Chapter 10

Acetylcholine: a novel regulator of airway smooth muscle remodelling?

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Abstract
Increased airway smooth muscle (ASM) mass is a pathological feature that asthma and chronic obstructive pulmonary disease (COPD) have in common. This increase has gained renewed interest in view of recent developments showing that ASM, instead of solely being a contractile partner, is capable of interacting dynamically with its environment, especially under inflammatory conditions. ASM are able to proliferate, to migrate, and to secrete chemokines, cytokines, extracellular matrix proteins and growth factors, and most importantly, to adapt to these functions by changing its phenotype from contractile to proliferative / synthetic. Conversely, switching to a (hyper)contractile phenotype may also occur. A vast number of inflammatory stimuli regulate these functions and exert their effects via excitatory $G_q$ or $G_i$-coupled receptors. Since acetylcholine (ACh) activates muscarinic $M_2$ and $M_3$ receptors in the ASM cell membrane, which are coupled to $G_i$ and $G_q$ proteins, respectively, and since ACh release may be enhanced in airway inflammation, a pathophysiological role of ACh related to the above processes and exceeding contraction could be envisaged. In this review, evidence in favour of this hypothesis, based on recent data that show a role for muscarinic receptors in modulating ASM proliferation, contractility and contractile protein expression is discussed. Based on these findings, we postulate that endogenous ACh contributes to airway remodeling in asthma and COPD.

Introduction
Airway remodelling is a pathological feature observed both in asthma and in chronic obstructive pulmonary disease (COPD). The nature of this airway remodelling is different, however, as is the palette of inflammatory cells that are involved in the pathophysiology of these diseases. Comparative studies have demonstrated a prominent role for CD8$^+$ lymphocytes, neutrophils and macrophages in COPD; asthma on the other hand is best characterised by eosinophilic inflammation and CD4$^+$ lymphocytes [1;2]. Nevertheless, all of the mentioned inflammatory cells are potential sources of growth factors, proteases, cytokines and chemokines that generate structural changes in the airways [3;4]. In COPD, these structural changes include destruction of the lung parenchyma (leading to emphysema), fibrosis, epithelial metaplasia, mucus gland hypertrophy and increases in vascular and airway smooth muscle (ASM) mass [2]. As for COPD, asthma is characterised by mucus gland hypertrophy, subepithelial fibrosis and increases in ASM mass. However, in asthma the epithelium is fragile, the basement membrane is thickened and there is no emphysema. In addition, the increased ASM mass in asthma may be more pronounced in the larger airways, whereas in COPD this smooth muscle thickening occurs more prominently in the small airways [1;2;5].

Despite of differences in the pattern of ASM thickening, the observation that ASM mass is increased in both inflammatory diseases is interesting in view of its putative role in airway hyperreactivity and chronic airways obstruction. In addition, recent findings have shown that ASM is not only involved in contraction, but is also capable of dynamically interacting with its environment, especially in inflammatory conditions.
Thus, ASM cells can proliferate, migrate, secrete substances such as chemokines, cytokines, extracellular matrix proteins and growth factors, and importantly, adapt to these functions by changing its phenotype from contractile to proliferative / synthetic or even hypercontractile [3;4;6-8]. As such, ASM is now considered to play an active role in the regulation of airway remodelling in inflammatory airway diseases. The functions mentioned above are induced by growth factors and inflammatory mediators from the local environment and support the inflammatory response. Interestingly, a vast number of the acute inflammatory mediators (e.g. bradykinin, leukotrienes, histamine) exert their effect through G protein coupled receptors (GPCRs) present in the ASM cell membrane [9]. Since contractile neurotransmitters, including acetylcholine (ACh), also activate GPCRs present in ASM, their regulatory role in the airways is likely to exceed contraction. Nevertheless, the potential role of increased cholinergic activity in airway remodelling in asthma and COPD has thusfar received little attention.

