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The pharmacological rationale for combining muscarinic receptor antagonists and β-adrenoceptor agonists in the treatment of airway and bladder disease☆

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Muscarinic receptor antagonists and β-adrenoceptor agonists are used in the treatment of obstructive airway disease and overactive bladder syndrome. Here we review the pharmacological rationale for their combination. Muscarinic receptors and β-adrenoceptors are physiological antagonists for smooth muscle tone in airways and bladder. Muscarinic agonism may attenuate β-adrenoceptor-mediated relaxation more than other contractile stimuli. Chronic treatment with one drug class may regulate expression of the target receptor but also that of the opposing receptor. Prejunctional β_2-adrenoceptors can enhance neuronal acetylcholine release. Moreover, at least in the airways, muscarinic receptors and β-adrenoceptors are expressed in different locations, indicating that only a combined modulation of both systems may cause dilatation along the entire bronchial tree. While all of these factors contribute to a rationale for a combination of muscarinic receptor antagonists and β-adrenoceptor agonists, the full value of such combination as compared to monotherapy can only be determined in clinical studies.

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Introduction

Obstructive airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) and urinary bladder dysfunction such as the overactive bladder syndrome (OAB) are typically seen as unrelated conditions. However, both affect hollow organs and are characterized by an imbalance between contractile and relaxant smooth muscle stimuli. Moreover, the sympathetic and the parasympathetic nervous system plays important roles in both cases, although sympathetic innervation may be sparse [1]; accordingly muscarinic receptor antagonists and β-adrenoceptor agonists are important therapeutics for both organ systems. The present manuscript reviews the molecular, cellular and tissue rationale underlying the combined use of these two drug classes. We combine data from airways and urinary bladder to improve the robustness of emerging concepts.

Clinical background

COPD is a progressive disease associated mainly with tobacco smoking, air pollution or occupational exposure, which can cause obstruction of airflow in the lungs resulting in debilitating bouts of breathlessness. Inhaled bronchodilators (β_2 adrenoceptor agonists or M_3 muscarinic acetylcholine receptor antagonists) remain the mainstay of current management of COPD at all stages of the disease [2••]. Clinical advances in the treatment of COPD have centered on improvements of these existing classes of bronchodilators, by either increasing duration of action or by improving their selectivity profiles [2••]. The combination of a β_2-adrenoceptor agonist with a M_3 muscarinic receptor antagonist, into a fixed-dose combination therapy, is currently being pursued by several pharmaceutical companies.

The Global Initiative For Asthma defines asthma as a 'chronic inflammatory disorder of the airways in which many cells and cellular elements play a role' (www.ginasthma.org). In bronchi from asthmatic patients, contraction responses to muscarinic receptor agonists are enhanced and relaxation responses to β-adrenoceptor agonists are attenuated [3]. This airway hyperresponsiveness leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night

or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment. First-line treatment of asthma is based on low-to-medium doses of an inhaled glucocorticoid, but this yields inadequate symptom control in many patients. Short-acting muscarinic receptor antagonists and β-adrenoceptor agonists, often in combination, can be added as acute reliever medication. Long-acting β-adrenoceptor agonists are an option as additional controllers, but their safety when used as monotherapy has been questioned. Alternative/additional controller medications are needed [4] and the combination of a long-acting β-adrenoceptor agonist with a long-acting muscarinic antagonist is considered a possible option. However, the efficacy and safety of such a combination, or of monotherapy with a long-acting muscarinic antagonist, has not been fully evaluated and hence is not an approved use.

OAB is defined by the International Continence Society by the presence of urgency, with or without incontinence, usually accompanied by urinary frequency and nocturia [5]. For a long time muscarinic receptor antagonists have been the mainstay of OAB treatment [6], but recently β_3-adrenoceptor agonists are emerging as an alternative treatment option [7•,8•]; the combined use of both drug classes is currently undergoing clinical exploration.

Accordingly, COPD, asthma and OAB share a number of features but also exhibit important differences [1]. The most important one is that obstructive airway disease leads to considerable morbidity and even mortality, whereas OAB mainly adversely affects quality of life. Nevertheless, it appears helpful to look at all three conditions concomitantly as they share important features with regard to the roles of the sympathetic and parasympathetic system and its interaction. Such interaction can occur at the level of exposure to the sympathetic and parasympathetic mediators (which importantly includes non-neuronal acetylcholine release in both airways and bladder) and the level of smooth muscle tone.

Descriptive interaction studies between muscarinic and β-adrenergic agents

Several studies have explored how concomitant exposure to β-adrenergic and muscarinic receptor ligands affects the response to each other. While there always is a physiological antagonism between contractile and relaxant stimuli, it appears that this interaction is more pronounced between relaxation by β-adrenoceptor agonist and contraction by muscarinic receptor ligands than by contracting agonists acting upon other types of receptors. This section will describe the 'privileged interaction' between the β-adrenergic and muscarinic system in airways and bladder. Subsequent sections will explore the underlying mechanism for these interactions.

