sensitive receptors, although one study has reported the failure of pertussis toxin to fully inhibit IL-8-stimulated calcium mobilization mediated by CXCR1 activation (60). CCR7 is also expressed in human ASM and is capable of stimulating Ca^{2+} mobilization and cell migration (61). Expression of functional chemokine receptors on ASM is intriguing and begs the question of their physiological significance. Although chemokine receptors on vascular smooth muscle are known to play a role in migration and repair from injury, the need for a similar role for chemokine receptors in ASM is unclear. Although a potential role for migration of smooth muscle progenitors has been proposed (61), it is also possible that chemokine receptors serve other functions in ASM, for example, contributing to ASM hyperreactivity or mitogenic signaling under inflammatory conditions.

G_s SIGNALING AND FUNCTION IN ASM

G_s activates adenylyl cyclase in ASM to increase intracellular cAMP accumulation and PKA activation. PKA plays an important role in regulating ASM contractile state, via mechanisms that: (1) reduce the magnitude of G_q -coupled receptor-mediated increases in Ca^{2+} ; and (2) affect the calcium-dependent regulation of various proteins involved in pro-contractile signaling. Phosphorylation of both G_q -coupled receptors and PLC by PKA inhibits GPCR-PLC-mediated PI generation and thus Ca^{2+} flux, although this mechanism has not been specifically demonstrated in ASM. PKA phosphorylates the IP3 receptor on the sarcoplasmic reticulum, reducing its affinity for IP3 and limiting IP3-stimulated calcium mobilization. PKA phosphorylation of MLCK decreases MLCK's affinity for calcium calmodulin, thus reducing activity and MLC phosphorylation. Myosin light chain phosphatase is phosphorylated on Ser 695 by PKA, which serves to inhibit Rho kinase (ROCK)-dependent phosphorylation of MLCP (potentially inhibiting the inhibition of MLCP) (62). PKA also phosphorylates K_{Ca2+} channels in ASM, increasing their open-state probability (and therefore K^+ efflux) and promoting hyperpolarization.

A recently appreciated PKA substrate in ASM is heat shock protein 20 (HSP20). When phosphorylated on Ser 16 by either PKA or PKG (63), HSP20 appears capable of relaxing smooth muscle through an ill-defined mechanism possibly involving effects on the cytoskeleton or on actomyosin interaction (64). Dreiza and colleagues (65) demonstrated that treatment with phospho-peptide HSP20 analogs possessing a protein transduction domain peptide can relax vascular smooth muscle pre-constricted with serotonin. Preliminary data revealing similar actions in ASM has been reported (P. Komalavilas, unpublished data).

Figure 2 depicts the signaling events mediated by G_s -coupled receptor activation that serve to antagonize G_q -coupled receptor signaling that promotes ASM contraction.

Inhaled β -agonists, acting on G_s -coupled β_2 ARs on ASM, are widely used in asthma therapy and are the treatment of choice for acute asthma attacks. Other G_s -coupled receptors in

ASM include the PGE₂-stimulated EP2 and EP4 receptors (66), IP prostacyclin receptors (67), A2b adenosine receptors (36, 67), and VIP receptors (68). Each of these receptors has been shown to either signal in ASM cultures or relax contracted ASM *ex vivo* or *in vivo*. It is probable that endogenous prostaglandins, prostacyclin, adenosine, and VIP modulate ASM contractile state by activating their cognate G_s -coupled receptors on ASM, although the conditions under which such modulation occurs is largely speculative. PGE₂, acting via an EP2R- and PKA-dependent mechanism, mediates the relaxant effect of the cytokines IL-1 β and TNF- α on ASM contractile state (69).

Although regulation of contraction is the most extensively characterized effect of PKA activity in ASM, other G_s - and PKA-dependent effects in ASM include altered transcription of numerous genes that impact airway inflammation and remodeling (70, 71), inhibition of cell growth (12), and ion channel gating (72). In addition, cAMP/PKA-independent signaling by G_s -coupled receptors resulting in altered ASM functions has also been reported. Isoproterenol, cholera toxin, and PGE₁ induce actin depolymerization in ASM via two mechanisms, one sensitive to PKA inhibition, another sensitive to Src inhibition (73). Direct activation of Ca^{2+} -sensitive K^+ channels can occur via interaction with $G_{\alpha s}$ subunits in a manner independent of activation by PKA-mediated phosphorylation of the channel (74). Relaxation of contracted guinea pig ASM by β -agonist (but not that caused by VIP) lacks sensitivity to pharmacologic inhibitors of PKA (75), suggesting that some but not all G_s -coupled receptors can invoke a PKA-independent mechanism of bronchorelaxation.