**Acetylcholine release in airway inflammation**

The primary source of ACh in the airways is the vagal nerve. The release of ACh from the vagal nerve is regulated by a variety of prejunctial receptors, including auto-inhibitory muscarinic M\(_2\) receptors [10]. In animal models of allergic airway inflammation and asthma, muscarinic M\(_2\) auto-receptor dysfunction has been found to contribute to exaggerated ACh release from the vagal nerve both in vivo and ex vivo [11-13]. This muscarinic M\(_2\) receptor dysfunction is thought to be mediated by eosinophils that migrate to cholinergic nerves and release major basic protein, which acts as an allosteric muscarinic M\(_2\) receptor antagonist [14-16]. Muscarinic M\(_2\) receptor dysfunction may also be relevant in humans. Thus, muscarinic M\(_2\) autoreceptor function has been reported to be impaired in some, but not all patients with asthma [17;18]. Taken into consideration that muscarinic M\(_2\) autoreceptor function is more prominent in the larger airways [19] and that muscarinic M\(_2\) receptor dysfunction is mediated by eosinophils, this mechanism may be more prominent in asthma when compared to COPD. Indeed, muscarinic M\(_2\) autoreceptors have been reported to be still functional in patients with stable COPD [20], although it should be noted that this does not exclude a dysfunction in acute exacerbations.

In addition to effects on auto-inhibition, eosinophil-derived polycations like major basic protein are known to cause epithelial shedding, exposing sensory nerve endings to the airway lumen [21]. Together with muscarinic M\(_2\) autoreceptor dysfunction, this may lead to increased cholinergic reflex activity in response to inhaled stimuli and contribute to allergen-induced airway hyperreactivity [22]. Afferent sensory nerve endings are also involved in central reflex bronchoconstriction upon stimulation by inflammatory mediators such as histamine, bradykinin, serotonin, adenosine and endothelin [23-25]. Tachykinins (neurokinin A, substance P) that originate from non-myelinated C-fibres are also involved in peripheral reflex mechanisms by enhancing ganglionic cholinergic transmission [25]. Furthermore, substance P can possibly induce major basic protein release from eosinohils, causing M\(_2\) dysfunction as described above [26]. In addition to reduced
M₂ autoreceptor function, inflammation-derived prostanoids including PGD₂, PGF₂α and TxA₂ can augment ACh release from cholinergic nerve endings by prejunctional facilitation [25]. Interestingly, ASM itself also represents a potential source of PGD₂, PGF₂α and TxA₂ [27].

Taken together, the above data indicate that vagal release of ACh during periods of airway inflammation may be increased by various mechanisms. Although the above data suggest an important role for exaggerated ACh release in asthma, anticholinergics are primarily used by patients with COPD, since in contrast to asthma, vagal tone appears to be the only reversible component of airways obstruction in these patients [28;29]. Nevertheless, mechanisms of increased cholinergic activity are thusfar unclear, although it could be envisaged that airway inflammation in COPD augments vagal neurotransmission as well.

Acetylcholine, excreted from non-neuronal tissues has been less well explored. Nevertheless, bronchial epithelial cells, T and B lymphocytes, mast cells, monocytes, granulocytes, alveolar macrophages and ASM cells all contain ACh and/or express its synthesizing enzyme, choline acetyltransferase (ChAT) [30-32]. At present, the role of ACh as an autocrine or paracrine hormone in inflammatory airways diseases has not yet been established. However, patients with atopic dermatitis, a condition often associated with bronchial asthma, express increased levels of ACh in non-neuronal cells in the skin, which may suggest a primed role for non-neuronal ACh in allergic inflammation [32;33].

**Cholinergic signalling in airway smooth muscle**

In order to better understand the established and potential effects of ACh on ASM, insight in the signal transduction that underlies muscarinic receptor activation is essential. ASM expresses both G_i-coupled muscarinic M₂ and G_q-coupled muscarinic M₃ receptors, the former being the predominant population, comprising ~80% of the total muscarinic receptor population [34;35]. G_q-coupled muscarinic M₃ receptors are not present, whereas the presence of G_i-coupled muscarinic M₄ receptors may be species specific. Thus, muscarinic M₄ receptor mRNA and protein have been observed in bronchiolar airway smooth muscle in the rabbit lung, but not in human bronchiolar as well as bronchial smooth muscle [36-38]. Therefore, a selective focus on signalling induced by muscarinic M₂ and M₃ receptors seems appropriate. These receptors are part of complex intracellular signalling networks that allow cross-talk with a variety of signalling cascades, including those primarily activated by growth factors, such as mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI 3-kinase) pathways, relevant for airway remodeling.

