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Review

Signaling and regulation of G protein-coupled receptors in airway smooth muscle

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Abstract

Signaling through G protein-coupled receptors (GPCRs) mediates numerous airway smooth muscle (ASM) functions including contraction, growth, and "synthetic" functions that orchestrate airway inflammation and promote remodeling of airway architecture. In this review we provide a comprehensive overview of the GPCRs that have been identified in ASM cells, and discuss the extent to which signaling via these GPCRs has been characterized and linked to distinct ASM functions. In addition, we examine the role of GPCR signaling and its regulation in asthma and asthma treatment, and suggest an integrative model whereby an imbalance of GPCR-derived signals in ASM cells contributes to the asthmatic state.

Introduction

G protein coupled receptors (GPCRs) comprise a superfamily of proteins capable of transducing a wide range of extracellular signals across the plasma membrane of the cell into discrete intracellular messages capable of regulating numerous, diverse cell functions. Over 800 GPCRs have been cloned to date and over 1000 are suspected in the human genome [1]. The majority of all prescribed drugs target either activation of GPCRs or their downstream signals. This holds true for drugs used in the management of airway diseases such as asthma; it is generally accepted that GPCRs on airway smooth muscle (ASM) are the direct targets of the majority of anti-asthma drugs.

Until recently most research efforts examining GPCR expression, function, and regulation in ASM have focused on those receptors capable of dynamic regulation of ASM contractile state and consequently, airway resistance. However, the growing appreciation of ASM as a pleiotropic cell capable of regulating airway resistance via "synthetic functions" has provided a much wider context in which to consider the relevance of numerous ASM GPCRs. GPCRs whose activation has little or no direct impact on contractile state may instead modulate ASM growth or the secretion of various cytokines, chemokines, eicosanoids, or growth factors that orchestrate airway inflammation through actions on both mesenchymal and infiltrating cells. These effects may ultimately influence airway resistance by: 1) promoting airway remodeling that impacts the mechanics of ASM contraction in vivo; or 2) regulating the inflammatory response to either disrupt the balance of local pro-contractile/relaxant molecules or alter electro- or pharmaco-mechanical coupling in ASM. Accordingly, it is no longer permissible to judge the relevance of a given ASM GPCR based on its ability to dynamically modulate ASM contractile state and airway resistance. Indeed, our

newfound appreciation of multiple experimental endpoints defining ASM function has aided efforts to identify relevant ASM GPCRs and their signaling properties.

In this review we will summarize the signaling and functional effects of various GPCRs that have been identified in ASM cells. In addition, we will consider how the regulation (or *dys*regulation) of GPCR signaling potentially impacts asthma pathogenesis and treatment.

Models for analyzing GPCR signaling in ASM

Models for analyzing GPCR signaling in ASM run the spectrum of integrative to reductionist approaches, each having certain advantages and disadvantages. Integrative in vivo models in which GPCR ligands are administered systemically or through inhalation can suggest the presence of ASM GPCRs capable of mediating bronchoconstrictive or relaxant effects. Such experiments can provide important insight into the role of a given GPCR in regulating lung resistance, and suggest the utility of targeting a receptor in order to control bronchospasm. However, the direct target cell of delivered agents is often unclear, and frequently the response of ASM is secondary to actions on other cell types. For example, inhaled agents can provoke the release of bronchoreactive substances from multiple cell types that in turn engage ASM GPCRs, or regulate autonomic control of ASM contraction through actions on pre- or post-ganglionic neurons or reflex arcs [2–4].

A more controlled environment in which to characterize ASM GPCRs is provided by ex vivo analyses of tracheal or bronchial smooth muscle isolated as strips or as part of a complex including cartilaginous ring. This approach reduces, but does not eliminate, neural or paracrine effects on ASM that can dominate functional ASM responses in vivo. Such effects can persist because preparations still include autonomic effector and sensory nerve fiber endings, epithelium, fibroblasts, and blood cells capable of releasing constricting/relaxing agents in response to exogenous agents or, possibly, mechanical forces [5]. Consequently, intelligent design of such ex vivo analyses can help clarify the in vivo effects of numerous agents and identify their target cells. For example, immunohistochemical analysis and tissue bath mechanics of excised ASM strips suggest that the pronounced bronchoconstriction elicited by inhaled adenosine or adenosine monophosphate in asthmatic subjects or sensitized animals can be attributed primarily to histamine release from mast cells in close proximity to or imbedded in ASM tissue [6–11].

Arguably, the development of ASM cell cultures has provided the most reliable system for identifying and characterizing ASM GPCRs. Typically generated by enzymatic dissociation of ASM cells from sections of tracheae or bronchi, ASM cultures provide a pure population of ASM cells that can be greatly expanded, and thus are amenable to extensive pharmacological, biochemical, and molecular analyses not possible in vivo or with tissues [12,13]. Cells of ASM cultures of several species (including human, canine, bovine, guinea pig, and mouse) have been shown to be morphologically and functionally similar to ASM in vivo; they stain for smooth muscle-alpha-actin and myosin heavy chain, and exhibit signaling and functional responses that are consistent with ASM function observed or suspected in vivo [12–15].

The power of ASM cultures as an experimental model capable of verifying existing and identifying new signaling paradigms, while also establishing their physiologic relevance, is under-appreciated. This power is largely attributed to the fact that ASM cells possess physiologic levels of most signaling components (e.g., receptors, effectors, and downstream signaling intermediates), yet many signaling pathways are readily characterized with robust signal to noise ratios. Most importantly, numerous ASM cell functions (including growth, synthesis/secretion of autocrine/paracrine factors, and to a limited extent, contraction) are also easily quantified and can be linked to their associated signaling events. In many other cell culture systems such linkage of signaling to relevant cell function cannot be achieved. For example, the majority of studies revealing novel receptor-mediated signaling paradigms have utilized expression systems such as COS or HEK293 cells to express recombinant receptors or signaling components in order to delineate pathway interactions and their modes of regulation. It is unclear whether such paradigms occur under relevant conditions in which most signaling components are expressed at low levels and their actions may be constrained by compartmentalization [16,17]. Moreover, whether such signaling has any relevance to cell function is unclear, because such cells typically either lack discrete measurable functions or their functions are known to be dysregulated (e.g., physiologic regulation of growth cannot be studied in a transformed cell). Recent studies [18–20] have begun testing the applicability and physiologic relevance of various GPCR signaling paradigms in cultured ASM cells.

However, ASM cultures as a model system are far from perfect. That ASM cells in culture lack the context of the in vivo condition is not only a strength but also an inherent limitation of this reductionist model. Moreover, like most primary cells grown in culture, ASM cells undergo a degree of de-differentiation that coincides with a loss or increase in various signaling elements and functional apparatus [3]. Specific changes in ASM cells relevant to GPCR signaling that are known to occur in culture include a rapid and progressive decrease in the expression of Gq-coupled receptors such as the m3 muscarinic acetylcholine recep-

tor (m3 mAChR) [21] and the cysteinyl leukotriene type 1 receptor (CLT1R; Stuart Hirst, personal communication). In addition, contractile function of cultured ASM cells is rapidly diminished, coinciding with reduced expression of smooth muscle alpha-actin and myosin heavy chain, calponin, h-caldesmon, beta-tropomyosin, and myosin light chain kinase (MLCK) [22]. However, Shore, Fredberg, and colleagues have developed a model for examining agonist-induced changes in stiffness of cultured ASM cells that has provided useful information linking regulation of GPCR signaling with ASM contractile state [23]. Interestingly, Stephens [24], Halayko, Solway [25–27], and colleagues have demonstrated that prolonged serum starvation of cultured canine ASM cells can beget a subpopulation of cells that reacquire high m3 mAChR and contractile/cytoskeletal protein expression and thus contractile function. These findings suggest a potentially powerful strategy for delineating elements critical to Gq-coupled receptor signaling and pharmaco-mechanical coupling in ASM.

Gq-coupled receptors

Although numerous GPCRs have the ability to couple to more than one heterotrimeric G protein, a given GPCR is typically classified based on the G protein subfamily (e.g., Gs, Gi/o, or Gq/11) it preferentially activates. A diagram of Gq-coupled receptor signaling, and the associated functional outcomes in ASM, is provided in Fig. 1. Signaling via Gq-coupled receptors in ASM is of particular interest due to its prominent role in promoting ASM contraction. Transmembrane signaling occurs in the classical GPCR-G protein-effector protein paradigm. An agonist-bound receptor undergoes a conformational change that promotes its association with and activation of the heterotrimeric G protein Gq. The extreme C-terminus of the G alpha subunit is the receptor recognition domain and dictates receptor-Gα specificity. Receptor-Gα association promotes the release of GDP from Gα and binding of GTP. The active GTP-bound Gα dissociates from Gβγ and in turn activates an effector molecule. The Gβγ heterodimer (numerous combinations of 7 different β and 12 different γ subunits exist) also has the capacity to regulate the activity of various effectors and numerous other signaling elements (discussed below). The duration of one cycle of receptor activation of effector is dictated by the GTPase activity of Gα, as the hydrolysis of GTP to GDP promotes reconstitution and membrane localization of the Gαβγ trimer. Traditionally, alpha subunit GTPase activity was presumed "intrinsic", but it is now appreciated that this activity can be regulated by GTPase proteins (GAPs) in a manner similar to that demonstrated for small G proteins [28]. Phospholipase C (PLC) is the principal effector of Gq-mediated signaling. Eleven different isoforms of PLC exist and exhibit distinct patterns of regulation; members of the PLCβ subfamily tend to mediate the actions of activated Gq [29]. Activated PLC hydrolyzes phosphoinositol 4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The net effect of increased IP_3 and DAG levels is to increase intracellular Ca^{2+} through release from internal stores and influx from membrane-bound channels [3], and in ASM to activate the cell's contractile machinery through both Ca^{2+} and protein kinase C (PKC) -dependent mechanisms [30–33] (see Fig. 1 Legend for details).

