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Crosstalk between beta-2-adrenoceptor and muscarinic acetylcholine receptors in the airway

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Abstract

The M_3 and M_2 muscarinic acetylcholine receptors (mAChRs) and beta-2-adrenoceptors (β2ARs) are important regulators of airway cell function, and drugs targeting these receptors are among the first line drugs in the treatment of the obstructive lung diseases asthma and chronic obstructive lung disease (COPD). Cross-regulation or crosstalk between mAChRs and β2ARs in airway smooth muscle (ASM) helps determine the contractile state of the muscle, thus airway diameter and resistance to airflow. In this review we will detail mAChR and β2AR-signaling and crosstalk, focusing on events in the ASM cell but also addressing the function of these receptors in other cell types that impact airway physiology. We conclude by discussing how recent advances in GPCR pharmacology offer a unique opportunity to fine tune mAChR and β2AR signaling and their crosstalk, and thereby produce superior therapeutics for obstructive lung and other diseases.

Introduction

The contractile state or "tone" of airway smooth muscle (ASM) is the principal determinant of airway diameter and thus resistance to airflow. Under physiological conditions ASM has relatively little tone; airways are patent and airway resistance does not limit breathing. Under conditions of lung inflammation that occur with obstructive lung diseases such as asthma, increased presentation of agents that promote contraction by activating various G protein-coupled receptors (GPCR) on ASM increase ASM tone and thus airway resistance. Although multiple agents and their cognate receptors can contribute to increased ASM contraction, the M_3 muscarinic acetylcholine receptor (mAChR) is arguably the most important, being activated by acetylcholine (ACh) released from nerves of the parasympathetic nervous system. Conversely, the beta-2-adrenoceptor (β2AR) is arguably the most important GPCR capable of antagonizing ASM contraction. The importance of

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both the M_3 mAChR and β2AR in regulating ASM contraction is underscored by the importance of mAChR *antagonists* and β2AR *agonists* as bronchodilators in airway diseases. The signaling crosstalk between mAChRs (both M_3 and M_2) and β2ARs plays a prominent role in determining ASM contractile state, and is critical to the both the efficacy and limitations of those asthma therapeutics targeting these receptors. In this review we will detail mAChR and β2AR signaling and crosstalk, focusing on events in the ASM cell but also addressing the function of these receptors in other cell types that impact airway physiology. We will conclude by discussing how recent advances in GPCR pharmacology offer a unique opportunity to fine tune mAChR and β2AR signaling and their crosstalk, and thereby produce superior therapeutics for obstructive lung and other diseases.

GPCR signaling and function in airway cells

M_3 mAChR signaling and function

M_3 muscarinic acetylcholine receptors are coupled to heterotrimeric G_q proteins. The binding of ACh to the receptor induces a conformation change in the receptor, which promotes association with and activation of the G_q protein by exchanging GTP for GDP on the G_α subunit. The subsequently released G_α subunit activates phospholipase C which hydrolyzes PIP_2 into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors on the endoplasmatic reticulum, releasing Ca^{2+} from intracellular stores whereas DAG activates protein kinase C (PKC).

M_3 mAChRs expressed on ASM cells are the principal mediators of ASM contraction or tone under physiological conditions, and the majority of studies posit them as significant in mediating the pathological contraction (bronchoconstriction) associated with asthma and chronic obstructive pulmonary disease (COPD) (for a comprehensive analysis of G_q-coupled receptor signaling that mediates ASM contraction, see [1;2] and references therein). Briefly, the initial ACh-induced increase in intracellular Ca^{2+} is followed by a more sustained increase in Ca^{2+} mediated by the Ca^{2+}-sensitive ryanodine receptors (RyR) on the endoplasmic reticulum, and by Ca^{2+} influx from the extracellular space involving store-operated Ca^{2+} channels and further promoted by a PKC-dependent increase in the open probability of Ca^{2+} channels on the cell membrane (reviewed in [3]). Formation of the Ca^{2+}/calmodulin complexes activates myosin light chain kinase (MLCK) which phosphorylates myosin light chain and allows activation of myosin ATPase resulting in generation of force through cross-bridge cycling. Concomitantly-activated PKC and Rho kinase (the downstream effector of RhoA), which are activated by not only the M_3 mAChR but other Gq-coupled GCPRs in ASM, serve to augment this contractile signaling by inhibiting myosin light chain phosphatase (MLCP). Both PKC and Rho kinase activate CPI-17, an endogenous inhibitor of MLCP. MLCP serves as a brake on contraction by reversing MLCK-induced phosphorylation of MLC. MLCP inhibition results in increased MLCK phosphorylation/activity at any given level of intracellular calcium. MLCP inhibition is thus a key mechanism mediating “calcium sensitization,” enabling maintenance of ASM contraction as intracellular calcium levels wane, and increasing ASM responsiveness to contractile agents (a key mechanism mediating airway hyperreactivity in the asthmatic). PKC may also promote contraction by directly phosphorylating MLCK and MLC, however,