Finally, a potential downstream effector of G_s in ASM is Epac (exchange protein directly activated by cAMP). Epac is a guanine-nucleotide-exchange factor for the small G protein Rap1 that can be activated by cAMP in both a PKA-dependent and -independent manner. In various cell types, Epac has been shown to mediate numerous cellular functions including cell adhesion, insulin secretion, and neurotransmitter release (76). Although mRNA for Epac1 is expressed in human ASM cultures and the Epac-selective activator 8-pCPT-2'OMe-cAMP appears capable of regulating Akt phosphorylation (unpublished observations), a definitive role for Epac signaling in ASM has yet to be identified.

$G_{12/13}$ SIGNALING AND FUNCTION IN ASM

Signaling in ASM via activation of the $G_{12/13}$ family is poorly understood, and unfortunately very little progress has occurred in recent years to clarify this situation. Rat bronchial smooth muscle tissue has been shown to express $G_{\alpha 12}$ and $G_{\alpha 13}$ protein, and levels are elevated by repeated antigen challenge (77). ASM expresses numerous GPCRs (including the thromboxane [TP], PAR, and EDG receptors) that are known to couple to $G_{12/13}$. The lack of studies delineating $G_{12/13}$ signaling in ASM can be attributed to difficulties in employing molecular approaches to primary ASM cells, which would be useful for selective inhibition of $G_{12/13}$ (or other G proteins) activation or of proximal downstream effectors. The complement of effector molecules for G_{12} and G_{13} is not well established, with the exception of members of a family of guanine nucleotide exchange factors (GEFs) for the small G protein Rho (78). Rho and Rho kinase are important mediators of ASM actin polymerization (32), as well as contractile sensitization (79, 80) mediated by numerous agents including cytokines and GPCRs. However, whether $G_{12/13}$ activation is involved in these Rho/Rho kinase-dependent effects in ASM has not been established, whereas in some instances G_i (32) and G_q (32, 81) have.

Figure 2. Competitive G_q and G_s signaling in ASM regulates contractile state. Activation of G_{α_s} and PKA by G_s -coupled receptors antagonizes pro-contractile signaling by G_q at multiple junctures. See text for details. Not depicted in figure is the PKA-dependent regulation of G_q -coupled receptors, G_{α_q} , and PLC.

PHYSIOLOGIC REGULATION OF HETEROTRIMERIC G PROTEINS

Virtually every protein involved in heterotrimeric G protein signaling, ranging from the upstream activating GPCR to the most downstream signaling molecule in the signal transduction pathway, is subject to regulation via either alterations in its expression, localization, or activity. A comprehensive discussion of such regulation relative to ASM has been previously presented (4). This review will focus on regulation at the G protein locus and discuss specific findings relevant to ASM.

Heterotrimeric G protein signaling can be affected by regulation of expression of the α subunit. Because G_{α_s} is ex-

pressed in low abundance in ASM (and in most cell types), heterologous overexpression of G_{α_s} increases basal and β -agonist-stimulated cAMP accumulation (58). Perhaps owing to the difficulty in accurately quantifying G_{α_s} protein in ASM, few studies have reported modulation of G_{α_s} expression. Finney and coworkers (82) reported reduced G_{α_s} expression in rat lung after chronic administration of β -agonist, although expression in ASM was not assessed. Kalavantavanich and Schramm (83) demonstrated increased G_{α_s} protein in bovine ASM after treatment with dexamethasone, which was associated with increased β_2 AR expression and an increase in high-affinity isoproterenol binding, suggesting a mechanism of cooperativity by combined

β -agonist and steroid treatment, which is currently the preferred therapy for moderate and severe asthma.