Studies of agonist-induced increases in airway resistance, smooth muscle contraction ex vivo, and receptor binding and second messenger analyses of cultured ASM cells have helped identify numerous Gq-coupled receptors in ASM (Table 1). Resting ASM tone in vivo is determined primarily by parasympathetic cholinergic innervation acting on ASM m3 mAChRs. Other ASM Gq-coupled receptors capable of inducing significant ASM contraction (in vivo or ex vivo) include the H1 histamine receptor, CLT1R, B2 bradykinin receptors, and ET-A endothelin receptor. Additional Gq-coupled receptors such as the A3 adenosine, NK-1, NK-2 (Neurokinin-1 and -2) and P2 purinergic have been identified, but their importance in mediating contraction under physiologic or pathologic conditions is unclear. In some cases the evidence for their expression in ASM is either indirect or is difficult to interpret given the labile nature of Gq-coupled receptor expression in ASM cultures.

However, as noted above ASM cells do more than contract and studies of other functional outcomes in ASM suggest a potentially important role for numerous Gq-coupled receptors in modulating ASM synthetic functions. Both thrombin (capable of activating Gq through protease-activated receptors (PARs) [34]) and lysophosphatidic acid (LPA) (capable of activating Gq through endothelium differentiation gene (EDG) receptors) are strong stimulators of cultured ASM DNA synthesis and cell proliferation. These effects appear in part Gq-dependent (Billington and Penn, unpublished observations) and may be mediated by the capacity of Gq signaling to stimulate the p42/p44 MAPK (via PKC-mediated phosphorylation of Raf-1) and p70S6K pathways and therefore induce promitogenic transcription factor activation, cyclin D1 induction, and upregulate the translational machinery necessary for cell cycle progression [36,37]. Moreover, numerous Gq-coupled receptor agonists including thrombin, lysophosphatidic acid, leukotriene D4 (LTD4), endothelin, histamine, thromboxane (activating Thromboxane A2 / Prostaglandin (TP) receptors)[19], and sphingosine-1-phosphate (SPP) (activating EDG receptors) have been shown to potentiate the mitogenic effects of receptor tyrosine kinase signaling, although it has not been established that Gq activation per se mediates this effect.

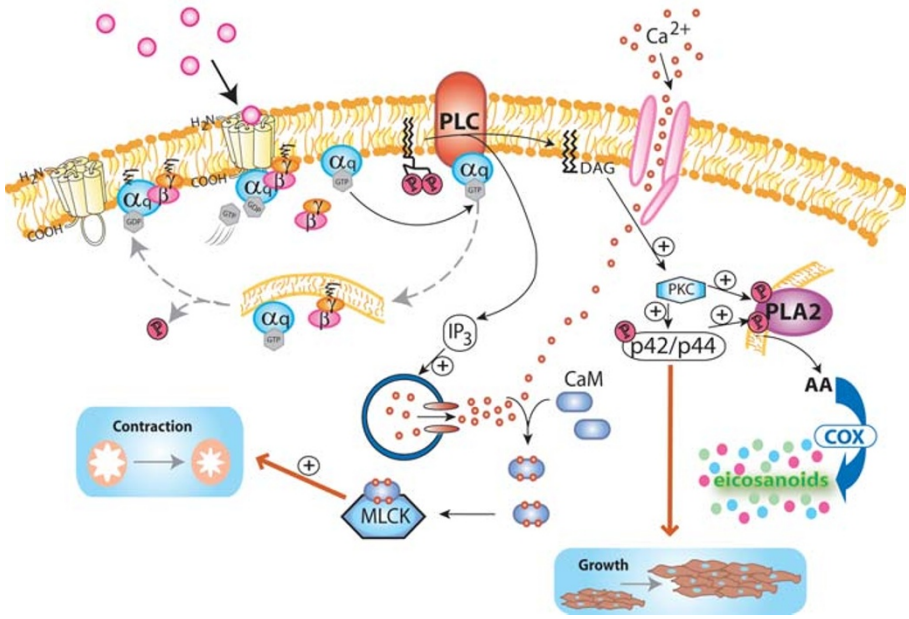

Figure 1

Gq-coupled receptor signaling in airway smooth muscle. Airway smooth muscle (ASM) is innervated by postganglionic parasympathetic nerves that release acetylcholine (acting on m3 mAChRs) to control resting ASM tone. In addition to the m3 muscarinic acetylcholine receptor (mAChR), other Gq-coupled coupled receptors are expressed in ASM (see Table 1), and can similarly mediate contraction and other depicted ASM functions. Transmembrane signaling of G protein-coupled receptors (GPCRs) involves sequential activation of receptor, G protein, and effector. Upon agonist binding, the receptor undergoes a conformational change exposing a high-affinity binding site for a G-protein in its GDP-bound inactive state. The receptor specifically interacts with the C-terminus of the α subunit of the G-protein heterotrimer. G-protein binding to receptor releases the nucleotide leaving an empty nucleotide binding pocket readily occupied by GTP, which exists at a higher cytosolic concentration than GDP. This exchange of the G-protein-bound GDP for GTP induces a conformational change in the switch region of Gα and causes the dissociation of Gα from the G$\beta\gamma$ dimer. The Gβ and Gγ subunits are tightly associated and remain anchored into the lipid bilayer due to the prenylation of the Gγ subunit – a permanent lipid modification. In the case of Gαq, the GTP-bound Gα q-protein's effector interaction domain is exposed and activates phospholipase C (PLC). PLC promotes the hydrolysis of phosphoinositol 4,5-bisphosphate (PIP2) into the intracellular messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG remains membrane bound and promotes the translocation of protein kinase C (PKC) from the cytoplasm to the membrane and its subsequent activation. Activated PKC is capable of phosphorylating a number of substrates including calponin; PKC-mediated phosphorylation of calponin results in a loss of calponin's ability to inhibit actomyosin ATPase [30,269]. PKC also phosphorylates intermediates of MAPK signaling pathways, which activate various gene transcription factors involved in promoting ASM growth. Gq-coupled receptors are also able to impact receptor tyrosine kinase-induced ASM growth via a synergistic activation of p70S6K. Both PKC and p42/p44 MAPK phosphorylate and stimulate the catalytic activity of phospholipase A2 (PLA2). Calcium binding to PLA2 triggers its association with the plasma or nuclear membrane and the subsequent cleaving and release of arachadonic acid (AA). The conversion of AA to prostaglandins and thromboxanes is facilitated by cyclo-oxygenase-2, a highly regulated enzyme upregulated by pro-inflammatory agents including lipopolysaccharide, cytokines and growth factors. The other product of PIP_2 hydrolysis, IP_3, translocates and binds to IP_3 receptors located on sarcoplasmic calcium stores. Activation of IP_3 receptors results in the opening of Ca^{2+} channels and calcium efflux into the cytosol. Intracellular calcium stores are the major source of elevated calcium mediating ASM contraction, although influx from receptor-operated calcium channels can contribute. The rise in intracellular calcium promotes calcium binding to calmodulin forming calcium-calmodulin complexes that activate 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. Via its interaction with various guanine-nucleotide exchange factors for Rho (RhoGEFs), Gq has also recently been shown to activate the small G protein Rho [270]. In ASM, Gq-mediated activation of Rho has been implicated in regulating actin cytoskeletal rearrangement [40]. Rho is also a key mediator of calcium sensitization – a phenomenon observed following stimulation with numerous GPCRs whereby heightened contractile effects can be induced for a given level of calcium mobilization. Rho activates Rho kinase, which in turn phosphorylates the myosin binding subunit of myosin light chain phosphatase (MLCP) to inhibit phosphatase activity, resulting in net increased phosphorylation of myosin light chain (MLC) and an associated increase in cross-bridge cycling [271]. Although activation of $G_{12/13}$ is most commonly associated with Rho activity, studies of ASM suggest that Gq and Gi can also participate in Rho-mediated functions [40,272,273].

Table 1:

GPCR	References	Couples to	Functions in ASM[1]	Comment
5-HT	[125,126,213–215]	Gi[2]	CXN, GP	5-HT2c identified, other subtypes likely
A1 adenosine	[42,216,217]	Gi	CXN	Low levels suggested in human ASM
A2b adenosine	[42]	Gs[3]	RLXN	Mediates effects of autocrine and paracrine adenosine
A3 adenosine	[218,219]	Gq[4]	unclear	
α-1 adrenergic	[220–223]	Gq	unclear	Only response in lung or ex vivo occurs with βAR antagonist present
β_2 adrenergic	[56,185,224,225]	Gs	RLXN, Cyt, GI	
BK bradykinin	[39,226–231]	Gq	CXN	Robust activation of PLC and PLA2 in cultured ASM;putative B3 yet to be cloned
CLT1R	[232–236]	Gq	CXN, GP	CLT1R antagonists most therapeutic of all GqCR antagonists
ET-A/B	[237–242]	Gq	CXN, GP	
EDG 1–7	[38,243–245]	Gq, Gi, $G_{12/13}$	GS, Cyt	Most subtypes exhibit promiscuity toward G proteins
EP2	[20,246,247]	Gs	RLXN, GI, Cyt	Indirect evidence for expression of EP1, EP3, and EP4
H1 histamine	[248,249]	Gq	CXN, GP	Exhibits homologous and heterologous desensitization
IP Prostacyclin	[41,250]	Gs	GI	Responsive to autocrine PGI2 induced by cytokines via COX-2 induction
m2 muscarinic	[21,251,252]	Gi	unclear	Mediator of acute adenylyl cyclase inhibition, chronic sensitization
m3 muscarinic	[251–256]	Gq	CXN, GP	Rapid reduction of expression in culture
NK-1/2	[257–260]	Gq	CXN, GP	
PAR-1,2,3	[34,261,262]	Gq, Gi, $G_{12/13}$	GS, GP	Thrombin most mitogenic GPCR agonist; subtype promiscuity towards G proteins
P2 purinergic	[218,219]	Gq	unclear	P1 may also be expressed
TP	[41,263–266]	Gq, Gi, $G_{12/13}$ (?)	CXN, GP	Coupling specificity poorly characterized in ASM
VIP	[43,267,268]	Gs	GI	

[1] Abbreviations: CXN – contraction; Cyt – regulation of ASM cytokine/chemokine synthesis; GI-inhibition of ASM growth (DNA synthesis/cell proliferation); GP – potentiation of growth stimulated by polypeptide growth factors; GS – growth stimulation ; RLXN – relaxation [2] Coupling to Gi is suggested by sensitivity of signal transduction or functional effects to pertussis toxin [3] All receptors noted to couple to Gs have been shown to stimulate cAMP production in ASM. [4] Coupling of receptors to Gq is suggested by either agonist-stimulated phosphoinositide production or calcium flux.