G_q-coupled muscarinic M₃ receptors in ASM activate phospholipase C, causing hydrolytic conversion of phosphatidylinositol 4,5-biphosphate (PIP₂) into inositol 1,4,5-trisphosphate (InsP₃) and sn-1,2-diacylglycerol (DAG) [39]. InsP₃ is involved in the mobilization of Ca²⁺ from intracellular stores, which generates a rapid and transient increase in [Ca²⁺]. DAG generated through muscarinic M₃ receptor
activation activates protein kinase C (PKC). Both Ca\textsuperscript{2+} and PKC are involved in the regulation of ASM contraction. Different PKC isozymes exist, most of which being expressed in ASM. The precise functions of these individual isozymes are not fully known, but they may relate to receptor-specific effects [40]. PKC can activate the p42/p44 MAP kinase signalling cascade through direct phosphorylation of the MAP kinase kinase kinase Raf-1 [41]. This PKC-dependent pathway may be involved in muscarinic agonist-induced p42/p44 MAP kinase activation in bovine tracheal smooth muscle (BTSM), as shown by its sensitivity to the PKC inhibitor 2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide (GF109203X; Figure 10.1). Nevertheless, methacholine-induced p42/p44 MAPK activation is not fully inhibited in the presence of GF109203X, which indicates that additional signalling pathways induced by the muscarinic receptor agonist activate the MAP kinase cascade independently of PKC. In this regard, activation of the Ca\textsuperscript{2+}-dependent non-receptor protein tyrosine kinase Pyk2 could play a role, presumably by inducing transactivation of growth factor receptors (receptor tyrosine kinases) [42;43] (Figure 10.2).

![Figure 10.1](image)

**Figure 10.1** Methacholine-induced p42/p44 MAPK activation in BTSM is concentration- and PKC dependent. A: Intact strips were stimulated with increasing concentrations of methacholine (5 min; 37 °C), homogenised and immunoblotted against phosphorylated p42/p44 MAP kinase. Unstimulated strips were used as a control (C). Shown is the densitometric analysis of 4 blots. B: Intact strips were stimulated with methacholine (10 µM) or vehicle for 5 min, after 30 min preincubation with GF109203X (10 µM) or vehicle (C). Subsequently, proteins were separated using electrophoresis and immunoblotted for phosphorylated p42/p44 MAP kinase. Shown is the densitometric analysis of 6 blots. * P<0.05 compared to unstimulated; # P<0.05 compared to the absence of GF109203X.
In addition, p42/p44 MAP kinase activation in response to muscarinic M<sub>2</sub> receptor activation has been reported in canine tracheal smooth muscle [44]. Presumably, this occurs via α<sub>i</sub> mediated activation of Ras [45], or through βγ mediated activation of PI 3-kinase, which can transactivate receptor tyrosine kinases [46-48] (Figure 10.2). PI 3-kinase can also modulate transcriptional regulation through activation of protein kinase B (PKB) [49]. Activation of PI 3-kinase is also achieved by activation of Rho in airway smooth muscle [50]. This could imply the involvement of both muscarinic M<sub>2</sub> and M<sub>3</sub> receptors in the activation of PI 3-kinase, since both receptor subtypes are known to activate the RhoA/Rho-kinase signalling pathway [51]. Therefore, both Rho-dependent, PI 3-kinase-dependent and MAP kinase-dependent pathways may be activated in response to muscarinic agonists in ASM. As elaborated on below, all of these pathways are involved in effects that could underlie airway remodelling, including the regulation of ASM contractility and contractile protein expression, proliferation, secretory function and migration.