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the relevance of PKC-mediated MLCK phosphorylation needs to be confirmed in ASM whereas the contribution of PKC phosphorylation sites on MLC to contraction is uncertain. Although the above-described receptor-mediated "pharmaco-mechanical coupling" is viewed as the predominant pathway mediating contraction, recent studies have indicated an important role for ACh-activated RhoA in promoting ASM contraction through dynamic actin remodeling [4].

As alluded to above, the important role of M_3 mAChRs in mediating increased airway resistance and airway hyperresponsiveness (AHR) in obstructive lung diseases is underscored by the use of mAChR antagonists in the treatment of both asthma and COPD [5;6]. In COPD, the increased cholinergic ASM tone is the *major* reversible component of airway resistance [7–9] and mAChR antagonists are used as first line treatments. Ipratropium, a nonselective (targeting M_1, M_2 and M_3 mAChRs) mAChR antagonist, has been a COPD treatment for almost 40 years, and has recently been supplanted by the long-acting tiotropium, whose binding kinetics render it more selective for M_3 mAChR antagonism [10;11].

In addition to their role in mediating aberrant ASM tone, M_3 mAChRs have also been implicated in airway remodeling and inflammation. M_3 mAChRs have been shown to augment growth factor-induced proliferation of ASM [12;13] as well as the release of IL-8 in response to TNF-α or cigarette smoke [14;15]. The M_3 mAChR also stimulates the release of neutrophil-attracting chemokines from airway epithelial cells [16] and alveolar macrophages [17].

More recent studies also indicate cooperativity between M_3 mAChRs and TGF-β signaling in increasing contractile protein expression in ASM cells. Using guinea pig precision cut lung slices, Oenema et al. [18] determined that M_3 mAChR- (as well as histamine- and KCl-) -mediated bronchoconstriction results in the release of active TGF-β which promotes ASM remodeling. These findings suggest a mechanism explaining the findings of Grainge et al. [19] in which bronchoconstriction alone (in the absence of an inflammatory stimulus) was sufficient to induce airway remodeling in asthmatics.

M_3 mAChRs also promote mucus secretion from submucosal glands in the conducting airways, which are innervated by the vagus nerve. M_3 mAChR stimulation under pathophysiological conditions in diseased airways, by either neuronal or non-neuronal sources of ACh, leads to mucus hypersecretion as well as goblet cell hyperplasia which contribute to increased airway resistance [8;20]

Animal models employing pharmacological or genetic inhibitory strategies provide the most compelling evidence to date implicating the M_3 mAChR in airway remodeling and inflammation. The mAChR antagonist tiotropium prevents ASM (and vascular smooth muscle) hypertrophy, goblet cell hyperplasia and eosinophilic inflammation a guinea pig model of chronic allergic asthma [21;22]. Similarly, in a guinea pig model of COPD, tiotropium inhibits neutrophilic inflammation, goblet cell hyperplasia and airway fibrosis [23]. A recent study from the Gosens lab [24] provides conclusive evidence for the role of M_3 mAChRs as allergen-induced ASM (and vascular smooth muscle) remodeling, goblet

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cell metaplasia and airway fibrosis were all attenuated in M_3 mAChR knockout mice relative to wild type and M_2 mAChR knockout mice. Importantly, M_3 mAChR knockout did not affect the development of eosinophilic inflammation exhibited in wild type mice, again supporting the findings from Grainge et al. [25] suggesting that inflammation is not required for the development of airway remodeling.