Airway studies

Physiological resting tone in airways is mediated by parasympathetic innervation of airway smooth muscle, via muscarinic receptors. Muscarinic receptor subtypes M_2 and M_3 are expressed at a 4:1 ratio [9] but the contraction response is mediated predominantly if not exclusively by the M_3 subtype [10–12]. Regulation is disturbed under pathological conditions [13,14]. In addition to agonists of the muscarinic pathway, other contractile mediators are released during pathological conditions, including histamine and bradykinin, receptors for which (H_1 and B2, respectively) are located on airway smooth muscle [15–17].

Airway smooth muscle relaxation is primarily mediated by β-adrenoceptors, in humans and most other mammals their β_2-subtype [3,18]. This relaxation provides a physiological antagonism of the contraction induced by mediators such as carbachol and histamine. However, there is a disparity between contractile agonists in their ability to attenuate β_2-adrenoceptor-mediated relaxation, even when matched for initial extent of contraction. For instance, the inhibitory potency of isoprenaline (pEC_{50}) to cause relaxation in canine airways was 8.0 against histamine but only 7.0 against acetylcholine; even 100 μM isoprenaline did not fully reverse acetylcholine-induced contraction [19]. The relative resistance of muscarinic contraction to β_2-adrenoceptor-induced relaxation was confirmed in human airway preparations [20–22]. Whether the resistance to β_2-adrenoceptor-mediated relaxation was caused by activation of an M_2 or M_3 receptor has not been resolved conclusively [21,23,24] but it may be mediated by PKC [25]. Thus, a privileged interaction exists between β_2-adrenoceptors mediating relaxation and muscarinic receptors mediating contraction, whereby muscarinic receptor-induced contraction is more resistant to β_2-adrenoceptor induced relaxation, than that induced by agonists acting independent of muscarinic receptors. This may explain why combined administration of a muscarinic antagonist and a β_2-adrenoceptor agonist causes greater airway relaxation than monotherapy [26–30]. Moreover, while a long-acting muscarinic antagonist had no significant effect by itself, it enhanced the ability of a long-acting β_2-agonist to antagonize histamine-induced bronchoconstriction [31]. Moreover, in some of these studies combination treatment not only reduced elevated smooth muscle tone but also had greater anti-inflammatory effects than monotherapy.

Bladder studies

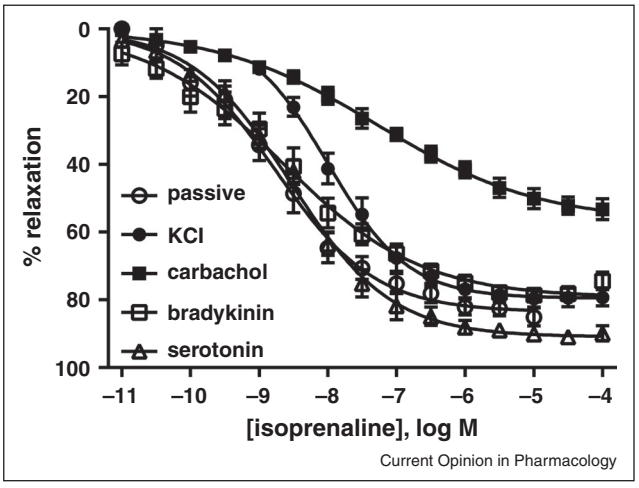
Muscarinic receptors are the primary mediator of urinary bladder contraction during physiological voiding but, in contrast to humans, non-cholinergic mediators can significantly contribute to bladder contraction in the healthy bladder of various animal species [32]. However, in both animals and humans, non-cholinergic mediators such as ATP or bradykinin become increasingly important under

pathological conditions [33–35]. Despite the much greater expression of M_2 than M_3 receptors in the bladder of humans and most other mammalian species (see 'Receptor expression patterns in airways and bladder' section), the direct contractile effects of muscarinic agonists is mediated primarily if not exclusively by the minor population of M_3 receptors [36]. The primary mediator of bladder relaxation is β-adrenoceptors; in humans this occurs primarily if not exclusively via the β_3-subtype, but in other species, for example, rats, additional subtypes may be involved [37]. However, it should be noted that the tone of detrusor smooth muscle is not only regulated directly by autonomic receptors expressed by these cells but also indirectly via muscarinic and β-adrenergic receptors located on the urothelium and the afferent nerve endings [38••].

In porcine bladder and urethra the presence of isoprenaline reduced the E_{max} and pEC_{50} of carbachol-induced contraction [39,40]. In a follow-up study from the same group isoprenaline caused parallel right-ward shifts of the carbachol concentration–response curve but did not affect maximum contraction; in urothelium-denuded bladder strips isoprenaline caused greater right-ward shifts than in the presence of urothelium, indicating that the β-adrenoceptor agonist may in part act on the urothelium [41]. In murine bladder isoprenaline reduced the potency and efficacy of contractions by the muscarinic agonist oxotremorine [24]. However, in M_3 receptor knock-out mice oxotremorine elicited only a small contractile response, which was considerably enhanced in the presence of α,β-methylene ATP and isoprenaline, an effect not observed in M_2/M_3 double knock-out mice [42].

