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in airway remodeling

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Chapter 1

General Introduction

Airway smooth muscle and asthma

Asthma is an inflammatory airway disease characterised by exaggerated bronchoconstriction to neurotransmitters, inflammatory mediators and inhaled contractile stimuli [1-5]. Stimuli that are bronchoconstrictive for asthmatics, may even be hardly effective or ineffective in healthy individuals [6-8]. This airway hyperresponsiveness may in part be explained by increased shortening of the airway smooth muscle (ASM) layer in the airway wall, caused by the presence of inflammatory mediators that augment ASM contraction to other contractile agonists [4]. These inflammatory mediators can be released in the airways following the recruitment of inflammatory cells, but can also be released from structural cells, including ASM itself, providing a mechanism to adapt acutely to the pro-inflammatory environment.

Asthma is, however, chronic of nature. Chronically inflamed airways are subjective to structural changes (airway remodeling), which are thought to play an important role in the development of chronic airway hyperreactivity and decline of lung function. These include thickening of the basement membrane, subepithelial fibrosis, epithelial damage, increases in ASM mass, bronchial microvascular remodeling and mucus gland hypertrophy [9;10] (Figure 1.1).

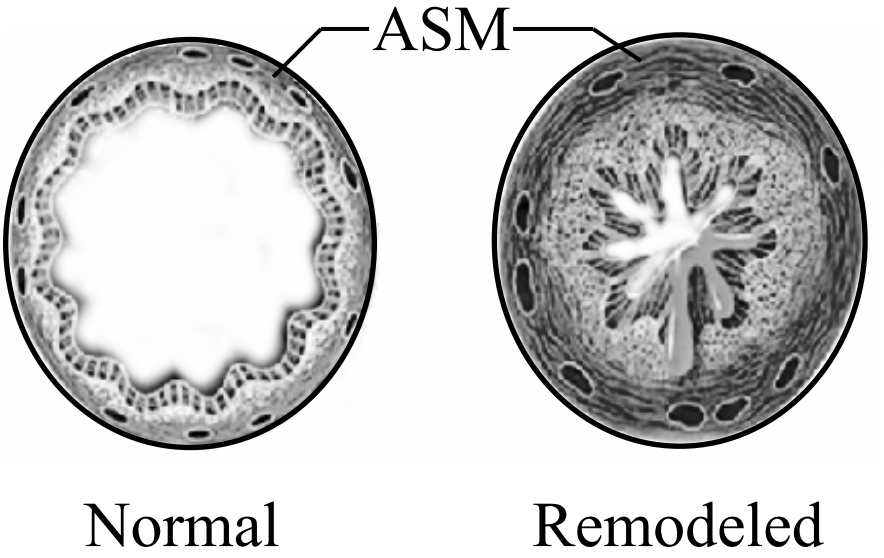

Figure 1.1 *Schematical cross-sections of a normal and a remodeled airway. Shown are increases in ASM mass, fibrosis of the subepithelial layer, mucus hyperplasia and bronchial microvascular remodeling, all characteristic for airway remodeling in asthma. (after Jeffery, [1]).*

Due to airway remodeling, the elastic forces of the tissue surrounding the airway are diminished through uncoupling of ASM from its parenchymal recoil, which may contribute to exaggerated constriction [9;11]. In contrast, fibrosis of the subepithelial layer may stiffen the airway and protect against excessive airway narrowing [9]. The benefits of thickening of the airway wall are limited, however, as this will result in changes in airway diameter, sufficient to limit airway capacity to the extent seen in asthmatics [12]. In addition, myofibroblasts have been observed in the subepithelial layer in patients with chronic and severe asthma [13], which may contribute to constriction of the airway as a whole. The deposition of extracellular matrix proteins can be mediated by subepithelial fibroblasts, but recent reports suggest that ASM cells are also capable of producing matrix proteins such as fibronectin and collagen [14-16]. It is not completely clear whether matrix protein production by ASM represents a significant contribution to the subepithelial fibrosis seen in asthmatics. Nevertheless, the composition of matrix proteins in which the ASM is embedded may have a major impact on its contractile function [17;18].