M_2 mAChR signaling and function

M_2 mAChRs are expressed on various cell types in the airways, although their function in ASM and parasympathetic pre-synaptic nerves appears to have the greatest impact on airway function. M_2 mAChRs are coupled to the G_i heterotrimeric protein, and early studies in S49 lymphoma cell membranes demonstrated the ability of activated $G_{i\alpha}$ subunit to bind and inhibit adenylyl cyclase activated by the $G_{s\alpha}$ [26]. Thus, as will be discuss below, M_2 mAChR activation constrains the signaling and bronchorelaxant effect of β2ARs by antagonizing β2AR/G_s activation of adenylyl cyclase. However, M_2 mAChRs expressed pre-synaptically on parasympathetic nerve endings, when activated exert negative feedback on neuronal ACh release, thereby limiting bronchoconstriction. The dysfunction of the presynaptic M_2 mAChR receptors has been proposed as a pathophysiological mechanism of AHR in asthma. Several animal model studies have shown that allergen challenge as well as viral infections or noxious insult to the lung may result in the dysfunction of the presynaptic M_2 mAChR receptor, likely mediated by eosinophilic inflammation, and contribute to AHR [27].

The capacity of M_2 mAChRs expressed on ASM cells to promote contraction through pharmacomechanical coupling is not apparent based on studies comparing mice in which each of the (M_1-M_5) mAChR knockout mice have been compared [28;29]. However, a role for M_2 mAChRs in $G_{\alpha i}$-dependent activation of RhoA and subsequent stress fiber formation through actin polymerization has been proposed in ASM [30;31] and may contribute to tension development.

A role for M_2 mAChRs in regulating airway remodeling has also been suggested. M_2 mAChR stimulation in fibroblasts results in increased proliferation and collagen synthesis, a finding which suggests potential for contributing to airway fibrosis [32;33]. A recent study also shows that ASM cell proliferation induced by prolonged TGF-β_1 stimulation is augmented by M_2 mAChRs [34].

Collectively, several studies suggest that the M_2 mAChR may have a role in mediating increased ASM contraction through both direct and indirect actions on ASM, and also mediate other features of obstructive airway disease. However, the relative importance of these mechanisms is unclear.

β2AR signaling and function

β2ARs are coupled to G_s heterotrimeric G proteins. Binding of an agonist (e.g., endogenous epinephrine or exogenous beta-agonist) to the receptor promotes $G\alpha_s$ activation which in turn activates adenylyl cyclase which hydrolyzes adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). The binding of cAMP to the regulatory subunits of cAMP-dependent protein kinase (protein kinase A (PKA)) releases the catalytic subunit of

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the PKA, and activated PKA exerts myriad effects in ASM by phosphorylating numerous intracellular targets. cAMP-/PKA- dependent signaling promoted by the β2AR inhibits M_3 mAChR (and other pro-contractile G_q-coupled receptors in ASM) signaling at multiple steps and constitutes the "crosstalk" that antagonizes ASM contraction (see below).

In addition to the pro-relaxant capability of β2ARs, immunomodulatory functions of the β2AR have been described, including the ability to induce IL-6 and TSLP expression, and inhibit cytokine-induced GM-CSF, RANTES and eotaxin release from ASM cells [35–37]. The immunomodulatory role is also evident in other cell types including bronchial epithelial and mast cells in which beta-agonists increase release of TSLP and inhibit histamine release, respectively [38;39]. In type 2 T cells, beta-agonists augment cytokine-induced cell accumulation by prolonging T-cell survival [40]. PKA-mediated regulation of gene expression occurs through phosphorylation of the transcription factor CREB and phosphorylation of GPCRs and receptor tyrosine kinases [1]. The anti-mitogenic and anti-migration properties of beta-agonists in ASM cells [41;42] have also been shown to be mediated by PKA, with PKA being directly implicated via heterologous expression of PKA-inhibitory peptides.

In addition to the well characterized PKA-mediated β2AR signaling, it is now appreciated that the regulatory proteins *arrestins* can promote β2AR signaling events distinct from those of PKA and independent of β2AR-G_s coupling [43]. Arrestins were originally identified as important in regulating β2AR both desensitization and resensitization by promoting β2AR internalization to either recycling or degradative pathways. Subsequent studies determined that arrestins also serve as scaffolds and initiators of signaling to numerous pathways including p42/p44, JNK, and NF-κB (see below). Arrestin-dependent signaling in ASM or any airway cell remains poorly defined, but in mice with beta-arrestin-2 knockout, both lung inflammation and AHR due to allergen sensitization and challenge is significantly attenuated [44]. As will be discussed below, future studies distinguishing between G protein- and arrestin- dependent signaling events and consequences in airway cells should ultimately greatly increase our understanding of GPCR control of airway function in health and disease, and likely lead to a new generation of drugs capable of exploiting this diverse signaling capacity.