The opposite experiment, that is, testing effects of a muscarinic agonist on bladder relaxation by a β-adrenoceptor agonist, was largely performed in rats, a species where relaxation involves not only β_3-adrenoceptors but also other subtypes [37]. Isoprenaline-induced relaxation of rat bladder strips was less potent and less efficacious against tone induced by carbachol than that induced by KCl (pEC_{50} 5.32 vs. 7.24, remaining tone 35% vs. full relaxation) [43]. In another rat study relaxant responses to isoprenaline were significantly less potent and less efficacious against carbachol than against passive tension, KCL, bradykinin or serotonin [44] (Figure 1). In a follow-up study from the same group it was found that both M_2 and M_3 receptors contributed to the attenuation of the isoprenaline response by muscarinic agonists [45•]. Other follow-up work from these investigators reported that relaxation responses to the β_3-selective agonist KUC-7322 were also weaker against carbachol than against the other responses (Cernecka, Sand and Michel; unpublished observation). Another β_3-selective agonist, TRK-380, was less efficacious against carbachol than against KCl in human detrusor strips [46]. Similarly, the phosphodiesterase inhibitor papaverine was less potent in

Figure 1



Relaxation of rat bladder strips with passive tension or precontracted with KCl, carbachol, bradykinin or serotonin by the β-adrenoceptor agonist isoprenaline. Note that both the potency and the efficacy of isoprenaline against carbachol were significantly smaller than against all other conditions.
Taken from [44].

causing relaxation against carbachol- than against KCl-induced tone in guinea pig [47], rat [48] and human bladder [49]. Similarly, isoprenaline-induced relaxation was enhanced in M_2 receptor knock-out mice [24,42]. However, some conflicting data have been reported as relaxation by a single high isoprenaline concentration was similarly effective against KCl and carbachol-induced contraction in canine bladder [50].

In conclusion most bladder data indicate that muscarinic receptor agonists inhibit relaxation by β-adrenoceptor agonists more than contractile stimuli acting independent of muscarinic receptors. A stronger inhibition of β-adrenoceptor responses by muscarinic agonists than by other contractile stimuli has also been reported in esophagus [51], ileum [24,52], colon [53] and the iris sphincter [54]. These findings support the concept of a privileged interaction between muscarinic and β-adrenergic pathways in control of bladder smooth muscle tone and support the combined use of a muscarinic antagonist and β-adrenoceptor agonist also in the bladder. In support of this hypothesis the potency and efficacy of relaxant effects of the β_3-selective agonists CL 316,243, mirabegron and solabegron in rat bladder against field stimulation was enhanced in the presence of muscarinic receptor antagonists [55].

Receptor expression patterns in airways and bladder

The expression pattern of subtypes of muscarinic and β-adrenergic receptors in airways and bladder has been studied at the mRNA and protein level. While mRNA

detection techniques are unequivocal, their predictive value for corresponding functional receptor protein remains uncertain. Expression at the protein level can be assessed using antibodies in immunoblot or immunohistochemistry studies, but most available receptor antibodies lack suitable specificity [56]. It can also be tested using radioligands in tissue homogenates or autoradiography; while this works well for β_2-adrenoceptors, radioligands for β_3-adrenoceptors are just emerging [57] and those for muscarinic receptors typically lack subtype-selectivity. Despite these limitations, the combined mRNA, protein and functional data allow a reasonably clear picture on the expression of these receptors in airways and bladder. Of note, expression in nerve terminals is typically not detected in most studies as they represent only a minor fraction of the overall expression.

Airway studies

Muscarinic receptors are unevenly distributed in the lung, exhibiting a greater expression in submucosal glands and airway ganglia than in airway smooth muscle [58]. The receptor present on smooth muscle from both large and small airways was described as being entirely of the M_3 subtype in early studies, while the M_1 receptor was exclusively expressed in alveolar walls [9]. Recent studies in human lung found the M_3 receptor more abundantly expressed in segmental than subsegmental bronchus and entirely absent in the parenchyma, whereas the M_2 subtype was widely distributed throughout the lung, and M_1 was found only in parenchyma [59•].

Expression of lung β-adrenoceptors was also reported to be higher in epithelium, alveolar walls and submucosal glands than in airway and vascular smooth muscle [60]. The subtype responsible for labeling airway smooth muscle was entirely β_2, whereas co-expression of both β_1 and β_2 was observed in bronchial submucosal glands and alveolar walls, with the β_1 subtype dominating, as also confirmed in human lung [59•]. Interestingly, the expression level of β_2 increased along the airways, with levels being lowest in the segmental bronchus and highest in the parenchyma [59•,60]. Thus, the relative roles of muscarinic and β-adrenergic receptors appear to differ, with the former more prominent in the more proximal and the latter in the more distal airway segments. Therefore, maximal bronchodilation in all regions of the human lung may require a combination of a muscarinic antagonist and a β_2-adrenoceptor agonist. Regulation of receptor expression in animal models of [61] and patients with obstructive airway disease [62•] may contribute to the pathophysiology and treatment responses and may additionally support the use of such combination treatment.