Increased ASM mass may increase the force produced by bronchoconstrictor agents and therefore increase airway responsiveness to these stimuli. The physical obstruction of inward growing tissue may also contribute to changes in airway reactivity. Importantly, these changes in airway structure worsen with duration of disease, which could add to disease-induced chronic increase in severity of airway narrowing [19]. Studies in animal models of asthma have shown that the increase in ASM mass is most prominent in the larger airways and declines progressively towards the periphery [20]. Increased ASM mass may be explained in part by increases in cell number (hyperplasia), as confirmed in studies that determined changes in ASM cell number after repeated allergen challenge [21;22]. In asthmatics, however, both hyperplasia and increases in cell size (hypertrophy) have been noted [23]. The increase in ASM mass caused by either hypertrophy or hyperplasia is considered sufficient to comprise a major cause of exaggerated airway narrowing [24;25]. Thus, the increase in ASM in the central airways of allergen challenged rats was found to correlate with the increase in hyperreactivity to methacholine [20]. Although this would suggest a central role for ASM growth in chronic airway hyperreactivity, others have shown that the time-profile of the progressive increase in airway hyperreactivity does not match the time-profile of the increase in ASM mass [26]. It should also be noted that these studies do not take into account that ASM can adapt its phenotype, which may superimpose on changes in ASM mass.

Airway smooth muscle phenotype

Accommodating the elements that comprise the contractile machinery, has for a long time been considered the prominent function of ASM. However, recent findings have focused on plasticity in ASM function under pathophysiological conditions [27-30]. By changing its phenotype to hypercontractile, ASM shortens more rapidly [31;32], which may result in exaggerated airway narrowing [33]. *In vitro*, this

(hyper)contractile phenotype can be induced by growth arrest and is characterised by increases in contractile protein expression, such as smooth muscle-specific actin and myosin [34]. Also, muscarinic M_3 receptor expression and contraction regulatory protein expression (e.g. myosin light chain kinase, calponin) are known to increase under these conditions [34;35].

Nevertheless, it should be noted that the occurrence of a hypercontractile ASM phenotype in asthma is still subject of debate. In favour, isolated asthmatic ASM cells have been reported to contract more profound and more rapidly *in vitro* [36]. Furthermore, passive sensitization of human bronchi with atopic serum increases maximal contractility and agonist-sensitivity *in vitro* [37]. Interestingly, this effect is associated with serum IgE [38], suggesting a relationship between allergic sensitization and increased contractile responsiveness. Studies using a canine model of allergic sensitization have revealed similar effects after active sensitization [39;40]. This increase in contractility is accompanied by increases in MLCK expression [41], which has been demonstrated in sensitized human ASM as well [42]. However, although some have reported increased contractility or agonist-sensitivity of asthmatic ASM *in vitro*, a vast amount of reports on this subject suggest no major changes in contractility *in vitro* (cf. [43] for review). At the moment, it is therefore not certain whether hypercontractility to some extent is an artefact, caused by extremely favorable culturing conditions, or (patho)physiologically relevant indeed.

Paradoxically, switching to a less contractile phenotype may also be relevant in the pathophysiology of asthma and this may in part explain the controversy that exists about contractility changes in asthmatic ASM *in vitro*. As in other smooth muscle cell types, ASM is able to switch to a less contractile phenotype in a reversible fashion [27;29;44]. Switching to a less contractile phenotype generally occurs when smooth muscle cells, kept in culture, are exposed to high concentrations of fetal bovine serum. Under these conditions, ASM cells reduce their ability to contract due to diminished contractile protein expression [34]. In addition, M_3 receptor expression is strongly reduced [45]. Although a less contractile phenotype may seem favourable in airway diseases such as asthma, it is important to note that smooth muscle cells in culture proliferate faster and growth factor receptor expression is increased [46-49]. Also, organelles involved in synthesis and secretion such as the Golgi apparatus are increased under these conditions [50]. This has led to the hypothesis that smooth muscle cells reversibly switch between a contractile and a proliferative/secretory phenotype [46]. The latter state may contribute to the increase in ASM mass, seen in asthmatics. Indeed, cultured ASM cells obtained from asthmatics proliferate faster in response to the same mitogen and cannot be made quiescent by deprivation of fetal bovine serum [51].

Increases in ASM secretory function have also been postulated to contribute to airway inflammation and airway remodeling as ASM are potent producers of cytokines, chemokines and extracellular matrix proteins [52-56]. Moreover, passively sensitized ASM cells produce more extracellular matrix when compared to cells obtained from healthy controls and can therefore be considered hypersecretory [57].

Furthermore, the profile of extracellular matrix proteins produced by asthmatic ASM cells supports the induction of a hyperproliferative ASM phenotype [58]. Therefore, ASM phenotype switching may contribute to the pathophysiology of asthma by augmenting inflammation and proliferation during periods of allergen exposure and by augmenting contractile responses in the periods in between. This could increase ASM mass and contractile function in a cumulative fashion.