Crosstalk among GPCRs in the airway

β2ARs regulating M_3 mACHRs

The physiological consequence of crosstalk between β2ARs and M_3 mAChRs in ASM is described as functional antagonism. Although certain pro-relaxant mechanisms promoted by β2AR stimulation (phosphorylation of heat shock protein 20 (HSP20) [45;46] and K_{Ca2+} channels [47]) do not involve inhibiting M_3 mAChR or G_q–coupled receptor signaling *per se*, β2AR signaling effects bronchorelaxation by targeting M_3 mAChR signaling at the plasma membrane and at numerous downstream intracellular junctures (Figure 1).

β2AR signaling limits M_3 mAChR-mediated IP_3 production by several distinct mechanisms, most presumed to involve PKA. Activated PKA can phosphorylate the M_3 mAChR (1), G_q subunit (2) or PLC (3); promoting receptor desensitization (1 & 2) and decreased IP_3

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production (1–3). Although M_3 mAChR has a PKA phosphorylation site [48], a study using overexpressed M_3 mAChR in CHO cells did not show a role for PKA in M_3 mAChR phosphorylation [49].

Downstream of PLC, PKA inhibits Ca^{2+} release from the intracellular Ca^{2+} stores by phosphorylating the IP_3R (4) and RyR (not shown), while hyperpolarizing the cell through phosphorylation of K_{Ca2+} channels (5). Moreover, PKA limits contraction by inhibiting the activation of CPI-17 (6) which otherwise promotes PKC- and Rho kinase-mediated Ca^{2+} sensitization. PKA also inhibits RhoA-mediated actin polymerization (loss of stress fibers) via an unknown mechanism (not shown). In addition, PKA phosphorylates HSP20 (7) which promotes relaxation, possibly by regulating actin dynamics and actin-myosin binding.

Although PKA has long been presumed the key effector of the β2AR mediating the functional antagonism of pro-contractile signaling, it is important to remember that little if any direct evidence implicating PKA in this regard exists. We simply know that the β2AR activates PKA, and that multiple agents capable of activating PKA (e.g., EP2/4 receptors, forskolin, and phosphodiesterases) can also relax contracted ASM. Recently, another cAMP effector, Exchange protein directly activated by cAMP (Epac) (8), has recently been proposed as sufficient to inhibit ASM contraction, and perhaps required in the effect of known bronchorelaxants such as beta-agonists. Two different groups have shown the capacity of Epac to induce relaxation of pre-contracted smooth muscle tissues [50;51]. However, it remains difficult to ascertain conclusively to what extent Epac contributes to relaxation mediated by β2ARs or other physiological inducers of cAMP, as pharmacological Epac inhibitors are not available and most studies simply demonstrate the *sufficiency* of Epac to promote signaling and functional consequences through the use of Epac-selective cAMP analogs.

Cross-talk in the other direction: M_3 and M_2 mAChR regulation of the β2AR

In addition to the induction of Ca^{2+}-sensitization (detailed above), several studies have indicated that M_3 mAChR activation promotes desensitization of the β2AR in a PKC-dependent manner (Figure 2). Using bovine ASM tissue strips, Boterman et al. have shown that M_3 mAChR stimulation promotes heterologous (i.e. independent of beta-agonist-induced change in β2AR conformation) as well as homologous (i.e. beta-agonist-induced) desensitization of the β2AR resulting in diminished capacity to relax muscle [52;53]. A role for M_3 mAChR-induced PKC-mediated heterologous β2AR desensitization has also been reported earlier in a study using co-expression of M_3 mAChR and β2AR in CHO cells [54]. Direct activation of PKC by the DAG analog phorbol-12-myristate-13-acetate has been shown to inhibit AC, however it is still unknown whether this occurs in response to M_3 mAChR activation [55].

Incubation with an M_2 mAChR-selective muscarinic receptor agonist potentiates β2AR-mediated relaxation of tissues pre-contracted with MCh (but not those pre-contracted with histamine) [56;57], although some *ex vivo* studies have also reported that decreased tone post-M_2 mAChR antagonist may also contribute to this effect and have questioned the role of M_2 mAChR in functional antagonism [58]. The discrepancy may be a consequence of

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variation in pre-contracted tissue tone, mode of tension measurement (isometric versus isotonic) and species differences.