Bladder studies

Studies in whole human bladder have largely detected mRNA for M_2, M_3 and M_4 receptors and much less M_1 expression [63]. This apparently applies similarly to smooth muscle [64] and urothelial cells [65,66]. Radioligand binding studies confirm that muscarinic receptors in the bladder of humans and animals largely belong to the M_2 subtype, with a smaller contribution of M_3 and even smaller one of other subtypes [67–69].

Studies in whole human bladder have reported that β_3-adrenoceptors contribute about 95% of total β-adrenoceptor mRNA [70], whereas other subtypes may be more prominently expressed in experimental animal species such as rats [71]. Moreover, the relative contribution of β-adrenoceptor subtypes at the mRNA level may be different in human urothelium [66]. The relative contribution of subtypes to total bladder β-adrenoceptor expression at the protein level has been more difficult to determine due to a lack of suitable radioligands or antibodies [72], but some radioligand binding studies have suggested that mostly β_3-adrenoceptors may be present [73]. On the basis of more recently emerging antibody validation data [74•], the presence of β_3-adrenoceptors in the human bladder has also been demonstrated by immunohistochemistry, surprisingly showing an apparently greater abundance in urothelium than in smooth muscle [75,76]. Despite these uncertainties, there is overwhelming functional evidence that relaxation of human detrusor smooth muscle occurs predominantly if not exclusively via the β_3-subtype, but in other species such as rats additional subtypes may contribute [37].

Prejunctional modulation of transmitter release

Smooth muscle tone is regulated by both the parasympathetic and sympathetic nervous systems but the exact contribution of each of these systems in maintaining tone in physiology and disease is unclear in airways [77••] and bladder. Transmitter release from parasympathetic and sympathetic nerve endings can be modulated by prejunctional auto- and hetero-receptors, with M_2 (and perhaps M_4) receptors typically inhibiting transmitter release from both types of nerve terminals and M_1 muscarinic and β_2-adrenergic receptors facilitating it [78]. Thus, prejunctional receptors provide an additional level for an interaction between the two systems. Because of sparse sympathetic innervation there has been limited attention to modulation of noradrenaline release in airways [79] or bladder [80], but several studies have explored the modulation of neuronal acetylcholine release. As in many other tissues, acetylcholine release can also come from non-neuronal sources in airways [81••] and bladder [82]. While such non-neuronal release is considered important, particularly in disease, little is known about its regulation by muscarinic or β-adrenergic receptors; hence it will not be discussed here.

In the airways direct assessment of β-adrenoceptor effects on acetylcholine release has yielded conflicting results. The facilitation of transmitter release by

autoreceptors on sympathetic nerves was also demonstrated for the heteroreceptors on parasympathetic nerves in equine [83,84] and guinea pig airways [85]; in one of these studies, however, such facilitation was only detectable when inhibitory muscarinic autoreceptors were blocked [84]. In contrast, inhibition of acetylcholine release by β-adrenoceptor agonists was observed in rat and guinea pig [86] and in bovine airways [87]. Except for the inhibition in rat (apparently β_1-adrenoceptor-mediated), all facilitating and inhibitory effects on acetylcholine release were β_2-mediated.

Indirect evidence in this regard comes from studies in which the inhibition of airway contraction induced by either electrical field stimulation of exogenously applied acetylcholine was compared. Isoprenaline and several β_2-adrenoceptor agonists inhibited the response to field stimulation more potently and/or effectively than that to acetylcholine in equine [83,84] and human airways [88,89]. On the other hand, isoprenaline was similarly potent against both contractile stimuli in guinea pig trachea [85]. Interestingly, the inhibition of acetylcholine release may involve not only cAMP but also BK_{Ca} [87]. Thus, the functional role of prejunctional β-adrenoceptors on parasympathetic nerves in the airways has not yet been fully resolved. Species differences are possible but technical differences in the preparations being employed may also have contributed, particularly blockade of muscarinic autoreceptors or presence of functional epithelium [85,87]. Nevertheless, it has been argued that both the facilitatory and the inhibitory effect would be in favor of combining a muscarinic antagonist and a β_2-agonist [90]. If it is facilitatory, the muscarinic antagonist will overcome the mitigation of direct smooth muscle effects of β-agonist; if it is inhibitory, the combined effect at the smooth muscle effect will be stronger than either agent alone.

Studies in the bladder have not focused on β-adrenoceptors but rather on muscarinic autoreceptors regulating acetylcholine release. Irrespective of the use of direct measurements of acetylcholine release or of modulation of contraction induced by field stimulation, these studies have unequivocally demonstrated a role for facilitatory M_1 receptors and inhibitory M_2 and M_4 receptors in rat [63,91–93], mouse [42], rabbit [94] and human bladder [95]. As the non-subtype-selective atropine enhanced acetylcholine release in several of those studies, the net effect of the various muscarinic autoreceptors appears to be inhibitory. This may limit the usefulness of a muscarinic antagonist (unless it has very low M_1 affinity) and further supports the concept of combination treatment with a β-adrenoceptor agonist.