**Figure 10.2** Putative mechanisms of activation of p42/p44 MAP kinase, Rho and PI3-kinase by muscarinic M<sub>2</sub> and M<sub>3</sub> receptors in airway smooth muscle. These signalling pathways provide potential mechanisms for muscarinic receptors to cross-talk with growth factor-induced signal transduction, relevant for airway remodelling.

**Cholinergic regulation of airway smooth muscle remodelling**

**Phenotype, contractility and contractile protein expression**

Accommodating the elements that comprise the contractile machinery, has for a long time been considered the prominent function of ASM. This does not imply incapability to self-regulation, however, considering recent findings focusing on plasticity in ASM function under pathophysiological conditions [6;7;52;53]. ASM may be induced to change its phenotype to hypercontractile in response to prolonged growth arrest or in response to insulin (Chapters 2 &3, [54;55]). This hypercontractile phenotype is characterised by more rapid and extensive shortening and by increased expression of contractile and contraction regulatory proteins, such as...
smooth muscle-specific actin, myosin and myosin light chain kinase (MLCK). In addition, muscarinic M3 receptor expression is thought to increase under these conditions, since reconstitution of the contractile phenotype in culture also induces functional re-coupling of muscarinic M3 receptors in canine ASM cells [56]. Conversely, ASM can also switch to a less contractile phenotype, characterised by decreased contractility, decreased contractile protein expression and decreased muscarinic M3 receptor expression (Chapters 2, 6, [7;53]). Switching to a less contractile phenotype generally occurs when airway smooth muscle is stimulated to proliferate in response to growth factors or fetal bovine serum (FBS) and is dependent on p38 and p42/p44 MAP kinase and on PI 3-kinase (Chapters 2 and 4). Thus, the less contractile phenotype is thought to be associated with an increase in proliferative capacity and could as such contribute to the increase in ASM mass, seen in asthma and COPD.

Contractility of ASM preparations obtained from patients suffering from asthma and/or COPD has been reported increased in some [57-60], but not all patients [61-64]. Moreover, isolated cells obtained from asthmatics are hypercontractile [65], yet proliferate faster in culture [66]. Passive sensitization of human airway smooth muscle in vitro is also known to increase contractility [67]. Furthermore, passively sensitized human ASM cells have been found to produce more extracellular matrix proteins when compared to cells obtained from healthy controls and may therefore be considered hypersecretory [68]. These seemingly paradoxical results may be explained by the dynamics of phenotype switching, dependent on the inflammatory conditions in the airways, which can be controlled in vitro, but not in lung tissue obtained from patients.

The effects of ACh on ASM phenotype are complex as muscarinic receptors may both induce and reduce contractility. As described above, muscarinic receptor stimulation activates RhoA and Rho kinase, which may be involved in induction of contractility. Thus, Rho-kinase has been found to be important in maintaining bovine tracheal smooth muscle contractility (Chapter 4) and is known to direct serum response factor to the nucleus, which regulates smooth muscle specific gene expression in airway smooth muscle [69;70]. Indeed, carbachol has been noted to increase smooth muscle specific myosin heavy chain and SM22 protein expression in M3 transfected cultured canine ASM cells through Rho and Rho-kinase dependent pathways [71]. Cholinergic activation of PKC on the other hand has been found to temper carbachol-induced expression of SM22 and myosin in the same cells [72], which implies a role for PKC in reducing contractility, possibly as an auto-inhibitory feedback mechanism.