Gq-dependent activation of PKC and p42/p44 also promotes phosphorylation and activation of phospholipase A2 (PLA2), which contributes to rapid eicosanoid synthesis in ASM cells stimulated with bradykinin (acting on B2 bradykinin receptors)[39]. Other effects reported to involve Gq activation by ASM GPCRs include actin polymerization induced by LPA, endothelin, or carbachol, which appears to occur via a Rho-dependent mechanism [40]. This suggests that effectors other than PLC can be directly activated by Gq in ASM.

Gs-coupled receptors

Whereas Gq-coupled receptors are the principal mediators of ASM contraction, Gs-coupled receptors on ASM play a central role in promoting relaxation of contracted ASM and in conferring prophylactic "bronchoprotection". Inhaled beta-agonists, which activate the Gs-coupled beta-2-adrenergic receptor (β_2AR) on ASM, are the most widely used agents in asthma therapy and are universally recognized as the treatment of choice for acute asthma attacks. Several other Gs-coupled receptors, including the E-Prostanoid 2 (EP2) prostaglandin E2 (PGE_2) [20], IP prostacyclin ([41] and Pascual and Penn, unpublished observations), A2b adenosine [42], and vasoactive intestinal peptide (VIP)[43] receptors have been identified in ASM and represent intriguing, albeit elusive, therapeutic targets (Table 1).

Gs-coupled receptor signaling and its regulation have been extensively characterized in numerous cells types, including ASM [44]. The overwhelming majority of studies delineating the basic tenets of Gs-coupled receptor signaling have examined β_2AR signaling, based on the prevalence of endogenously expressed β_2ARs, the established relevance of β_2ARs in the function of several organ systems, the existence of highly selective β_2AR ligands, and the early cloning of the β_2AR enabling heterologous expression of the receptor in various cell systems. Figure 2 depicts the most prominent features of Gs-coupled receptor signaling and functional consequences in ASM cells.

Adenylyl cyclase (AC) is the principal effector of Gs-coupled receptor transmembrane signaling. Nine isoforms (type I through IX) of AC are known to exist [45]. RT-PCR has identified transcripts of all AC subtypes except III and VIII in human ASM cultures, although immunoblot anal-

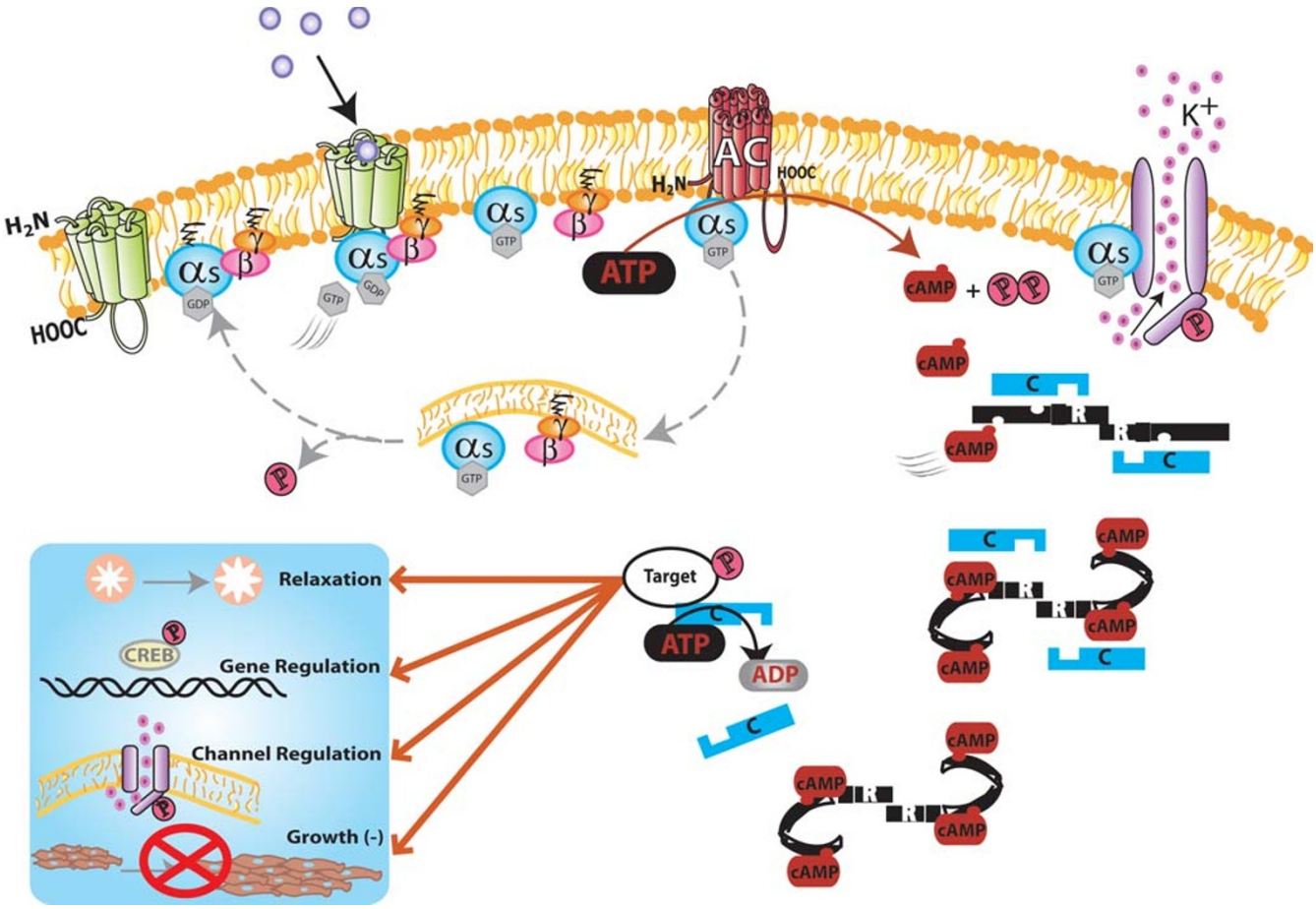

Figure 2

Gs-coupled receptor signaling in airway smooth muscle. Gs-coupled receptors on airway smooth muscle (ASM) are activated by endogenous agents such as circulating catecholamines, prostaglandins and iso-prostanes, adenosine and vasoactive intestinal peptide (VIP). Activated Gαs binds to and activates membrane bound adenylyl cyclase (AC). AC is comprised of eight membrane-spanning α-helices, and two cytosolic domains which are required for catalytic activity and integrate various regulatory signals. The cytosolic domains possess specific binding sites for the G-protein subunits Gαs, Gαi, and Gβγ. Of the nine know AC isoforms, AC V and VI appear be expressed and functionally important in human ASM. Adenylyl cyclase activation catalyzes the formation of cyclic AMP from cytoplasmic ATP. Cyclic AMP is a ubiquitous second messenger whose principal function is to activate protein kinase A (PKA). Inactive PKA exists as a complex comprising two regulatory and two catalytic subunits. The high affinity binding of cyclic AMP to domains in the regulatory region induces a conformational change forcing the release of the active catalytic subunits. PKA-mediated phosphorylation of various intracellular proteins has widespread effects in ASM. PKA can phosphorylate certain Gq-coupled receptors as well as phospholipase C (PLC) and thereby inhibit G protein-coupled receptor (GPCR) -PLC-mediated phosphoinositide (PI) generation, and thus calcium flux. PKA phosphorylates the inositol 1,4,5-trisphosphate (IP_3) receptor to reduce its affinity for IP_3 and further limit calcium mobilization. PKA phosphorylates myosin light chain kinase (MLCK) and decreases its affinity to calcium calmodulin, thus reducing activity and myosin light chain (MLC) phosphorylation. PKA also phosphorylates K_{Ca++} channels in ASM, increasing their open-state probability (and therefore K+ efflux) and promoting hyperpolarization. Through its phosphorylation of the transcription factor CREB and its (typically inhibitory) effects on GPCR and receptor tyrosine kinase signaling, PKA regulates the transcription of numerous genes. Recent studies suggest that cAMP/PKA mediates regulation of the expression of numerous immunomodulatory proteins in ASM including IL-6, RANTES, eotaxin, and GM-CSF [53,54,274–276]. Although poorly characterized, the growth inhibitory effect of Gs-coupled receptor activation in ASM is consistent with the known effects of PKA on mitogenic signaling. These effects include inhibition of p42/p44 MAPK signaling via phosphorylation and inhibition of the upstream intermediate raf-1, and via inhibition of promitogenic transcriptional regulation mediated by phospho-CREB. Lastly, Gs-coupled receptor activation is also believed to promote PKA-independent effects, including gating of K_{Ca++} channels directly by Gαs [56], and actin polymerization via an unestablished mechanism [55].

ysis suggests the presence of only V/VI (existing antibodies do not distinguish between type V and VI), and analyses of AC regulation in human ASM cultures (discussed below) are consistent with the expression of AC V and VI [46,47]. Interestingly, AC subtype expression in ASM cultures may be species specific, as regulatory features of AC in bovine, canine, and guinea pig ASM suggest prominent expression of AC II [48–51], whereas a minimal [46] or no [47] level of AC II transcripts were detected in human ASM (see below).