Intra-cellular signaling cross-talk

The prototypical primary signaling pathway of M_2 and M_3 muscarinic receptors is inhibition of adenylyl cyclase and stimulation of phospholipase C (PLC), respectively, the latter leading to formation of inositol phosphates and diacylglycerol, which in turn mobilize Ca^{2+} from intracellular stores and activate protein kinase C (PKC), respectively [96]. Additionally, coupling to a phospholipase D and, as a downstream event of all of the above, myosin light chain phosphorylation and activation of rho kinase have been demonstrated. Given the role of Ca^{2+} in initiating smooth muscle contraction, it seems plausible that the PLC activation is the molecular basis of muscarinic receptor mediated smooth muscle contraction in airways and bladder, but this view has been challenged.

The prototypical signaling pathway of all β-adrenoceptor subtypes is stimulation of adenylyl cyclase leading to formation of cAMP, which can activate protein kinase A (PKA) [97]. More recently it became clear that cAMP may alternatively also activate the exchange protein activated by cAMP (Epac) pathway [98••]. While various cAMP-elevating agents such as the direct adenylyl cyclase activator forskolin or phosphodiesterase inhibitors can induce airway and bladder relaxation, many studies have questioned whether cAMP formation indeed underlies relaxation induced by β-adrenoceptor agonists. Moreover, β-adrenoceptors can couple to activation of several potassium channels, mostly large conductance, Ca^{2+}-activated channels (BK_{Ca}). An overview on the signal transduction pathways of muscarinic and β-adrenergic receptors in smooth muscle cells is shown in Figure 2.

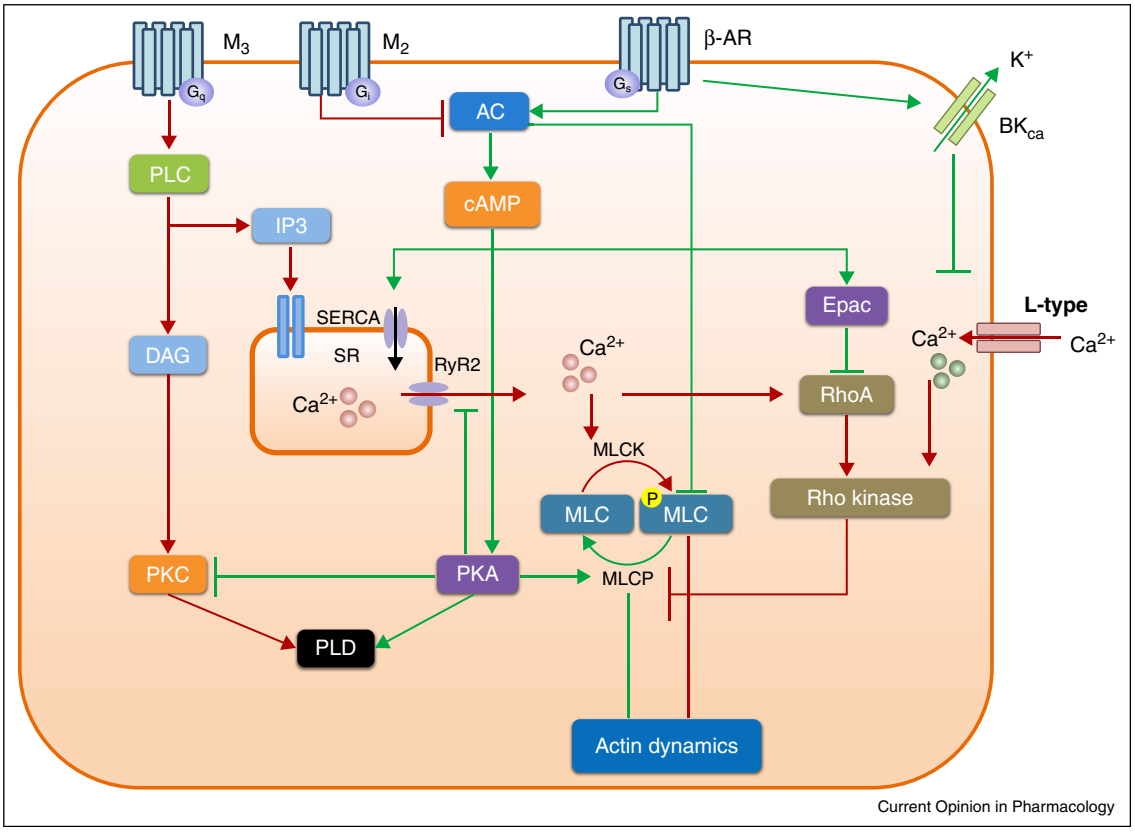
Airway studies

While the involvement of PLC and PKC in muscarinic receptor-mediated airway contraction is plausible, there is only little experimental proof. However, in its support PKC inhibition enhanced the ability of methacholine to contract bovine trachea [25].