However, prolonged (8 days) exposure of organ cultured BTSM strips to high concentrations of methacholine results in strongly reduced contractility and contractile protein expression (actin, myosin), which is dependent on muscarinic M3 receptors, but independent of PKC and only partially dependent on p42/p44 MAP kinase and PI 3-kinase (Chapter 6). This does not represent a phenotypic change comparable to that induced by growth factors, however, since the proliferative
capacity of the tissue was not concomitantly increased. Importantly, this also demonstrates that changes in contractility or contractile protein expression do not necessarily have to be interpreted as phenotype ‘switching’. The mechanism responsible for this decreased contractility most probably is the prolonged rise of intracellular Ca\(^{2+}\) (Chapter 6), which is known to negatively regulate contractility in the organ cultured rat tail artery and guinea pig ileum [73-75]. It is not clear how the balance of this inhibitory mechanism and the above described Rho/Rho-kinase dependent stimulatory mechanism relates to cholinergic regulation of contractility in vivo. The phenotypic starting-point may be of critical importance to the outcome, as the highest serum response factor-mediated smooth muscle specific gene transcription is observed in synthetic, not contractile smooth muscle cells [69].

Very recently, we found evidence showing that tracheal smooth muscle contractility and contractile protein expression in lung homogenates has been increased in repeatedly allergen-challenged guinea pigs, which could indicate a role of allergen-induced phenotype-switching in the development of (chronic) airway hyperresponsiveness. Importantly, the increase in contractility and contractile protein expression was reduced by treatment with tiotropium bromide, a long-acting muscarinic receptor antagonist used for the treatment of COPD as well as for asthma (Chapter 9). These results for the first time indicate that endogenous ACh may be involved in allergen-induced airway remodelling in vivo. Further experimentation is required to find out whether the muscarinic contribution to allergen-induced airway remodelling is caused by affecting contractility and/or by inducing increased ASM mass. Also, the effects of tiotropium bromide on airway remodelling in asthma and COPD warrants investigation.

**Airway smooth muscle proliferation**

The increases in ASM mass observed in asthma and COPD could in part be mediated by peptide growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) [76]. These growth factors have all been implicated in airway inflammation as they can be released from inflammatory cells, such as eosinophils and macrophages. In addition, they can be derived from the epithelium, extravasated plasma and the airway smooth muscle itself [3,27]. Mechanistically, these growth factors rely on activation of MAP kinases and PI 3-kinase (and downstream targets) for their proliferative responses [77-80], which can be activated by muscarinic receptor agonists as well (Figure 10.2). Nevertheless, muscarinic receptor stimulation alone is not sufficient to induce an increase in cell proliferation or \(^{3}H\)thymidine uptake in bovine (Chapter 7) and human [81] ASM cells. This may be explained by the incapability of cholinergic agonists to induce prolonged p42/p44 MAP kinase activation, which is required to induce proliferative responses [78,82]. However, muscarinic receptor stimulation has been described to interact with peptide growth factor signalling, causing synergistic induction of mitogenesis in bovine (Chapter 7) and human [81] ASM cells. This potentiation can be quite effective, as combined administration of non-mitogenic concentrations of methacholine and PDGF induce approximately 45 % of the maximal control
response to PDGF. Despite the complex signalling network that may be activated by muscarinic M2 and M3 receptors, this potentiation was found to be mediated solely by muscarinic M3 receptors in bovine tracheal smooth muscle cells (Chapter 7).

Mechanistically, the synergistic induction of mitogenesis by methacholine and PDGF in BTSM could be explained by synergistic activation of p70 S6 kinase but not of p42/p44 MAP kinase, as reported for the combination of carbachol and EGF in human airway smooth muscle cells [81]. Even though PKC activity has been associated with p42/p44 MAP kinase activation (as described above), PKC may still be functionally involved in the observed synergism, however, by activating other pathways. For instance, we have recently demonstrated that activation of Gq coupled bradykinin B2-receptors induces synergistic activation of mitogenesis when combined with EGF, which was dependent on conventional PKC isoforms (Chapter 8). In addition, the G protein-coupled receptor agonist lysophosphatidic acid is synergistic with EGF by activating Rho [83]. Since muscarinic M3 receptors activate both Rho and conventional PKC isoforms, these pathways may be important in muscarinic receptor induced synergism with growth factors. Additional research is therefore needed to clarify the role of these pathways.