Adenylyl cyclase isoforms are subject to multiple forms of regulation (discussed below), although dynamic activation of AC under physiologic conditions occurs almost exclusively by interaction with Gαs [52]. Gαs activation of AC catalyzes ATP to cyclic AMP (cAMP), which in turn binds to the regulatory subunits of the cAMP-dependent protein kinase (protein kinase A or PKA). The cAMP-bound regulatory subunits then dissociate from and thereby activate the catalytic subunits of the enzyme, which in turn phosphorylate and regulate the activity of numerous proteins, including the transcription factor CREB. PKA activity is presumed responsible for the majority of cellular actions elicited by Gs-coupled receptor activation, which in ASM include relaxation, altered transcription of numerous genes that impact airway inflammation and remodeling [53,54], inhibition of cell growth, and ion channel gating [3]. However, cAMP/PKA -independent signaling by Gs-coupled receptors has also been proposed and may have important functional consequences in ASM. These include beta-agonist-induced actin depolymerization [55], direct activation of Ca^{2+}-sensitive K^+ channels by Gαs subunits [56], and possibly other ill-defined signaling events that promote relaxation and are unaffected by exposure of ASM to pharmacological inhibitors of PKA [57].

Gi-coupled receptors

The majority of known GPCRs preferentially couple to members of the Gi family, and Gi appears to be the most abundantly expressed heterotrimeric G protein in most cell types. Members of the Gαi family expressed in ASM include Gαi-1, Gαi-2, and Gαi-3 [58,59]. Gαi activation is typically associated with inhibition of Gαs-stimulated AC activity (for certain AC isoforms) and thus reduced cAMP generation, the functional consequences of which should be predictable but are often difficult to identify in a wide range of experimental models [2,60]. However, numerous other signaling events elicited by Gαi activation, with clear functional consequences, have recently been identified (Fig. 3). Gi appears 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 [40,61]. Whether Gi activation of Rho is mediated by α or βγ subunits is unclear. βγ subunits released due to Gi activation are believed to promote many of the βγ effects identified to date, perhaps reflecting the relatively high levels of Gi in most cells that could provide the levels of free βγ required for its signaling effect [52]. In in vitro systems βγ subunits have been shown to enhance the activity of selected AC isoforms stimulated by Gαs. Moreover, βγ may also mediate, through what may be an indirect mechanism [62], the AC sensitization observed in neuronal cells chronically exposed to opioids (contributing to tolerance to morphine [63–65]) and in human ASM cells chronically treated with carbachol and other ligands capable of activating Gi-coupled receptors [46]. The purpose of such AC sensitization in ASM is unclear, but may involve the need to maintain a degree of Gs-coupled receptor signaling in the face of persistent Gi-coupled receptor activation.

A role for Gi-coupled receptors in modulating growth in ASM is suggested by studies that demonstrate that pertussis toxin (which ADP-ribosylates and inhibits Gαi) partially inhibits ASM DNA synthesis stimulated by numerous GPCR ligands including carbachol (activating the m2 mAChR), LPA, SPP, endothelin, and thrombin [18,38]. The mechanism mediating Gi-stimulated growth of ASM is unclear, although actions of both α and βγ subunits may be involved. Gβγ has the potential to stimulate p42/p44 MAPK via activation of PLC and PKC, and can also mediate p42/p44 activation through Src-dependent transactivation of the epidermal growth factor (EGF) receptor [66]. However, none of these mechanisms has been established in ASM. On the contrary, transactivation of the EGF receptor is not induced by thrombin, carbachol, or LPA in human ASM cultures, and increased p42/p44 MAPK signaling does not appear to mediate the synergistic effect of several GPCR agonists on EGF-stimulated ASM growth [18,19]. These latter findings suggest potentially novel mitogenic signaling events and define cooperativity between GPCRs and receptor tyrosine kinases in mediating ASM growth.

$G_{12/13}$ coupled receptors

Signaling via activation of the $G_{12/13}$ family has not been characterized as extensively as has that by other heterotrimeric G proteins. The effector molecules that interact directly with G_{12} and G_{13} are not well established, with the exception of members of a family of guanine nucleotide exchange factors for the small G protein Rho [67]. The GPCRs capable of activating G_{12} or G_{13} are also unclear. Immunoblot analysis demonstrates $G\alpha_{12}$ and $G\alpha_{13}$ protein in rat bronchial smooth muscle tissue, and levels are elevated by repeated antigen challenge (see below)[68]. In ASM cells, those GPCRs activating $G_{12/13}$ have not been characterized, although SPP/LPA-activated EDG receptors, thrombin-activated PAR receptors, and TP receptors

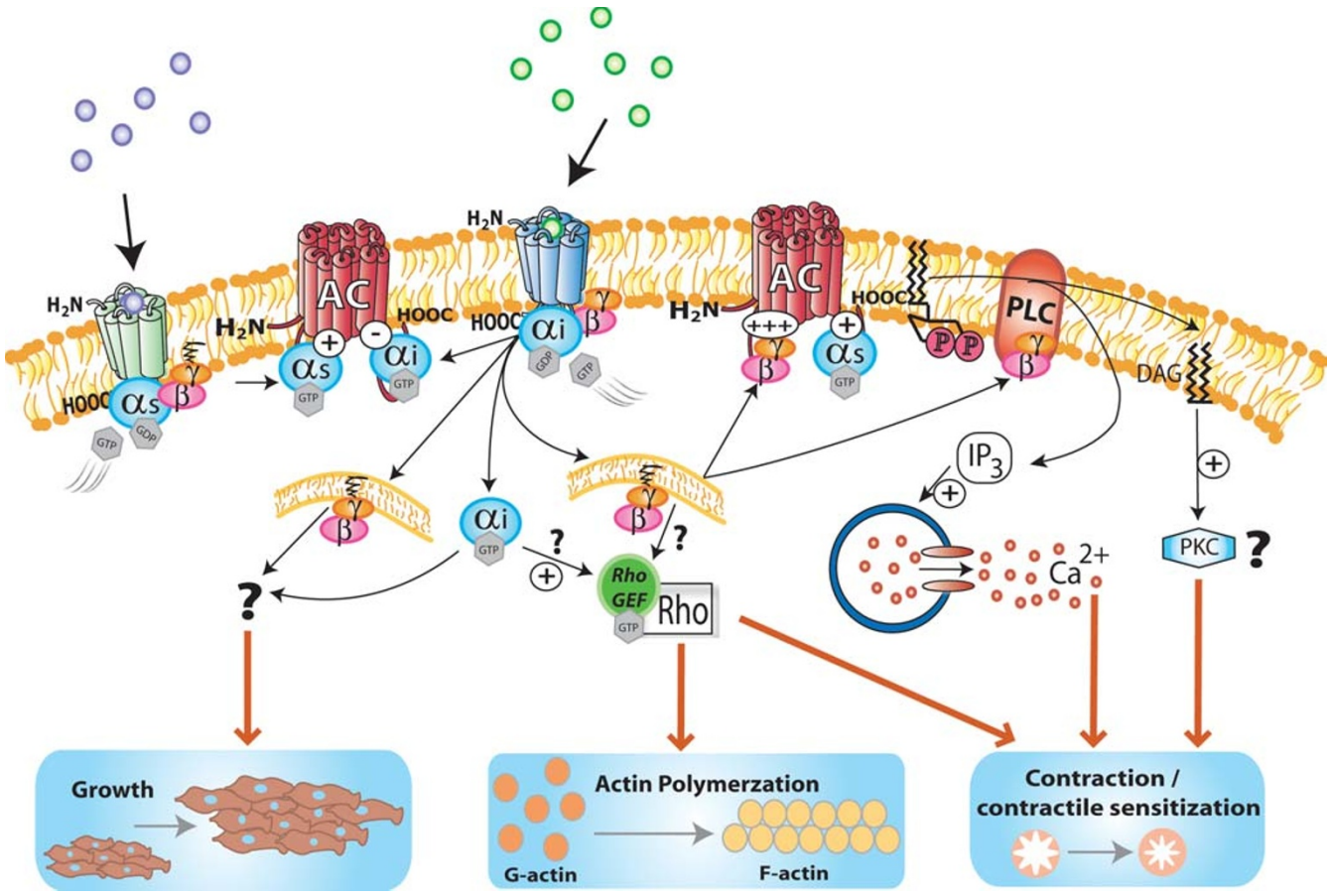

Figure 3
Gi-coupled receptor signaling in airway smooth muscle. Gi-coupled receptors have the capacity to initiate or modulate signaling through the actions of both Gi-derived α and βγ subunits. Activated Gαi dissociates from the heterotrimeric complex and binds to adenylyl cyclase (AC) V and VI to act as a negative modulator of Gαs-induced signaling. Gβγ subunits modulate AC activity in an isoform-specific manner, inhibiting AC type I but enhancing Gαs-induced activation of AC II, IV and VII. Gβγ can also activate phospholipase C beta (PLCβ) isoforms, resulting in phosphoinositide generation, protein kinase C (PKC) activation via 1,2-diacylglycerol (DAG), and calcium mobilization. Through ill-defined mechanisms, Gi-coupled receptor activation can also promote airway smooth muscle (ASM) growth [18], and cooperate with both other G protein-coupled receptors (GPCRs) [277,278] and receptor tyrosine kinases [19,243] to synergistically stimulate growth. Lastly, Gi activation in ASM can contribute to Rho-dependent changes in actin polymerization [40,279,280] and calcium sensitization [273], although the mechanism of Rho activation by Gi in ASM (or other cell types) is not well established.

are candidates. The profound effect of inhibitors of Rho and Rho kinase on GPCR-mediated changes in contractile sensitization [69,70] and actin polymerization [40] strongly suggest a physiologic role for $G_{12/13}$ signaling in ASM.