Functional antagonism mediated by M_2 mAChR regulation of the β2AR has also been demonstrated in mice and is further exacerbated in GRK5–/– mice. β2AR treatment is less effective in relaxing either carbachol- or KCl-induced pre-contracted tracheal tissue of GRK5–/– mice [59]. This is presumably due to increased M_2 mAChR G_i signaling inhibiting AC activity (potentially via GRK5 knockout alleviating M_2 mAChR desensitization), as treatment with the M_2 mAChR selective muscarinic receptor antagonist methocramine normalizes β2AR effectiveness to levels observed in WT mouse tissues. A role for GRK3 has similarly been suggested in the regulation of muscarinic receptor responses in the murine lung, as GRK3 ablation results in enhanced MCh constrictor responses [60]. Although the muscarinic receptor subtype responsible for the increased bronchoconstrictor response in this study has not been identified, the GRK3 locus has been implicated as a potential regulator of functional antagonism. Interestingly, although acute M_2 mAChR stimulation inhibits AC activity, prolonged (18 h) treatment of human ASM cell cultures paradoxically results in increased basal and stimulated AC activity [55]. Opposing actions of M_2 mAChR (stimulatory) and β2AR (inhibitory) activation on lung fibroblast proliferation and collagen synthesis implies a role for this antagonism in lung fibrosis [32;33;61].

Relevance to disease and therapy

Obstructive airway diseases are characterized and perhaps driven by dysfunction of GPCR-mediated signaling in the lung. Overpresentation of ACh drives a pathogenic increase in muscarinic (pro-contractile, pro-remodeling) signaling. It is unclear to what extent changes in receptor crosstalk per se contribute to the pathogenesis, but given the signaling between mAChRs and β2AR is competitive, an increase in mAChR signaling coupled with no change or a decrease in β2AR signaling (as a consequence of desensitization) alters the balance of signaling and results in greater ASM tone. There is evidence that approaches focusing on restoring the balance by regulating signaling to affect mAChR- β2AR competition are effective [62;63].

Beneficial effects of combined mAChR antagonists and beta-agonists relative to those of a (monotherapy) bronchodilator in COPD have been determined for SAMA/SABA [64] and more recently for LAMA/LABA [65;66]. Recent clinical trials provide evidence to support the longstanding off-label use of tiotropium as add-on therapy to LABA/inhaled corticosteroid (ICS) in asthma [67–69]. In patients with poorly controlled asthma, tiotropium improved lung function [67;68] and reduced exacerbations when added on to LABA/ICS [68]. Similarly, add-on of tiotropium in patients with COPD and concomitant asthma results in improved bronchodilatation and lung function [70].

A number of combination therapy inhalers are currently under development [5]. There is now ample clinical/scientific evidence of the benefits LAMA/LABA combination therapy. However, the nature of the cooperativity, in what cell types, contributing to this effect remains unclear; effects on ASM contractility are probably significant. An increasing

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number of studies in cell-based and animal models of lung disease combining LAMA and LABA are currently being performed and should be published in the near future. The data at the functional level are encouraging and the molecular mechanisms require further exploration [71;72].

In addition, the current combination therapies of LABA plus inhaled corticosteroids (Advair, Symbicort) also affect the balance of mAChR- β2AR signaling by activating β2ARs while reducing inflammation-induced cholinergic discharge [8].

The idea of mAChR- β2AR crosstalk promoting functional antagonism and controlling ASM contractile state provides a convenient paradigm for explaining the roles of these receptors in disease pathogenesis and therapy. However, recent studies in GPCR biology suggest that mAChR and β2AR (and probably all GPCR) signaling is more complex and extends beyond the canonical G protein signaling known for each receptor. If true, we are likely unappreciative of the full impact of GPCRs on various diseases, including effects on pathobiology and in their capacity to function therapeutically.