β-Adrenoceptor agonist-induced smooth muscle relaxation in airways involves activation of potassium channels, mostly BK_{Ca} channels [99]. Activation of such channels and relaxation may involve partly cAMP/PKA-dependent and partly cAMP-independent pathways in airways [100,101], possibly involving direct coupling of β-adrenoceptor-activated $G_{s\alpha}$ to BK_{Ca} [100].

Several studies have explored how β-adrenoceptor activation affects contraction-relevant signaling by muscarinic receptors in the airways (corresponding bladder data are largely lacking). Whether β-adrenoceptor agonists and other cAMP-elevating or mimicking agents suppress muscarinic receptor-mediated inositol phosphate formation has remained controversial. Lack of inhibition was reported by some investigators in canine [102] or bovine tracheal smooth muscle [15,103], but inhibition was observed in porcine [104] and canine tracheal smooth muscle by others [105,106]; interestingly, the inhibition at the 24 hours time point in the dog study was abolished by the protein synthesis inhibitor cycloheximide.

Figure 2



Schematic representation of assumed signal transduction pathways involved in the regulation of smooth muscle contraction by muscarinic and β-adrenergic pathways. AC, adenylyl cyclase; AR, adrenoceptor; DAG, diacylglycerol; IP3, inositol-tris-phosphate; MLC, myosin light chain; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; SR, sarcoplasmic reticulum. Red and green lines and arrows represent pathways activated by muscarinic and β-adrenergic receptors, respectively.

On the other hand, inhibition of muscarinic agonist-induced intracellular Ca^{2+} elevation in airway smooth muscle by β-adrenoceptor agonists or other cAMP-related agents was consistently observed in bovine [103,107], porcine [108], murine [109] and canine preparations [105,110], although it was reported to wane over time in the latter [106]. Several mechanisms have been proposed how β-adrenoceptor agonists may attenuate Ca^{2+} elevations: firstly, cAMP/PKA-mediated inhibition of L-type Ca^{2+} channels [111]; secondly, reductions of Ca^{2+} oscillations [108,112], which have been linked to reducing Ca^{2+} release from internal stores under control of inositol phosphate receptors [109]; thirdly, activation of the sarcoplasmatic reticulum Ca-ATPase (SERCA) [18]; fourthly, reduction of the detectable number of inositol-1,4,5-trisphosphate binding sites [113]. Moreover, β-adrenoceptor stimulation apparently reduces not only Ca^{2+} elevations but also the Ca^{2+} sensitization of contractile filaments induced by muscarinic agonists [114] or histamine [112]. On the other hand, in contrast to most other cell types, β-adrenoceptor agonists not only suppress Ca^{2+} elevations or lower basal Ca^{2+} concentrations [115] but at least in some cases can also increase it in airway smooth muscle cells [107] and this effect may differ between subcellular compartments [116]. Similarly, they can both activate phospholipase D in porcine tracheal smooth muscle and inhibit such activation caused by muscarinic stimulation [104]. However, it remains difficult to understand how β-adrenoceptor-mediated Ca^{2+} elevations or phospholipase D activation can be related to smooth muscle relaxation, unless they are restricted to subcellular compartments not linked to the contractile machinery.

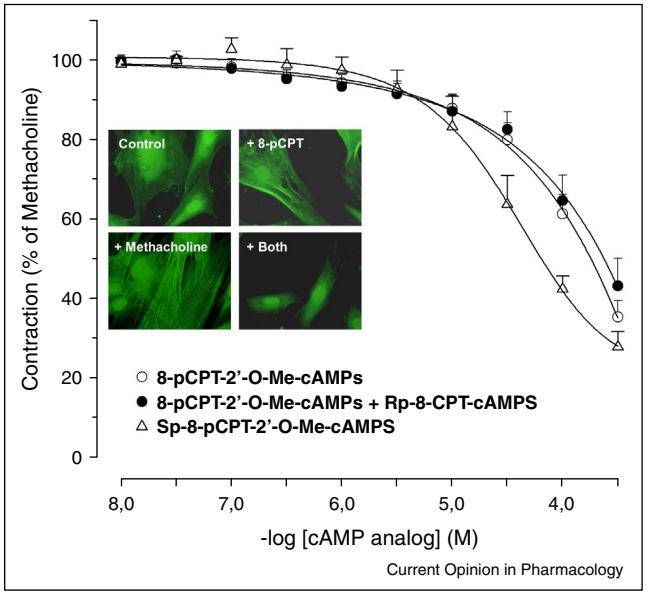
Other studies have explored how muscarinic receptor activation affects relaxation-relevant signaling by β-adrenoceptors. Although direct contractile effects of muscarinic stimulation occur almost exclusively via the M_3 subtype, attenuation of relaxation involves both M_2 and M_3 receptors based on knock-out mouse data [24]. Muscarinic receptor-mediated inhibition of cAMP accumulation is a bona fide M_2 response and well documented in airway smooth muscle [117–119]. Additional evidence comes from experiments in bovine airway

smooth muscle where isoprenaline or the cAMP-mimetic 8-bromo-cAMP lowered basal Ca^{2+} concentration; carbachol abolished such lowering but did not affect Ca^{2+} lowering by release of caged cAMP, indirectly indicating that this interaction occurred through inhibition of adenylyl cyclase by muscarinic receptors [115]. While an obvious explanation for adenylyl cyclase inhibition is an effect mediated by M_2 receptors acting via G_i, M_3 receptors may also be involved. Elevation of Ca^{2+} inhibited isoprenaline-stimulated adenylyl cyclase in human bronchial smooth muscle cells, apparently acting on the cyclase isoform AC6 which was also shown to colocalize with β_2-adrenoceptors [120].