Regulation of GPCR signaling

Signaling by GPCRs is a highly regulated process. One critical way in which a cell controls its response to extracellular GPCR ligands is through regulation of the expression and activity of each component of the GPCR-G protein-effector pathway. Either a loss (desensitization) or increase (sensitization) in responsiveness of transmembrane signaling components can be evoked to presumably preserve the cell/organism from excessive signals or ensure detection and reaction to infrequent or minimal signals. In ASM, studies of regulation of GPCR signaling have focused on changes that occur in receptor and G protein expression and second messenger generation in cells, or on altered contractile/relaxant effects on ASM in vivo or ex vivo. No studies to date have considered the effect of desensitization or sensitization of GPCR signaling on GPCR-mediated functions in ASM other than contraction.

Regulation at the receptor locus

Changes in expression or activity of the receptor represent a powerful means of regulating GPCR signaling. Altered GPCR responsiveness can occur via altered receptor density (*up-* or *down- regulation*), modifications of the receptor such as phosphorylation that diminishes receptor-G protein interaction (*uncoupling*), and trafficking of receptor away from G protein (*sequestration/internalization*) that enables either recycling of receptor to a responsive form or facilitates receptor loss by lysosomal degradation (Figure 4). These mechanisms have been characterized extensively in studies of the β_2AR. The degree to which they apply to other GPCRs is both receptor- and cell-dependent [44]. In ASM cells, upon exposure to their agonist, both the β_2AR and A2b adenosine receptor undergo rapid desensitization [42,71,72], which is defined by a loss in agonist-stimulated cAMP generation, (*agonist-specific* or *homologous* desensitization). Rapid beta-agonist-promoted desensitization of the ASM β_2ARs is mediated primarily by receptor phosphorylation by G protein-coupled receptor kinases (GRKs)[44], which specifically recognize the agonist-occupied form of GPCRs. Numerous GPCRs in various cell types including ASM [72] have been shown to be regulated by GRKs, and GRKs themselves are subject to multiple forms of regulation, some of which may influence GPCR function in certain disease states (reviewed in [1]). GRK phosphorylation of GPCRs partially uncouples the receptor from Gα, and also promotes binding of arrestin molecules to the receptor, which more effectively uncouple the receptor from G protein by sterically inhibiting the receptor-Gα interaction [73]. For numerous GPCRs, GRK-mediated arrestin binding also initiates receptor internalization/sequestration, which occurs via the association of the receptor-arrestin complex with components of clathrin-coated pits [74,75]. GPCR internalization is not required for GPCR desensitization, but is required for resensitization, as demonstrated for the β_2AR in ASM [72]. Interestingly, agonist-stimulated arrestin-dependent internalization of both the β_2AR and A2b adenosine receptor is observed in human ASM cells, whereas ASM EP2 receptors do not readily bind arrestin, do not appear to be phosphorylated by GRKs, and do not undergo rapid agonist-stimulated internalization [20]. Although ASM EP2 receptors do exhibit desensitization with chronic PGE_2 treatment, they are much more efficacious in stimulating cAMP generation and promoting PKA-dependent functional effects in ASM cells than are either β_2ARs or A2b adenosine receptors ([20] and Pascual and Penn, unpublished observations). These findings demonstrate the receptor-specific nature of mechanisms of homologous desensitization, and also show that susceptibility to desensitization at the receptor locus can be a major determinant in establishing the effect of GPCR ligands and their receptors on cellular functions.

GPCRs are also subject to phosphorylation and desensitization by PKA and PKC. Accordingly, any agent capable of activating cellular PKA or PKC (e.g., other GPCR agonists, phosphodiesterase inhibitors) can diminish GPCR responsiveness. PKA and PKC-mediated phosphorylation causes a degree of receptor uncoupling from G protein, but it does not promote arrestin binding to receptor and rapid internalization. Such *heterologous* desensitization of a given GPCR is typically not as profound as homologous desensitization. Cultured ASM cells exposed briefly to either PGE_2, adenosine, forskolin (all stimulators of cAMP production and PKA activation) or phorbol ester (a PKC activator) exhibit diminished isoproterenol-stimulated cAMP production [42,46,71,72]. Similarly, chronic exposure of ASM cells to interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), or transforming growth factor beta (TGF-β) also results in heterologous desensitization of the β_2AR, presumably via the induction of Cyclo-oxygenase-2 (COX-2) activity and the autocrine effect of induced PGE_2 [76–80]. The PGE_2- or IL-1β-mediated loss of beta-agonist-stimulated second messenger generation is associated with a loss in the relaxant effect of beta-agonist on carbachol-contracted ASM cells in culture [77]. The H1 histamine receptor exhibits both homologous [81] and heterologous [81] desensitization, the former presumably mediated exclusively by GRKs, the latter induced by phorbol ester in a PKC-dependent manner.

Down-regulation, defined as a loss in receptor density, occurs as a result of increased receptor degradation or reduced receptor synthesis. Recovery from GPCR down-regulation is a relatively slow process and requires new receptor synthesis. Virtually all GPCRs studied to date undergo some degree of downregulation when chronically exposed to their agonist. Other agents can promote a loss of GPCR density through either inhibition of receptor gene transcription, or via ill-defined mechanisms that promote receptor degradation.

Arrestin-dependent internalization of GPCRs has been identified as a pathway leading to lysosomal degradation of GPCRs [82]. Recently studies also suggest that β_2ARs and CXCR4 receptors are subject to ubiquitination that ultimately directs internalized receptor to lysosomes [83,84], or in the case of mu and delta opioid receptors, to proteosomal degradation [85]. Chronic exposure of ASM cells to beta-agonist, or ASM tissue to histamine results in down-regulation of the β_2AR [86] and H1 histamine receptor [87], respectively. The effects of a receptor's agonist and other agents (e.g., glucocortoids, cytokines, beta-agonists) on pre- and post-transcriptional regulation of new receptor synthesis have been characterized for numerous GPCRs in ASM or lung [81,87–97]. Although receptor degradation probably plays a prominent role in the down-

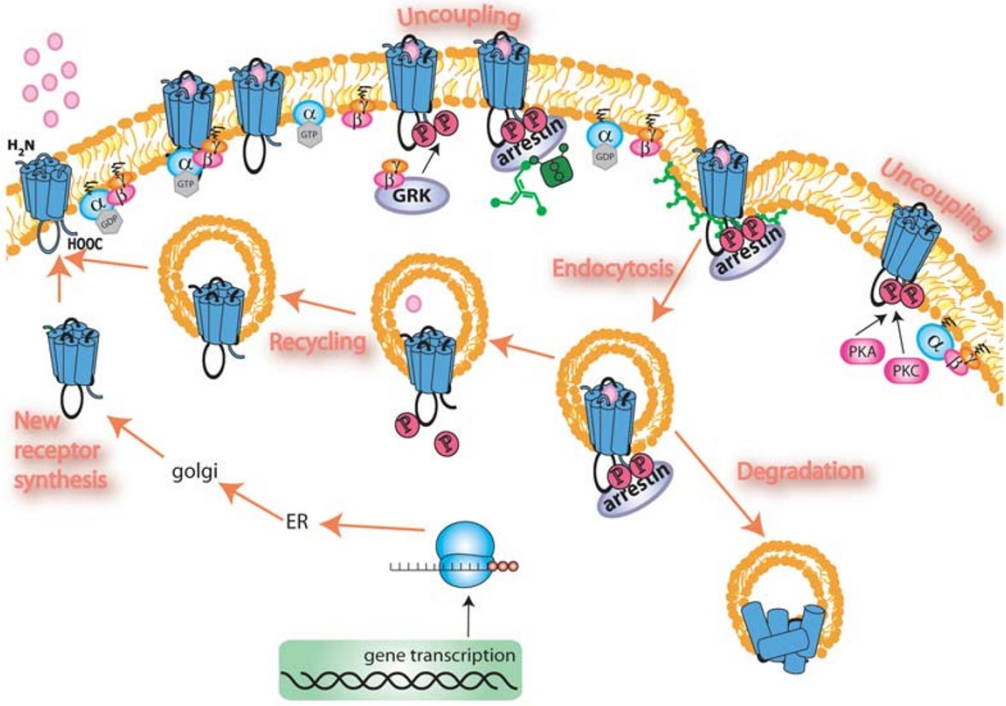

Figure 4
G protein-coupled receptor regulation in airway smooth muscle. Regulation of G protein-coupled receptor signaling at the receptor locus is effected by numerous mechanisms that establish the number and responsiveness of receptors at the cell surface. These mechanisms include new receptor synthesis, as well as modes of desensitization and resensitization that unfold after a receptor is activated by agonist. Receptor uncoupling occurs as a result of G protein-coupled receptor kinase (GRK) -mediated phosphorylation of agonist-occupied receptor, which promotes arrestin binding to phosphorylated receptor and steric inhibition of GPCR-G protein interaction. Arrestin binding to receptor also initiates internalization of receptor into clathrin-coated pits, after which receptors can traffick to lysosomes for degradation (downregulation) or be dephosphorylated and recycled back to the plasma membrane (resensitization). In addition, activation of intracellular kinases such as protein kinase A (PKA) or protein kinase C (PKC) can also phosphorylate GPCRs and promote a loss of GPCR-G protein coupling. See text for details.

regulation of GPCRs in ASM, the trafficking of GPCRs to their degradation fate has not been studied in ASM cells.