The β2AR has been shown to promote diverse and qualitatively distinct signaling events. In additional to canonical G_s/cAMP/PKA signaling, the β2AR has been shown to coupleto G_i via a “specificity switch” that occurs upon PKA-mediated phosphorylation of the β2AR [73]. G protein-independent signaling has also been demonstrated, occurring through an arrestin-dependent mechanism. Arrestin molecules, originally demonstrated to play a critical role in β2AR desensitization (and resensitization), are now known to function as scaffold proteins capable of initiating signaling to multiple pathways distinct from those involving heterotrimeric G proteins. Arrestins have been shown to mediate β2AR signaling to the p42/p44 [74], and p38 [75] MAPK pathways, to NF-κB [76], and to RhoA [77]. Although arrestins have been shown to mediate β2AR desensitization in ASM *in vitro, ex vivo,* and *in vivo* [62;78], arrestin-dependent signaling in airway cells is poorly understood, However, several studies suggest that arrestin is critical to the development of allergic lung inflammation and the asthma phenotype. Walker et al. [44] originally reported that mice lacking the beta-arrestin-2 gene fail to develop significant allergic inflammation and associated AHR after allergen sensitization and challenge.

The obvious question raised by Walker et al. involves the receptors and cell types in which arrestin function plays a pro-inflammatory and pro-asthmatic role. A recent study by Nichols et al. [79], utilizing protease-activated receptor 2 (PAR2) knockout and beta-arrestin-2 knockout mice, implicates PAR2-dependent arrestin signaling as important in the development of allergic lung inflammation. While it is conceivable that numerous GPCRs on numerous cell types promote inflammation in an arrestin-dependent manner, a series of studies by Bond and colleagues has led us to consider the β2AR as a critical mediator of arrestin-dependent pathology in allergic lung inflammation. Original studies from the Bond lab implicated β2AR agonism as required for the development of both lung inflammation and AHR in ovalbumin sensitized and challenged mice. β2AR knockout [80], or treatment of mice with the inverse agonist nadolol (but not the antagonist alprenolol) [81], inhibited mice from developing lung inflammation and AHR, leading the authors to conclude that *constitutive* β2AR agonism (receptor activity in the absence of ligand; only inverse agonists

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inhibit this activity) was permissive to pathology. Yet that interpretation was abandoned when it was demonstrated that depletion of systemic epinephrine had the same effect as β2AR knockout or chronic nadolol. But why did some "beta-blockers" work (e.g., nadolol, ICI 118,551) whereas others did not (alprenolol, carvedilol)? Moreover, results from clinical trials suggested one beta-blocker (nadolol) was effective in reducing AHR in asthmatics [82;83], while another (propranolol) was not [84]. The differential effects of these beta-blockers could be explained by their differing capacities to stimulate arrestin-dependent signaling. It is important to keep in mind that to date, β2AR ligands have been defined as agonists or antagonists based on their ability to stimulate, and block agonist-induced stimulation of, the G_s-adenylyl cyclase-cAMP pathway. However, multiple recent studies (reviewed in [85]) have demonstrated that β2AR (and other GPCR) ligands can exhibit different abilities to stimulate G protein- and arrestin- dependent signaling. Interestingly, nadolol and ICI 118,551 cannot stimulate (but can block) both G protein- and arrestin-dependent signaling, whereas the "beta-blockers" carvedilol and propranolol antagonize G protein but stimulate arrestin-dependent signaling [86;87]. Thus, the therapeutic efficacy of β2AR ligands for asthma may rely on their capacity to not activate/antagonize arrestin signaling, while perhaps stimulating the G_s signaling known to have a pro-relaxant effect on ASM.

With respect to the M_3 mAChR, signaling via G protein and arrestins has been shown to differentially affect cellular functions, raising the possibility that biased ligands for the M_3 mAChR may be therapeutically superior to existing drugs. Kong et al. [88] demonstrated that the early phase of glucose-induced insulin secretion in pancreatic islets cells was stimulated by G protein-dependent signaling, whereas the late phase was dependent G protein-independent/arrestin-dependent. Insight into the utility of biased M_3 mAChR ligands in the treatment of obstructive lung disease would first require an understanding of the role of M_3 mAChR-mediated arrestin signaling in airway and inflammatory cells, of which nothing is currently known.

In conclusion, crosstalk between mAChRs and β2AR in the airway, and specifically in ASM, plays a profound role in determining lung function in health and disease. Future studies further delineating the function of these receptors, particularly with respect to the consequences of G protein- and arrestin- dependent signaling, in the numerous cell types whose function/dysfunction contributes to airway diseases, will undoubtedly facilitate the development of new and better therapies.

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Highlights (for review)

Crosstalk between mAChRs and β2ARs dictates ASM contractile state and thus airway resistance and lung function.

mAChRs and β2ARs regulate multiple airway cell functions affecting airway inflammation and remodeling.