Airway smooth muscle secretory function
Airway smooth muscle secretory function has important implications for airway inflammation, as the number of molecules that can be secreted by ASM cells is considerable. As a potential source of pro-inflammatory cytokines (e.g. IL-5, IL-6, IL-13) and chemokines (e.g. eotaxin, IL-8), ASM cells could modulate inflammation in the airways, both directly and indirectly by affecting chemokinesis of inflammatory cells and the mediator production by these cells. In addition, ASM cells can produce inflammatory mediators (mainly prostanoids), growth factors (e.g. PDGF, IGF, bFGF) proteases (e.g. matrix metalloproteinase I) and extracellular matrix proteins (e.g. pro-collagen, fibronectin, laminin) [4;27;84;85]. In turn, these secretory components may have effects on ASM proliferation and phenotype. Extracellular matrix proteins for instance can affect ASM proliferation and contractility. Thus, human ASM cells coated on collagen I or fibronectin exhibit a proliferative phenotype, whereas cells coated on laminin switch to a more contractile phenotype [86]. Thus, ASM may contribute to various aspects of airway remodelling in asthma and COPD by dynamically interacting with its environment through both direct and indirect mechanisms.

Although the majority of studies has focussed on the regulation of ASM secretory function by cytokines (e.g. IL-4, IL-13, TNFα), some have addressed the possibility that these functions can be regulated by GPCR agonists [27]. Bradykinin for instance is capable of inducing IL-6 and IL-8 release from human ASM [87;88]. Importantly, bradykinin-induced IL-6 production by these cells is dependent on the short-lived p42/p44 MAPK activation by bradykinin, which could indicate that other GPCR agonists are capable of inducing IL-6 release as well. Indeed, histamine and endothelin-1 have been reported to induce IL-6 release in human airway smooth muscle cells [89]. Remarkably, cholinergic regulation of ASM secretory function has
Airway smooth muscle migration
Recent studies have demonstrated that ASM cells in culture have the capacity to migrate. By migrating to a more pro-mitogenic environment, for instance to the collagen-rich matrix in the subepithelial region, ASM migration has been postulated to contribute to hyperplasia [91]. Indeed, human ASM cell migration can be stimulated by pro-mitogenic stimuli, such as PDGF and bFGF [92]. However, the G protein-coupled receptor agonist thrombin was without effect in these cells, even though this agonist is a highly effective mitogen. This would imply that GPCR agonists do not affect migration by themselves. Nonetheless, \( \text{LTE}_4 \) can augment PDGF-induced migration of human ASM cells in which PI 3-kinase is the key signalling event [93]. Likewise, ACh could potentially have effects on ASM cell migration, although this has not yet been studied.

Figure 10.3 Proposed mechanisms by which ACh could affect ASM remodelling. ACh has been shown to affect ASM contractility, contractile protein expression, pro-mitogenic signalling and proliferation. In addition, like several other G protein-coupled receptor agonists, ACh could also be involved in ASM cell migration, extracellular matrix protein production and secretion of cytokines and chemokines. Altogether, these effects could contribute to airway remodelling in asthma and COPD.
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Concluding remarks
Muscarinic receptor antagonists such as ipratropium bromide and tiotropium bromide are often used for the treatment of COPD and represent an important co-treatment in severe asthmatics [94]. They are used as bronchodilators and are generally not considered to have beneficial effects on airway remodelling. Nevertheless, there is evidence that prolonged treatment with these anticholinergics may improve lung function in patients with COPD [95;96]. Although no direct evidence exists to suggest that these effects are due to improvement of airway remodelling, these studies are particularly interesting in view of the recently discovered effects of ACh on ASM remodelling. Thus, prolonged stimulation of muscarinic receptors on ASM may affect contractility, contractile protein expression, pro-mitogenic signalling and proliferation. In addition, other effects of ACh on ASM, including regulation of secretory function and migration, may be envisaged (Figure 10.3). Since prolonged neuronal and non-neuronal release of ACh may be induced by several inflammatory processes as observed in asthma and COPD, a role for ACh in airway remodelling could be postulated, a contention confirmed by recent observations using tiotropium bromide inhalations that muscarinic receptor signalling is involved in airway remodelling in allergen challenged guinea pigs.

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References