Up-regulation of GPCR expression is also observed for numerous GPCRs in numerous cell types and is an important physiologic means of conferring sensitization of GPCR signaling. Increased GPCR expression, mediated by increased gene transcription as well as post-transcriptional mechanisms, is frequently induced experimentally by chronic treatment of cells with antagonist. Antagonist-mediated up-regulation of GPCRs is relatively unexplored in ASM cells or tissue, although chronic treatment of rabbits with atropine has been shown to up-regulate both m2 and m3 mAChRs in the airway [98]. Transcription regulation of most GPCR genes in ASM cells is poorly understood, but should be greatly abetted by the increasing adroitness in applying molecular techniques to primary ASM cultures and by the emergence of models of ASM phenotype regulation [27,93].

One final means by which GPCR responsiveness is influenced is by receptor genotype. Single nucleotide polymorphisms (SNPs) that result in changes in β_2AR expression, cellular distribution, and signaling have been identified in both the promoter and coding region of the β_2AR gene [99,100]. SNPs identified in the β_2AR promoter have been shown to affect receptor expression [101,102]. Among those polymorphisms detected in the coding region, Arg→Gly16 exhibits enhanced agonist-induced desensitization (of beta-agonist-stimulated cAMP generation) and down-regulation, whereas Gln→Glu27 is decidedly desensitization- and down-regulation-resistant. Importantly, these properties are evident in β_2ARs expressed endogenously in ASM cultures [86]. SNPs identified in

other GPCRs (including the α2a- [103], α2b- [104], and β1-adrenergic receptors [105,106]) have also been shown to be of functional consequence, although their characterization has been performed primarily in either cell expression models or in the cardiovascular system.

The relevance of β_2AR SNPs to asthma and asthma therapy are discussed below.

Regulation at the G protein locus

Regulation of G protein expression and activity has the potential to modify GPCR signaling. Gα subunit GTPase activity is known to be regulated by recently discovered RGS (regulators of G protein signaling) proteins [107]. Experimental manipulation of RGS protein expression can alter GPCR signaling, but the physiologic role of RGS proteins is unclear. Interestingly, GRK2 has been recently shown to contain an RGS domain that can interact specifically with Gαq and quench its activity [108].

Overexpression of Gα subunits in various cell systems can enhance GPCR signaling, and the expression of certain Gα subtypes is altered in various disease state models (see below). In human ASM cells in culture, overexpression of Gαs increases both basal and Gs-coupled receptor-mediated cAMP production [46]. Whether altered Gα expression or localization impacts GPCR signaling under physiologic conditions is somewhat controversial. Endogenous expression of G proteins is typically much higher than that of GPCRs or effectors, suggesting that most GPCR-G protein-effector signaling is probably limited more by the expression/activity of the effector or GPCR than by that of the G protein [109]. However, a growing appreciation that GPCR signaling may be highly compartmentalized [17] suggests that even small changes in Gα subtype expression may regulate GPCR signaling. Consistent with this notion are observations that exposure of lung [110–112], ASM strips ex vivo [113,114], or ASM cultures [50] to various agents can elicit a loss of β_2AR function that is associated with increased expression of specific Gαi isoforms or decreased expression of Gαs.

Regulation at the effector locus

Although the study of endogenously-expressed GPCR effectors lags behind that of GPCRs and heterotrimeric G proteins, the recent cloning of numerous PLC and AC isoforms and their analysis in expression systems has facilitated insight into the tremendous complexity of effector regulation. Multiple mechanisms by which PLC activity is regulated have been demonstrated [115]. PLCβ activity is greatly influenced by substrate availability; the agonist-sensitive pool of PIP_2 is metabolized several times per minute [116], meaning that recycling of products of hydrolysis, and the activity of numerous enzymes involved in this process, is critical to PLC activity. Localization of PLC isoforms to the membrane appears to be regulated by interaction of pleckstrin homology domains in PLC with specific phosphoinositides and Gβγ subunits [117,118]. PLCβ2 and PLCβ3 isoforms can be phosphorylated by PKA, which results in reduced activity [119–121]. Other PLC isoforms can be phosphorylated by PKC, albeit with no apparent consequence [115,122]. Interestingly, activated PLCβ isoforms serve as GTPase-activating proteins for Gαq and thus participate in negative feedback control of their activation [123].

Unfortunately our understanding of PLC regulation is derived largely from studies using cell-free models or cellular expression systems. With the exception of work from Martin and colleagues [124–126] and Pyne and Pyne [127], few studies to date have examined PLC signaling and its regulation in ASM cells.

Studies of AC regulation have been limited by the extremely low levels of endogenous AC isoform expression, and by the unstable nature of the AC protein, which has rendered its purification and characterization problematic. Detection of endogenous AC protein with currently available antibodies is often difficult in many cell types (including ASM), despite the suggestion of specific isoform expression in parallel analyses of AC mRNA levels. However, expression of recombinant AC isoforms has helped identify some regulatory features of AC [45,128,129]. AC I, II, III, V, and VII are subject to phosphorylation by PKC, which results in their sensitization [130–134]. Conversely, phosphorylation of AC V and VI by PKA inhibits AC activity [135–137]. βγ subunits potentiate the stimulatory effect of Gαs subunits on AC II, IV, and VII [138–140]. Calcium/calmodulim is also a physiologic regulator of AC I, III, and VIII; isoforms whose expression tends to be restricted to the brain and olfactory epithelium [128].

Adenylyl cyclase (as well as other elements and regulators of Gs-coupled receptor signaling) and its activity appear to be concentrated in lipid rafts or caveolae, suggesting that compartmentalization serves to facilitate initiation or quenching of GPCR signaling [141,142]. Similarly, components of PLC signaling, but not PLC isoforms themselves, are also recovered in caveolin-containing membrane fractions [143].

In ASM, AC regulation is evident but appears species-specific. Stevens et al. [48] and Pyne and Pyne [127,144] demonstrated that bradykinin, platelet-derived growth factor (PDGF), and phorbol ester stimulate cAMP formation in guinea pig ASM, presumably via a PKC-dependent enhancement of AC II activation by Gαs. Chronic treatment of canine ASM cultures with carbachol reduced basal and agonist-stimulated AC activity, an effect that was

reversed by PKC inhibition [51]. Similar results were obtained in studies of bovine ASM [50]. In contrast, chronic treatment of human ASM cultures with carbachol (as well as numerous other agonists of Gi-coupled receptors) promoted AC sensitization but in a PKC-insensitive, pertussis-toxin sensitive manner [46]. This manner of AC sensitization has been observed in other cell types including neuronal cells treated chronically with opioids, and appears to be an adaptive response (tolerance) to counteract persistent Gi signaling [64,145,146]. In an analysis of heterologously-expressed AC isoforms in COS cells, Nevo et al [147] determined that chronic Gi activation resulted in sensitization of AC I, V, VI, and VIII, and reduced activity of AC II, IV, and VII. Thus, the profile of AC transcripts and regulatory features of AC in human ASM suggest a predominance of AC VI or V in human ASM, whereas PKC-sensitive isoforms, perhaps AC II, may be preferentially expressed in non-human ASM.

Aberrant GPCR signaling and airway hyperreactivity

Changes in airway structure and ASM contractile state are the principal causes of increased airway resistance in asthma. Altered airway composition and architecture affect airway resistance through mechanisms that are both independent of and complimentary to changes in ASM contractile state. Excessive mucous production and edema are physical impediments to conductance, whereas edema and increased ASM mass alter airway geometry to amplify the effect of ASM contraction on airway lumen diameter [148–154].

ASM contractile state can be viewed as a function of: 1) the net sum of GPCR-mediated signals that result in establishing the level of the key contractile signaling molecule, calcium; and 2) the response of the cell's contractile machinery to calcium. Figure 5 offers a model that proposes 3 levels at which regulation of GPCR-mediated ASM contraction is altered in asthma.

Altered GPCR agonist presentation

On one level we can consider the contribution of a disrupted balance of procontractile and prorelaxant stimuli accessible to ASM, whereby 1) an increase in procontractile stimuli in the asthmatic airway promotes greater activation of GPCRs (Gq- and Gi-coupled receptors) mediating contraction, or 2) a reduction in agonist levels serving Gs-coupled receptor activation diminishes prorelaxant signaling. It is well established that numerous GPCR agonists (e.g., acetylcholine, histamine, and thromboxane) capable of evoking ASM contraction are elevated in the airways of many asthmatics [155–159]. The source of these agonists may be neural cells (increased parasympathetic discharge caused by numerous factors) inflammatory cells (e.g., from mast cells, platelets), or possibly resident mesenchymal airway cells (including ASM itself). Exacerbating this condition in asthma is the sloughing of airway epithelium, which constitutes a loss of diffusion barrier and may increase ASM access preferentially to procontractile agonists [152,160,161]. These findings strongly suggest that exaggerated procontractile GPCR agonist presentation to ASM occurs with asthma and contributes to increased ASM tone. Less certain is whether the levels of prorelaxant GPCR agonists are suppressed in asthmatics. Such agents (e.g., catecholamines, certain eicosanoids) tend to have short half-lives and their local concentrations are not easily measured. However, it should be recognized that the loss of airway epithelium in asthma also constitutes a loss of relaxant factors that target either GPCRs (e.g. PGE_2) or other pathways (nitric oxide) in ASM [152,162–164].