Recently, Moir et al. have shown an increase in ASM mass in bronchioli of repeatedly allergen challenged rats 24 h after the last allergen challenge which was accompanied by a reduction in contractility of the muscle, when corrected for changes in ASM cross-sectional area [59]. Conversely, 35 days after the last allergen challenge this increase in ASM mass was no longer present, whereas corrected ASM contractility was increased. This could indeed indicate switching of proliferative and contractile ASM phenotypes, although it should be noted that the observed contractile protein expression profiles do not completely match this hypothesis. Even 35 days after the last allergen challenge, reductions in contractile protein expression were observed, which is not easily explained. Nevertheless, this study provided evidence for the first time that phenotypic plasticity of ASM relates to airway remodeling *in vivo*.

In view of this potentially important role for phenotypic plasticity in the regulation of ASM function in asthma, insight into the mechanisms that control these processes is warranted. Multiple stimuli have been considered responsible. Mechanical strain for instance is known to increase contractile protein expression in cultured ASM cells and to trigger ASM cells into the cell cycle [60-63]. Strain may also change extracellular matrix composition as both cardiac fibroblasts and rabbit aortic smooth muscle cells produce collagen in response to mechanical forces [64;65]. It is uncertain however what role this strain-induced remodeling plays in the pathophysiology of asthma [63].

An altered composition of the extracellular matrix may cause altered contractile and proliferative characteristics of ASM. This is matrix protein-specific, as some (eg fibronectin, collagen type I) facilitate proliferation of cultured human ASM cells and induce proliferative marker protein expression such as the Ki67 nuclear antigen [17]. In agreement with the induction of a proliferative and less contractile phenotype by these matrix proteins, contractile markers such as calponin, smooth muscle specific myosin heavy chain and actin are reduced. In contrast, laminin as well as matrigel (a solubilized basement membrane matrix) can reduce proliferation of human ASM cells and increase contractile protein expression [17]. Similar results have been obtained with matrigel in vascular smooth muscle cells [66]. In view of the focus of this thesis, the possible role of growth factors and GPCR agonists in the regulation of ASM contractility and proliferation are of specific importance. Their potential role in airway remodeling and the signal transduction mechanisms involved will be dicussed below.

Peptide growth factors

As mentioned above, both ASM proliferation and the induction of a switch to the less contractile phenotype can be stimulated by treatment with fetal bovine serum. In contrast, deprivation of serum induces growth arrest and return to a contractile phenotype or even a hypercontractile phenotype. Peptide growth factors, which bind to receptors with intrinsic tyrosine kinase activity are major constituents of serum and are considered capable of inducing both ASM proliferation and ASM phenotype switching [30]. Furthermore, the >10 kD fraction (suggesting the presence of peptide growth factors) of dialysed broncho-alveolar lavage (BAL) fluid obtained from asthmatics, induces ASM proliferation and activates signaling pathways critcial for proceeding the G1 phase of the cell cycle [67]. For this reason, it has been hypothesized that peptide growth factors are, at least in part, responsible for the increase in ASM mass in airway remodeling in asthma [29;68].

Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-ß (TGF-ß) have all been implicated in asthma based on increased immunoreactivity in lung tissue slices or protein expression in airway biopsies [69-72]. Although mitogenic for endothelial cells, the role of VEGF in ASM remodeling is not well characterised. EGF and bFGF, however, are considered mitogenic for ASM. The role of TGF-ß in ASM mitogenesis is less certain, as it has been associated both with induction and inhibition of ASM proliferation. Rather, TGF-β is generally linked to the production of extracellular matrix (ECM) proteins and fibrosis (Table 1.1).

Interestingly, EGF induces contraction of guinea pig tracheal smooth muscle, presumably through the production of lipid mediators, such as leukotrienes, thromboxane A_2 and prostaglandins [76;100]. Similar actions of EGF and other growth factors have been noted in vascular smooth muscle [101-103]. This suggests the possibility that growth factor-induced smooth muscle contraction is a general physiological event and raises questions regarding the direct contribution of growth factor-induced ASM contraction to allergen induced bronchoconstriction. Despite of being of interest to vascular biologists as early as 1986 [102], growth factor-induced contraction of human ASM still remains to be established.