Biased ligands of mAChRs and β2ARs may promote "therapeutic" while avoiding "pathogenic" signaling.

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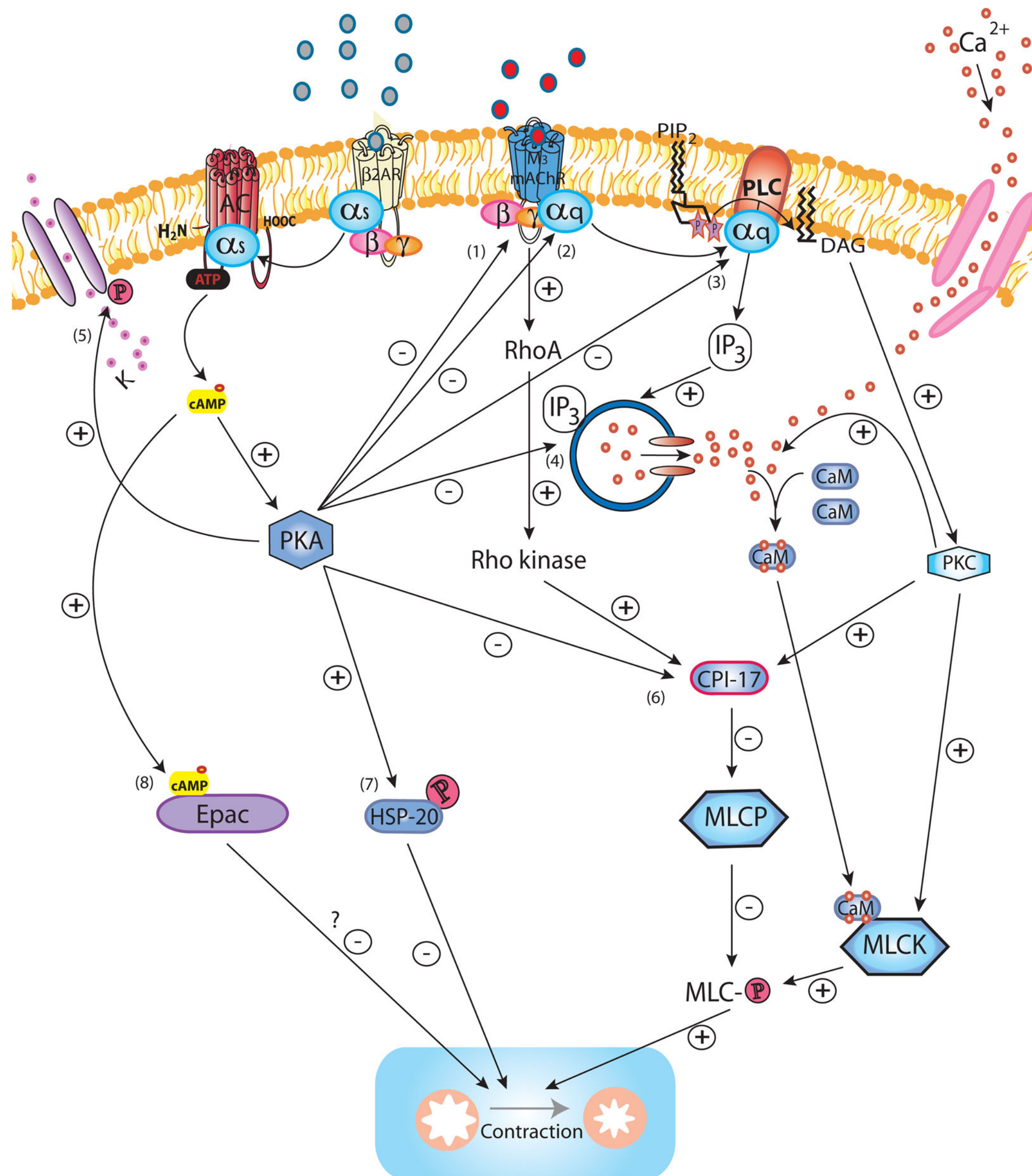