Altered GPCR responsiveness to agonist

On another level we can consider the contribution of altered GPCR responsiveness to a given level of agonist presented to ASM, such that the sum of GPCR-generated signals results in higher than normal increases in intracellular calcium. Such altered GPCR responsiveness may result from either sensitization of Gq- or Gi-mediated signaling that promotes increased calcium flux, or from desensitization of Gs-coupled receptor signaling that antagonizes signaling leading to elevated calcium. Numerous studies suggest that both of these phenomena occur and contribute to ASM hyperresponsiveness.

Recent findings demonstrate that GPCR-mediated contraction of ASM strips ex vivo is augmented by various "sensitization" strategies [165]. These strategies include sensitization to allergen in vivo [68,112,166,167] or prior exposure of ASM strips ex vivo to cytokines, serum from atopic asthmatics, or immune complexes [113,168–172]. Studies of ASM cells suggest that the observed ASM hyperreactivity results in part from an increased calcium flux mediated by sensitized Gq- or Gi-coupled receptor transmembrane signaling. Treatment of ASM cells with IL-1β or TNF-α causes a significantly greater increase in phosphoinositide generation and calcium flux elicited by carbachol, bradykinin, or thrombin [173–177]. Mechanistic studies suggest that up-regulated receptor or G protein expression may mediate this enhanced response. IL-1β and TNF-α are both able to increase B2 bradykinin receptor expression in ASM [174,175]. Treatment of ASM ex vivo with cytokines, rhinovirus, or asthmatic serum [114,171,178], in vivo with antigen or IL-1β [112,179], or ASM cells in culture with TNF-α [180], has been shown to increase expression of either Gq or specific Gαi isoforms in either lung or ASM. These latter findings are consistent with the observation in ASM cells that calcium mobilization stimulated by NaF (a nonspecific Gα activator) is increased following chronic treatment with TNF-α, and

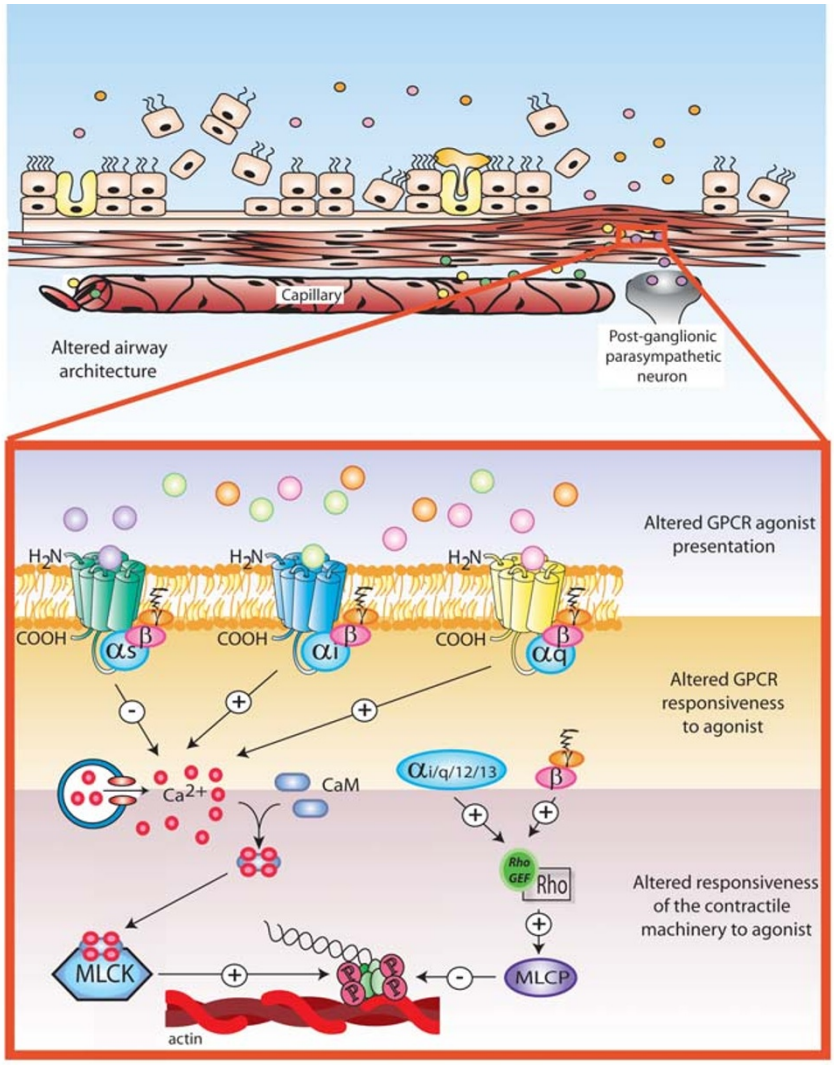

Figure 5

Model of aberrant G protein-coupled receptor signaling in airway smooth muscle contributing to elevated airway smooth muscle tone. Within the context of airway remodeling, G protein-coupled receptor (GPCR) signaling leading to airway smooth muscle (ASM) contraction may be altered at 3 different levels in the asthmatic airway. *First*, ASM may be exposed to greater levels of GPCR agonists that promote contraction (i.e., those activating Gq- or Gi-coupled receptors), or to lower levels of GPCR agonists that mediate prorelaxant signaling (Gs-coupled receptors). Increased levels of procontractile agonists can augment contraction, whereas combined stimulation of ASM with multiple Gq/Gi -coupled receptor agonists has a synergistic effect on contraction [277]. The sources of both procontractile and prorelaxant agonists include infiltrating inflammatory cells (e.g., mast cells releasing histamine, platelets releasing thrombin), postganglionic neurons with exaggerated cholinergic discharge resulting from stimulated reflex arcs or dysregulated m2 muscarinic acetylcholine receptor (mAChR) -mediated feedback inhibition [281], and resident airway cells such as epithelium, fibroblasts, and ASM itself. Access of these agonists to ASM may be increased by sloughing of airway epithelium. *Second*, Gq- or Gi-coupled receptor signaling may be sensitized, or Gs-coupled receptor signaling desensitized, resulting in an imbalance of signaling promoting increased phosphoinositide generation and increased calcium mobilization. *Third*, the contractile response to calcium may be exaggerated due to "augmented sensitization" manifested in increased myosin light chain phosphorylation caused by: 1) an imbalance of GPCR-derived signals; and 2) increased expression and activity of myosin light chain kinase (MLCK) associated with inhibited myosin light chain phosphatase (MLCP) activity, the latter a result of increased RhoA activity mediated by upregulated $G_{12/13}$ and RhoA expression. Not included in this model (for the sake of simplicity) are other potential regulatory features of ASM contraction including ASM strain and load, direct contribution of non-GPCR signaling pathways to phospholipase C (PLC) activation and calcium mobilization, calcium loading in intracellular stores, compartmentalization of calcium and calcium signaling, and ion channel and membrane pump activity that directly and indirectly affect intracellular calcium levels.

suggest a mechanism by which calcium flux stimulated by numerous GPCRs may be augmented [181]. However, it should be noted that the effects of cytokines on GPCR-mediated PLC activity can be receptor-specific; in the same model that demonstrates TNF-α-mediated augmentation of bradykinin-stimulated phosphoinositide production, the phosphoinositide response to histamine was depressed, presumably via a COX-dependent, PKA-mediated phosphorylation and desensitization of the H1 histamine receptor [175].

The contribution of desensitized prorelaxant Gs-coupled receptor signaling to airway hyperresponsiveness in asthma is unclear. To date, the β_2AR is the only Gs-coupled receptor whose role in asthma has received significant attention, and the preponderance of evidence suggests that β_2ARs on ASM are most responsible for the effect of beta-agonists on airway tone [182]. Whether β_2AR dysfunction, and specifically β_2AR dysfunction in ASM, plays a prominent role in asthma has been a hotly debated topic for over thirty years. Asthma triggers such as viral infections can diminish β_2AR function [183], and numerous animal models of airway inflammation, ex vivo analyses of ASM strips treated with cytokines or asthmatic serum [112,114], and limited data from ASM tissue from severe asthmatics [184,185] have all provided evidence that β_2AR-mediated relaxant effect and signaling are depressed in asthma. Several possible mechanisms by which the proposed diminished β_2AR function and signaling occurs can be proposed. The diminished capacity of beta-agonists to inhibit methacholine-induced contraction of ASM strips ex vivo may reflect an increased capacity of m2 mAChRs to inhibit beta-agonist-stimulated AC activity (note that asthmatic serum and cytokines upregulate Gαi expression). As noted above, several studies also demonstrate that numerous agents (e.g. cytokines, TGF-β, PGE_2, whose levels are elevated in the asthmatic airway) induce desensitization of the β_2AR in cultured ASM cells, typically by mechanisms suggestive of PKA-mediated β_2AR phosphorylation. Moreover, intratracheal installation of IL-1β in rats results in not only a loss of beta-agonist-mediated relaxation of methacholine-induced bronchoconstriction, but an increase in GRK activity, and GRK2 and GRK5 expression in the lung [112]. This is an intriguing finding and suggests that inflammation may modulate homologous GPCR desensitization in the airway. This may preferentially affect β_2AR signaling in ASM, in light of findings by McGraw et al. suggesting that low (endogenous) expression levels of GRKs in ASM cells account for relatively robust β_2AR signaling in ASM [186], and that such signaling may be sensitive to changes in GRK [72] or arrestin [20] expression.