Although K^+ channels, specifically BK_{Ca} critically contribute to β-adrenoceptor-mediated airway smooth muscle relaxation, muscarinic modulation of such activation has received only limited attention. While it would be expected that BK_{Ca} inhibition if anything should enhance smooth muscle contractility, the opposite was found in BK_{Ca} knock-out carbachol-contracted murine airways [121]. Concomitantly, relaxation responses to isoprenaline were enhanced. This paradoxical effect reduction of muscarinic and enhancement of β-adrenergic responses in BK_{Ca} knock-out mice was explained by a compensatory upregulation of the cGMP pathway.

Some studies have explored how muscarinic and β-adrenergic pathways interact at the level of the contractile machinery. In an early study in canine trachea it was found that forskolin raised cAMP levels and myosin light chain kinase phosphorylation but lowered myosin phosphorylation; in contrast, methacholine caused myosin phosphorylation but did not significantly affect cAMP content or myosin light chain kinase phosphorylation; when forskolin was added to methacholine, relaxation occurred which was accompanied by a lowered cAMP content, some reduction of myosin phosphorylation but no change in myosin light chain kinase phosphorylation [119]. Myosin light chain phosphatase activity was increased by isoprenaline in bovine tracheal smooth muscle, whereas carbachol lowered basal and isoprenaline-stimulated phosphorylation [18]. Activation of rho and rho kinase may link the proximal signaling of muscarinic receptors to changes in myosin light chain kinase activity. The carbachol-induced activation of rho and rho kinase in bovine trachea was not affected by pretreatment with isoprenaline or salmeterol, but adding the β-adrenoceptor agonist after carbachol reduced activities of rho, rho kinase, myosin light chain kinase and also reduced contractile tone [122]; these findings were interpreted as indication that some interaction between the muscarinic and β-adrenoceptor pathways can occur at the rho and rho kinase level, but the major part may occur at the myosin light chain kinase level. Experiments in guinea pig and human airways demonstrated that cAMP may cause relaxation of methacholine-contracted airways not only via the PKA but also via the Epac pathway [123•] (Figure 3). Epac activation reduced methacholine-induced rho A activation and Rac1 inhibition and also myosin light chain phosphorylation.

Figure 3



Epac as a novel effector of airway smooth muscle relaxation. Cumulative concentration response curves of the selective Epac activators 8-pCPT-2'-*O*-Me-cAMP (8-pCPT) and Sp-8-pCPT-2′-*O*-Me-cAMPS on methacholine (0.3 μM) precontracted guinea pig tracheal open ring preparations in the absence (control) or presence of 100 μM of the selective protein kinase A inhibitor Rp-8-CPT-cAMPS. Results are means ± SEM of 3-8 independent experiments. Stress fiber formation was measured by phalloidin staining in guinea pig airway smooth muscle. Results are expressed as percentage of stress fiber-positive cells relative to the total number of cells. Representative images of 5 experiments are shown. These data demonstrate that cAMP generated upon β-adrenoceptor stimulation may relax airway smooth muscle via the Epac pathway.
Taken from [123•].

Bladder studies

The muscarinic receptor subtypes involved in attenuation of β-adrenoceptor-mediated bladder relaxation have been studied based on pharmacological inhibitors [45•] and muscarinic subtype knock-out mice [24,42]. Both approaches have shown that, similar to airways, direct contractile effects of muscarinic stimulation occur almost exclusively via the M_3 subtype, but attenuation of relaxation involves both M_2 and M_3 receptors. The M_3 component of such attenuation was blocked by inhibition of PLC or PKC [45•], both of which had not attenuated M_3-mediated direct contractile responses in the bladder [124].

Surprisingly, multiple studies in rat, mouse and human bladder have demonstrated that muscarinic agonists induce contraction largely independent of PLC and rather rely on the opening of L-type Ca^{2+}-channels and

the activation of rho kinase, indicating that influx of extracellular Ca^{2+} through such channels and Ca^{2+} sensitization of contractile filaments may be more important than mobilization of Ca^{2+} from intracellular stores [124]. However, it should be noted that muscarinic receptor stimulation can not only directly cause smooth muscle contraction, largely via the M_3 subtype, but can also attenuate β-adrenoceptor-mediated relaxation, at least partly via the M_2 subtype, and that the latter may involve at least partly distinct signaling pathways.