Figure 1. β2AR regulation of M_3 mAChR signaling

Upon activation of M_3 mAChR by ligand binding, the $G_{\alpha q}$ subunit is released to bind and activate PLC, which hydrolyzes PIP_2 into IP_3 and DAG. IP_3 induces Ca^{2+} release from intracellular stores via the IP_3R whereas DAG activates PKC. The initial IP_3-induced release of Ca^{2+}, as well as cADP-ribose (not shown), both acting via RyR (not shown) contribute to a more sustained increase of intracellular Ca^{2+}, also promoted by additional influx of Ca^{2+} from the extracellular space through Ca^{2+} channels on the cell membrane. Ca^{2+} binds calmodulin to form the Ca^{2+}/calmodulin complex which activates MLCK to subsequently phosphorylate MLC allowing activation of myosin ATPase (not shown) which effectively leads to force generation by cross-bridge cycling (contraction). PKC promotes the influx of Ca^{2+} from the extracellular compartment by increasing the open probability of Ca^{2+} channels. PKC phosphorylation of the MLCP inhibitor CPI-17 mediates Ca^{2+} -sensitization

as MLCP limits contraction by dephosphorylating MLC. Similarly, M_3 mAChR activates RhoA and its downstream effector Rho kinase which also phosphorylates CPI-17 and thereby mediates Ca^{2+} -sensitization. PKC-mediated phosphorylation of MLCK may constitute an additional mechanism for Ca^{2+} -sensitization. Activation of the β2AR counteracts M_3 mAChR-induced signaling and contraction at several levels. Ligand-induced conformation change of the β2AR results in the release of the $G_{\alpha s}$ subunit which subsequently activates AC to hydrolyze ATP to cAMP. cAMP binds to the PKA regulatory units (not shown) which then release the catalytically active PKA. PKA phosphorylates the M_3 mAChR (1) (although relevance of this for desensitization in ASM is not certain), the $G_{\alpha q}$ subunit (2) and PLC (3) diminishing M_3 mAChR activation and IP_3 production (and possibly Rho kinase activation). The release of intracellular Ca^{2+} is inhibited through PKA-mediated phosphorylation of IP_3R (4) and RyR (not shown), whereas phosphorylation of the K_{Ca2+} (5) channels results in K^+ efflux and hyperpolarization of the cell. PKA abrogates Ca^{2+} -sensitization by phosphorylating CPI-17 (6). PKA phosphorylation of HSP20 (7) renders it capable of inhibiting contraction through ill-defined mechanisms possibly involving regulation of actin dynamics and actin-myosin binding. Another potential effector downstream of cAMP, Epac (8), has been suggested as an inhibitor of contraction, however its role in ASM as an effector downstream of β2AR remains to be determined.

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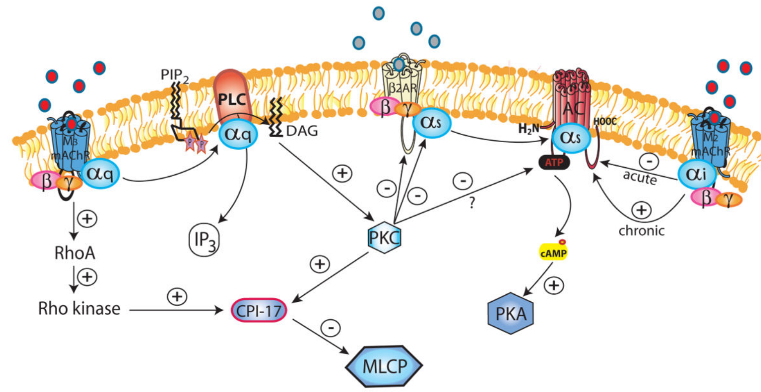

Figure 2. M_3/M_2 mAChR regulation of the β2AR

PKC is the major effector of M_3 mAChR-mediated regulation of the β2AR receptor. In addition to the PKC and Rho kinase-mediated Ca^{2+} -sensitization as detailed above, PKC phosphorylates both the β2AR and the $G_{\alpha s}$ subunit, thereby promoting β2AR desensitization; both directly (heterologous) and by increasing sensitivity to beta-agonist-induced (homologous) desensitization. The activated M_2 mAChR releases its $G_{\alpha i}$ subunit which inhibits AC, effectively limiting $G_{\alpha s}$-induced cAMP production. However, prolonged activation of M_2 mAChR can paradoxically sensitize AC and increase cAMP production, via an unknown mechanism. PMA-induced (receptor-independent) activation of PKC has been shown to inhibit AC, however, whether this holds true for M_3 mAChR-stimulated PKC still requires confirmation.