Although $G_{\alpha i}$ subunits in most cell types are highly expressed and presumed not limiting in G_i -coupled receptor signaling, regulation of their expression in either the lung or ASM strips *ex vivo* can be induced by cytokine treatment. Intratracheal instillation of IL-1 β in the rat induces expression of $G_{\alpha i1}$ and $G_{\alpha i2}$ (84), treatment of rabbit tracheal smooth muscle strips with IL-1 β or TNF- α up-regulates $G_{\alpha i2}$ and $G_{\alpha i3}$ (85), whereas exposure to serum from individuals with atopic asthma (86) or rhinovirus (87) increases expression of $G_{\alpha i3}$. Human ASM cultures treated with TNF- α exhibit an increase in both $G_{\alpha i2}$ and $G_{\alpha q}$ expression (88). The association of the induction of $G_{\alpha i}$ subunits in each of the systems with either a reduction in β -agonist-mediated relaxation or adenylyl cyclase activity implies a role for compartmentalized regulation of adenylyl cyclase by $G_{\alpha s}$ and $G_{\alpha i}$ subunits in ASM, and a possible role for increased $G_{\alpha i}$ expression in promoting cytokine-induced hypercontractility in these models. The recent study by McGraw and colleagues (11) confirms the regulatory effect of $G_{\alpha i}$ expression on β -agonist-mediated relaxation of ASM, yet questions the physiologic impact of inflammation-induced up-regulation of $G_{\alpha i}$ subunits. Instead of promoting pro-contractile signaling, increased $G_{\alpha i}$ expression that occurs with inflammation may serve to attenuate such signaling. When mice overexpressing IL-13 (which exhibit increased $G_{\alpha i2}$ expression) were treated with pertussis toxin, MCh-induced contraction was augmented, suggesting a protective effect of $G_{\alpha i2}$ induction, perhaps via the a reduction in PLC $\beta 3$ expression as demonstrated in $G_{\alpha i2}$ -overexpressing mice.

Regulation of $G_{\alpha 12/13}$ expression is not well understood, although as mentioned repeated antigen challenge in a rat model of airway inflammation was shown to cause increases in both $G_{\alpha 12}$ and $G_{\alpha 13}$ expression in bronchial smooth muscle (77). This up-regulation was associated with increased translocation of RhoA to the plasma membrane, and was speculated to contribute to the enhancement of ACh-induced Ca^{2+} sensitization of bronchial smooth muscle, and airway hyperresponsiveness (79) in this model.

In numerous cell types, lipid rafts and caveolae have been shown capable of sequestering and compartmentalizing GPCRs, G proteins, and effector molecules, thus serving to form "signalsomes" that enable localized signaling as well as another dimension of signaling regulation. Dynamic regulation of both entrance and egress of GPCR signaling molecules and their regulators occurs in caveolae (89). Although few studies exist examining the role of caveolae in G protein signaling in ASM, Gosens and coworkers (90, 91) and Halayko and colleagues (92) have begun to characterize caveolae structure in ASM via confocal and electron microscopy. Interestingly, caveolae and caveolin-1 protein are much more abundant in ASM cells of the contractile, as opposed to the "synthetic" phenotype. Moreover, the β_2 AR and m3mAChR associate with caveolin-rich fractions, whereas the EP2R does not. Lipid raft/caveolae disruption by cholesterol depletion decreases sensitivity of ACh-mediated mobilization of $[Ca^{2+}]_i$ and contraction of ASM, without affecting maximal ACh-stimulated contraction. These observations suggest that future studies will reveal caveolae as important regulators of G protein signaling in ASM.

As mentioned above, G_{α} subunit GTPase activity is known to be regulated by recently discovered RGS proteins (93). At least 20 different mammalian RGS proteins have been identified, divided into 9 subfamilies based on sequence similarity (94). RGS proteins function as GTPase-activating proteins (GAPs), stabilizing a conformation favoring GTP hydrolysis and accelerating the formation of the GDP-bound inactive state of

G_{α} subunits, thereby inhibiting G_{α} signaling. By binding to G_{α} subunits, they also have the ability to inhibit G_{α} signaling by steric inhibition of G_{α} -effector interaction. Most RGS proteins exhibit selectivity for both $G_{\alpha i}$ and $G_{\alpha q}$, although RGS2 exhibits greater selectivity towards $G_{\alpha q}$ in certain cell types (94, 95). Up-regulation of various RGS proteins occurs with heart failure (RGS3 [96] and RGS4 [96, 97]), addiction (RGS9 [98]) and neuropathic pain (RGS4 [99]). Down-regulation of RGS4 in rat hypothalamus was observed with chronic stress or glucocorticoid treatment (100). That RGS proteins are up-regulated under conditions of chronic GPCR activation suggests that their up-regulation serves as a negative feedback mechanism. RGS2 expression appears to be positively regulated by PKA. The RGS promoter contains a cAMP response element, and RGS2 has been shown to be induced by β -agonist or forskolin in cardiac myocytes (101) and osteoblasts (102–104). Other modes of RGS regulation, including post-translational modifications and spatiotemporal expression, have been described and represent a means of determining RGS selectively for specific G proteins (94, 105). To date, no studies have characterized the role of RGS proteins in ASM, although heterologous expression of RGS proteins has been employed to discriminate the role of specific G proteins in ASM functions (*see below*).