Although β-adrenoceptor agonists stimulate cAMP formation in the bladder, cAMP appears to play only a minor if any role in bladder relaxation mediated by these receptors [124]. Whether muscarinic receptors mediate inhibition of cAMP accumulation in the bladder has remained controversial [68,125].

On the other hand, similar to the airways, the β-adrenoceptor agonist-induced smooth muscle relaxation in bladder involves activation of potassium channels, mostly BK_{Ca} channels [124,126]. However, muscarinic modulation of such activation has received only limited attention. In mice with either constitutive or smooth muscle-specific inducible BK_{Ca} knock-out bladder contractions elicited by electrical field stimulation, a response largely mediated by muscarinic receptors, were enhanced [127]. This was accompanied by an enhanced suppression of such contractions by a β-adrenoceptor agonist. Interestingly, this suppression was more pronounced in the inducible than the constitutive knock-out, apparently reflecting reduced L-type Ca^{2+} current density and increased expression of cAMP-dependent protein kinase in the constitutive knock-outs. Collectively, these data demonstrate that muscarinic and β-adrenergic signaling opposes each other at multiple levels of their signaling cascade; however, they also illustrate that the molecular mechanisms underlying such interaction may differ between airways and bladder.

Chronic cross-regulation of receptor expression and desensitization

A key feature of long-term administration of receptor agonists and antagonists is that they may cause desensitization and sensitization, respectively, of their cognate receptors. Perhaps more importantly in the present context, chronic activation of one receptor may also affect the function of a physiologically opposing receptor. Such cross-regulation has extensively been studied in the heart, largely representing M_2 and β_1 subtypes [128•], but due to involvement of different receptors subtypes and physiological differences between cardiomyocytes and smooth muscle cells these cardiac findings have limited applicability to airways and bladder and will not be considered here.

Studies with extended exposure to agonists in airways and bladder have reported both sensitization and attenuation of the opposing pathway. An early study reported that a 28-day treatment of rabbits with albuterol enhanced the *in vitro* contractile response of main bronchi to methacholine [129]; as KCl responses were not altered, these findings already pointed to a specific interaction with the muscarinic receptors and their signaling. Prolonged β-agonist exposure may also sensitize the function of other pro-contractile receptors in the airways, for example, bradykinin or histamine receptors [130,131]. This concept has been further explored using mice which either lacked β_2-adrenoceptors or overexpressed them [132]; the former exhibited a reduced bronchoconstrictor response to methacholine and other agents, whereas the latter had an increased response, and both findings were related to a reduced or enhanced expression of PLC-β1. The intracellular Ca^{2+}-handling protein phospholamban was also identified as a target explaining increased bronchoconstrictor sensitivity upon β_2-adrenoceptor overexpression [133]. Using a similar approach, these investigators also explored consequences of overexpression of the G-protein $G_{i\alpha 2}$, which mediates signals of M_2 muscarinic receptors, or of a peptide inhibitor of this G-protein [134]; as expected, overexpression of $G_{i\alpha 2}$ attenuated bronchodilator responses to β_2-adrenoceptor agonists while inhibition enhanced them. On the other hand, overexpression of $G_{i\alpha 2}$ unexpectedly decreased contractile response to methacholine, whereas its inhibition enhanced them. The former was linked to a reduced PLC and the latter to an increased PKCα expression. A PKC activator was found to enhance agonist-induced desensitization of β_2-adrenoceptor function in bovine airways [135]. Much less data is available for the urinary bladder, but one recent study reported shown that rat bladder β-adrenoceptors can desensitize upon prolonged exposure to some agonists, which is accompanied by a reduced contractile response to carbachol [136•]. Taken together, these data show that chronic activation of one pathway may have effects on the opposing pathway, but the direction of such cross-regulation may differ among experimental models and also from the interaction seen upon acute agonist administration.

Conclusions and clinical implications

The above data demonstrate that the muscarinic and β-adrenergic systems in airways and bladder oppose each other at multiple levels, including mediator release, receptor signal transduction and receptor regulation, all funneling into functional antagonism at the level of smooth muscle tone. While there are distinct differences between airways and bladder in these interactions, both organs have pathologies characterized by too much muscarinic and too little β-adrenergic input. Therefore, the above data support the concept of combining muscarinic receptor antagonists and β-adrenoceptor agonists in obstructive airway disease and OAB. While such combinations have long been part of medical practice for short-acting drugs in obstructive airway disease and are guideline-recommended (www.ginasthma.org), the

combination of long-acting muscarinic antagonists and β-adrenoceptor agonists is currently undergoing clinical investigation [90]. Actually, such combinations may not only have beneficial direct effects on airway smooth muscle tone but also on airway inflammation [137•]. Less evidence for the use of such combinations is available for OAB treatment [55], but some clinical studies have been completed and are awaiting reporting (SYMPHONY study NCT01340027) or are ongoing. In both therapeutic areas additional clinical studies will be required to fully understand the role of combination treatment, particularly with regard to the use of long-acting compounds and long-term treatment outcomes.

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