In contrast to the evidence cited above, numerous studies have noted no appreciable loss of β_2AR function in asthmatics based on analyses of lung function, or tissues ex vivo (reviewed in [2,4,187]). Moreover, β_2AR blockade in normal subjects does not cause bronchoconstriction [188,189], and the Arg→Gly16 (desensitization-prone) β_2AR polymorphism is not over-represented in asthmatics [190]. These findings suggest that asthma is not defined by diminished β_2AR responsiveness. However, constitutive β_2AR signaling does appear to be important in the asthmatic subject, as administration of β_2AR antagonists is not well tolerated in many asthmatic subjects [189]. Predictably, diminished β_2AR function could influence disease severity. Results from both clinical trials and epidemiological studies suggest that β_2AR SNPs at codon 16 influence β_2AR responsiveness to both endogenous and exogenous beta-agonists and thereby influence disease severity and response to therapy [99,191,192]. Asthmatics homozygous for Gly16 may have fewer responsive β_2ARs as a result of greater down-regulation caused by endogenous catecholamines. Consequently, the effects of endogenous catecholamines and the initial response to exogenous beta-agonist may be diminished in these patients, as suggested by data from Martinez et al., demonstrating a significantly greater bronchodilator response in (beta-agonist naïve) Arg16 homozygotes [192]. Alternatively, continuous use of inhaled beta-agonists results in a progressive drop in morning peak flow only in patients homozygous for the Arg16 SNP [193], suggesting that the absolute loss of β_2AR responsiveness is greater in Arg16 homozygotes because of their greater capacity to down-regulate from the (naïve) untreated state.

Thus the collective evidence suggests that β_2AR dysfunction of any nature does not cause asthma, but the active disease state likely promotes a loss of β_2AR function that has a small impact on disease severity, at least in some subset of asthmatics. As a corollary, β_2AR polymorphisms that diminish β_2AR signaling are disease modifiers, but not disease predictors, and influence the response to therapy. In contrast, a more significant role in asthma is suggested for sensitized Gq or Gi-coupled receptor signal transduction that promotes a greater phosphoinositide generation and calcium mobilization in response to a given concentration of agonist.

Altered responsiveness of the contractile machinery to calcium

On a final level we can propose a role for altered responsiveness of ASM contractile machinery to calcium as a mechanism of airway hyperresponsiveness. Although Rho-mediated sensitization to calcium occurs within the context of ASM contraction under normal conditions, there is evidence that calcium sensitization mechanisms may be primed ("augmented sensitization") by inflammation. Chiba et al. noted that acetylcholine-induced isometric tension was greater in bronchial rings from

antigen-challenged rats compared to that from control rats, although no significant difference between the two groups in calcium mobilization is observed [194]. Similarly, when calcium concentrations were clamped to 1 μM in permeabilized bronchial rings, tension development was greater in rings from allergen sensitized/challenged rats compared to that from controls [70]. Changes in the expression of numerous proteins may underlie this augmentation of calcium sensitization in ASM. In tracheal and bronchial smooth muscle from ragweed-sensitized dogs, a constitutive increase in phosphorylation of myosin light chain 20 (MLC20) associated with increased content and activity of MLCK [195,196], and human bronchial rings sensitized with allergen ex vivo exhibit an ~3 fold increase in MLCK expression [197]. RhoA protein levels are increased, and acetylcholine-induced translocation of RhoA to the plasma membrane is significantly higher in bronchial smooth muscle from airway hyperresponsive versus control rats [70,198]. Finally, $G\alpha_{12}$ and $G\alpha_{13}$ (upstream regulators of Rho activity) levels in bronchial smooth muscle are also upregulated in hyperresponsive rats [68]. These data suggest that allergen-driven inflammation up-regulates multiple proteins in the pathway promoting Rho-dependent calcium sensitization, and that augmented calcium sensitization may be sufficient to confer airway hyperreactivity in asthma.

Altered GPCR responsiveness with therapy

To further complicate the relationship between GPCR responsiveness and asthma, evidence suggests that both glucocorticoids and beta-agonists, the two most widely used drugs in the treatment of asthma, also regulate GPCR responsiveness, primarily via changes in receptor expression and coupling. Glucocorticoids have been shown to up-regulate β_2AR and Gαs expression [89,91,199], counteract the β_2AR down-regulation induced by beta-agonist [92], and reverse increases in GRK activity and β_2AR desensitization induced in a rat model of airway inflammation [58]. Conversely, glucocorticoids inhibit expression of NK2 receptors in bovine ASM [94], inhibit m2 mAChR expression in the airway [200], and inhibit the IL-1β-mediated up-regulation of B2 bradykinin (BK) receptors in the airway [201]. Pretreatment of human ASM cells with glucocorticoids significantly inhibits histamine-stimulated phosphoinositide production [202]. Thus the sum of effects of glucocorticoids on GPCR signal transduction components tends to render ASM less responsive to procontractile stimuli and more responsiveness to beta-agonists.

Beta-agonist therapy, on the other hand, tends to promote the sensitization of procontractile GPCR signaling and desensitization of prorelaxant GPCR signaling, with uncertain clinical relevance. Although conflicting data exist as to whether beta-agonist therapy exacerbates bronchial hyperresponsiveness in asthmatics, Mak and colleagues have recently demonstrated that exposure of ASM ex vivo to beta-agonist up-regulates both NK2 [95] and H1 histamine [90] receptors, suggesting a mechanism whereby enhanced procontractile GPCR signaling promotes bronchial hyperresponsiveness.

Numerous studies have also demonstrated that repeated use of inhaled beta-agonists results in a loss of the prophylactic bronchoprotection conferred by beta-agonists [203–206]. In many respects this could be considered a normal and predictable response, consistent with a physiologic/teleologic role of β_2AR desensitization and the demonstration of homologous desensitization of ASM β_2ARs in multiple in vivo, ex vivo, and in vitro models. However, Finney et al. recently observed that lung GRK2 levels were elevated in rats chronically treated with beta-agonists [110]. Thus, in a manner similar to that invoked by IL-1β (see above), chronic beta-agonist therapy may up regulate the GPCR desensitization "machinery" to further limit the effect of therapy and possibly exacerbate disease.

Although the clinical relevance of the observed loss of bronchoprotection has been questioned [207], the collective evidence suggests that homologous β_2AR desensitization does occur as a consequence of beta-agonist therapy. Accordingly, therapies that minimize or counteract β_2AR desensitization, such as glucocorticoids and salmeterol, may benefit from this property. Glucocorticoids preserve or enhance β_2AR function in the airway through both their anti-inflammatory actions as well as their direct effects on ASM β_2AR expression and regulation noted above. These effects may explain in part the positive cooperativity exhibited by combined beta-agonist and glucocorticoids therapy. As a low intrinsic activity beta agonist, salmeterol has limited capacity to promote homologous β_2AR desensitization in in vitro models [208,209]; this property in addition to its lipophilic nature appears largely responsible for its long-lasting effect. Moreover, daily salmeterol treatment has little effect on the rescue or prophylactic ability of albuterol [203,210].

GPCRs in ASM: What lies ahead

Within the last decade the field of GPCR signaling has experienced an epiphany with the realization that GPCRs do more than subserve restricted functions in fully differentiated cells; they also play important roles in mediating diverse cell functions such as embryogenesis, tissue regeneration, and cell proliferation [211,212]. Interestingly, this realization coincided with a similarly profound discovery in the field of asthma research – that ASM not only contracts, but also performs numerous "synthetic" functions that modulate both airway structure and airway inflammation. Not surprisingly, ASM GPCRs are important regulators of many ASM synthetic functions.

The newfound respective focuses of GPCR signaling and ASM research suggest an exciting direction for the study of GPCRs in ASM over the next decade. The current challenge (or curse) confronting the student of ASM signal transduction extends beyond defining the myriad intracellular signaling pathways, their regulation, and their degree of "cross-talk" with each other, to understanding how these events occur within an equally complex, dynamic airway environment.

Such an understanding should not only greatly improve our knowledge of asthma pathogenesis, but also redefine asthma therapy. With the possible exception of steroids, asthma drugs have been developed and prescribed to prevent or reverse acute bronchospasm with little consideration of their effects on ASM synthetic functions and the chronic nature of asthma. As the roles of airway remodeling and ASM synthetic functions in asthma pathogenesis become more clearly established, agents that target the activation or signaling of various GPCRs that mediate these phenomena will undoubtedly receive greater consideration as prophylactic and therapeutic asthma drugs.

Abbreviations

AA = arachadonic acid

AC = adenylyl cyclase

ASM = airway smooth muscle

BK = bradykinin

β_2AR = beta-2-adrenergic receptor

CLT1R = cysteinyl leukotriene type I receptor

COX2 = cyclo-oxygenase-2

DAG = 1,2-diacylglycerol

EDG = endothelium differentiation gene

EGF = epidermal growth factor

GAP = GTPase protein

GEF = Guanine nucleotide exchange factor

GPCR = G protein-coupled receptor

GRK = G protein-coupled receptor kinase

IL-1β = interleukin-1β

IP_3 = inositol 1,4,5-trisphosphate

LPA = lysophosphatidic acid

LPS = lipopolysaccharide

LTD4 = leukotriene D4

mAChR = muscarinic acetylcholine receptor

MLC = myosin light chain

MLCK = myosin light chain kinase

MLCP = myosin light chain phosphatase

NK = neurokinin

PAR = protease-activated receptor

PDGF = platelet derived growth factor

PGE_2 = prostaglandin E_2

PI = phosphoinositide

PIP2 = phosphoinositol 4,5-bisphosphate

PKA = protein kinase A

PKC = protein kinase C

PLA2 = phospholipase A2

PLC = phospholipase C

RGS = regulators of G protein signaling

SNP = single nucleotide polymorphism

SSP = sphingosine-1-phosphate

TGF-β = transforming growth factor beta

TNF-α = tumor necrosis factor alpha

TP = thromboxane A2 / prostaglandin

VIP = vasoactive intestinal peptide

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