Another means by which G protein signaling can be regulated is by genetic variation (or epigenetic regulation) of any of the heterotrimeric G subunits. Known mutations of G protein subunits that cause striking clinical phenotypes are rare but worthy of note. Inactivating mutations of the gene encoding $G_{\alpha s}$ (GNAS1) can cause pseudohypoparathyroidism (PHP), a result of the inability of parathyroid hormone to transduce signals via the G_s -coupled PTH receptor (106). Not surprisingly, other G_s -coupled receptor pathways, including those mediated by thyroid-stimulating hormone (TSH), and growth hormone releasing hormone (GHRH), are affected in patients with PHP. Gain-of-function mutations are also observed, in which mutant $G_{\alpha s}$ functions as an oncogene to promote pituitary tumors (107). Similarly, gain-of-function mutations of GNAS1 that occur in McCune-Albright syndrome are associated with multiple endocrinopathies (106). Mutations in the gene encoding $G_{\beta 3}$ have been shown to be associated with an obesity interaction on hypertension risk, although the biological significance of $G_{\beta 3}$ variations is not known (108). Whether other variations in heterotrimeric G protein subunit genes that have more subtle effects on G protein subunit expression or function have any impact on ASM physiology is not known. Genes encoding G protein subunits tend to be large and possess several introns (unlike those of GPCRs, which are often intronless), rendering them relatively difficult to characterize in large populations. However, given the current rapid rate at which sequencing technology is advancing, it is likely that within 10 years we will have a significant appreciation of how gene variation in G proteins influence GPCR signal transduction, cell physiology, and the prevalence and severity of disease.

NOVEL THERAPEUTIC STRATEGIES FOR TARGETING HETEROTRIMERIC G PROTEIN SIGNALING

Previous reviews have discussed approaches for targeting numerous elements of the GPCR signaling pathways (4, 5); here we will focus on novel potential anti-asthma therapeutic strategies that specifically target G proteins in ASM.

Excluding β -agonists, which antagonize contractile G_q -dependent signaling at multiple pathway elements, most asthma therapies that target ASM contractile signaling do so by either limiting the amount of G_q -coupled receptor agonists (steroids) or antagonizing the binding of these agonists (e.g., CysLT1R

Figure 3. Therapeutic strategies involving GRK functional domains regulating G_s and G_q signaling. (A) X ray crystal structure of a $G\alpha_q$ -GRK2- $G\beta\gamma$ complex. Top view of the $G\alpha_q$ -GRK2- $G\beta\gamma$ complex as determined by Tesmer and coworkers (121). The kinase domain of GRK2 is yellow with olive β strands, the RGS homology (RH) domain is purple, and the PH domain is tan, while $G\alpha_q$ is cyan with orange β -strands, $G\beta$ is blue, and $G\gamma$ is green. The three switch regions (I, II, and III) and the N-terminal helix of $G\alpha_q$ are red and yellow, respectively. Reprinted by permission from Reference 121. (B) Mechanisms by which expression of GRK2CT and GRK2NT could augment G_s -coupled receptor signaling and inhibit G_q -coupled receptor signaling, respectively. GRK2CT binds to and sequesters $G\beta\gamma$, thus inhibiting $G\beta\gamma$ -mediated translocation of GRK2/3 to membrane/receptor. GRK2NT interdicts $G\alpha_q$ association with PLC, thus blocking PLC activation. GRK2NT can also function as a weak GAP. GRK2K22R, a kinase-dead GRK2 mutant containing both GRK2CT and NT domains, has the capacity to both block GRK-mediated desensitization and PLC activation, thus serving as an inhibitor of G_q -coupled receptor signaling and an enhancer of G_s -coupled receptor signaling. β_2 = β_2 -adrenergic receptor; m3 = m3 muscarinic acetylcholine receptor.

and mAChR antagonists). Steroids are arguably a more useful prophylactic given the global suppressive effect on inflammation and the induction of bronchoactive agents, whereas a given receptor antagonist typically blocks only one of many possible pro-contractile agonists. Effective inhibition of G_q in ASM would represent an attractive alternative to G_q -coupled receptor antagonists in that G_q inhibition would effectively block signaling transduced by numerous agonists induced in the inflamed airway.

Anesthetics represent a recently appreciated and intriguing means of regulating G proteins, and their therapeutic utility in asthma via effects on ASM G proteins is suggested by numerous studies by Kai and colleagues (81), Nakayama and colleagues (109, 110), Sakihara and colleagues (111), and Streiff and colleagues (112). Consistent with early observations that volatile anesthetics were an effective means of treating severe bronchospasm and avoiding mechanical ventilation of individuals with asthma, Kai and coworkers demonstrated that halothane

pretreatment reduced ACh-stimulated Ca^{2+} flux and tension development in canine ASM strips (81). Subsequent experiments in ASM and in COS-7 cells expressing m3mAChR and $G_{\alpha q}$ suggested that halothane selectively inhibits $G_{\alpha q}$ nucleotide exchange promoted by numerous GPCR agonists (110, 113, 114). Future studies that uncover the precise means by which anesthetics inhibit agonist-promoted $G_{\alpha q}$ nucleotide exchange could reveal a potentially useful strategy for therapeutic targeting of G_q in ASM.

When either microinjected or expressed in cells, peptides corresponding to the C-terminal amino acid sequence of G_{α} subunits have been shown to be effective and selective inhibitors of agonist-induced G protein activation (115, 116). They are presumed to work by blocking the site on the GPCR that normally binds the G protein. Expression of a C-terminal domain peptide of $G_{\alpha q}$ in cardiac myocytes has been shown to block the up-regulated MAPK activities and reduce the cardiac hypertrophy caused by experimentally-induced pressure overload (117). Although the efficacy of such peptides has not been tested in models of ASM functions, they represent an attractive therapeutic possibility should a suitable system for in vivo intracellular delivery be developed.

Another similar and potentially useful therapeutic approach involves expression or delivery of RGS proteins in/to ASM. RGS proteins that inhibit $G_{\alpha q}$ and possibly $G_{\alpha i}$ could be effective inhibitors of acetylcholine-stimulated contraction, and of contraction stimulated or augmented by the numerous GPCR agonists that are induced in the airway by inflammation. Moreover, ASM growth and other synthetic functions augmented by $G_{\alpha q}$ - or $G_{\alpha i}$ -coupled receptors could be similarly antagonized. As noted above, the utility of using RGS proteins in inhibiting $G_{\alpha q}$ signaling in ASM was demonstrated recently in Kong and colleagues (28) in which expression of the RGS-like N-terminus of GRK2 was able to mitigate the mitogenic effects of multiple agonists of G_q -coupled receptors. Conceptually more appealing yet untested to date in ASM is the (holo-) GRK2 protein lacking kinase activity (GRK2-K220R [118]). This protein includes both the RGS N-terminal domain and the $G_{\beta\gamma}$ -binding C terminal domain of GRK2, thus enabling inhibition of both $G_{\alpha q}$ signaling and $G_{\beta\gamma}$ effects. Because $G_{\beta\gamma}$ effects include activation of pro-mitogenic PI3K activity (27, 28) and enhancement of GRK-mediated desensitization of the $\beta_2\text{AR}$, kinase-dead GRK2 could conceivably shift the competitive balance of GPCR signaling by inhibiting the deleterious (pro-contractile, pro-mitogenic) signaling of G_q , while augmenting the (pro-relaxant, anti-mitogenic) signaling of the G_s -coupled $\beta_2\text{AR}$ (Figure 3).

In addition, because other RGS proteins appear to have greater GAP activity than that exhibited by GRK2NT, they have the potential to be more efficacious inhibitors of $G_{\alpha q}$ -mediated functions in cells.

Advantages of the commonly used GPCR ligands as therapeutics include their ease of targeted delivery to the airway and ability to bind their targets at the cell surface. The majority of the strategies discussed above for targeting G proteins, with the exception of anesthetics, require a means of delivery that will enable hydrophobic peptide/protein accumulation or expression within the cell. Presently, chimeric proteins possessing protein transfection domains (119, 120), liposome or nanoparticle carriers, and viral systems represent promising delivery systems for peptides or proteins, as well as siRNA, to lung cells. The inclusion of targeting moieties to delivery vehicles, or cell-specific transcription/translational control elements within delivered genes, can help target drugs/genes to ASM. The hope is that by the time these delivery systems come of age, we will have sufficient understanding of the regulation of G protein signaling in ASM, and will have identified molecules that antagonize

signaling events mediating bronchospasm, cell growth and migration, and immunomodulatory functions with sufficient efficacy and selectivity.

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