Insulin-like growth factor-1 (IGF-1) may also be relevant for asthma, although its levels have not been reported to be increased. The bioavailability of IGF-1 is negatively regulated through binding to IGF binding proteins (IGFBPs), the availability of which in turn is negatively regulated by IGFBP proteases. Together, these proteins constitute the IGF-axis [104]. In asthmatic airways, increased levels of the IGFBP protease MMP-1 have been demonstrated, which could increase the bio-availability of IGF-1 [105]. Although effects other than mitogenesis have not been studied in ASM [91;92], IGF-1 has profound effects on migration, phenotypic modulation and ECM production in vascular smooth muscle [104;106;107].

Table 1.1 *Sources and ASM effects of growth factors.*

Growth factor	*Source*	*Effect on ASM*	*References*
EGF	ASM Epithelium Macrophage Plasma	Proliferation Contraction ECM protein production	[54;70;73-77]
VEGF	ASM Epithelium	ECM protein production	[78-80]
bFGF	ASM ECM Macrophage	Proliferation Migration PDGFR expression ↑	[54;73;81-83]
TGF-ß	ASM Fibroblast ECM Macrophage Plasma T cell Eosinophil	Proliferation (↑↓) Hypertrophy ECM protein production Cytokine production (IL-6, IL-8, IL-11, LIF) Growth factor production (VEGF) COX-2 expression ↑ ß-adrenergic responsiveness↓ PDGFR expression ↓	[54;73;81;84-90]
IGF-1	ASM Macrophage Plasma	Proliferation	[73;91;92]
PDGF	ASM Epithelium Macrophage Plasma	Proliferation Phenotypic modulation Migration Cytokine production	[17;54;73;93-96]
CTGF	ASM	ECM protein production	[56;97]
AngII	Plasma	Contraction Hypertrophy Growth factor production (TGF-ß)	[85;98]
Insulin	Plasma	Proliferation	[99]

Platelet-derived growth factor (PDGF) is one of the most effective growth factors for ASM proliferation [92] and its effects are well characterised (Table 1.1). Although BAL fluid levels of PDGF are not increased in asthmatics when compared to healthy controls [108], increased PDGF and PDGF receptor expression have been reported in airway biposies in asthma [109]. In additon, strongly synergistic mitogenic interactions with other peptide growth factors have been reported [99]. The dominant mitogenic signaling induced by PDGF may therefore still be relevant to asthma pathology whether or not expression levels of the growth factor are increased. Although fetal bovine serum is an established stimulus with regard to the induction of ASM phenotype switching, surprisingly, PDGF is the only purified growth factor directly associated with phenotype switching of ASM to a less contractile phenotype [17].

Less well studied growth factors putatively relevant to asthma and other airways diseases include connective tissue growth factor (CTGF), angiotensin II and insulin. Recently, CTGF was found to be expressed in human ASM cells in culture and in human ASM cells in tissue slices in situ. Interestingly, TGF-ß induced a 70-fold increase in CTGF expression in asthmatic human ASM cells, compared to only 3-fold in healthy controls [56;97]. This mechanism may be very relevant to asthma, as CTGF is linked to ECM protein production by ASM cells [56].

Angiotensin II may also be of interest in view of increased plasma levels in acute severe asthma [110]. Moreover, intravenous administration of angiotensin II to mild asthmatics in concentrations similar to those endogenously present in severe asthmatics causes acute bronchoconstriction [98]. Also, antigen-induced airway hyperresponsiveness in guinea pigs has been found in part AT_1 receptor dependent [111]. Angiotensin II can also act as a hypertrophic growth factor in human ASM cells, presumably through the endogenous production of TGF-ß [85].

A potential role for insulin as a mediator in asthma is still under investigation: a lower prevalence of asthma and atopy symptoms in patients with type I diabetes mellitus has been reported in epidemiological studies [112;113] and has been a topic of discussion for many years [114]. Also in animal models of experimental diabetes, allergen-induced airway inflammation and dysfunction of inhibitory neuronal M_2 autoreceptors can be strongly reduced [115-117]. Although this does not prove a direct role for insulin, it is considered mitogenic for ASM [99] and activates signaling pathways also activated by other growth factors [118]. It could therefore be involved in airway remodeling.

Growth factor receptors

Most growth factors couple to and signal through single membrane-spanning receptors with intrinsic tyrosine kinase activity. Upon binding of the receptor with a peptide growth factor, dimerization of receptor subunits occurs, which is required to induce cellular signaling [119]. The intrinsic kinase activity, which is localized

intracellularly, then allows the receptor subunits to cross-phosphorylate each other at tyrosine residues, which is referred to as receptor autophosphorylation. The phosphorylated tyrosine residues act as docking sites for other kinases, such as the non-receptor tyrosine kinase Src and phosphatidyl inositol (PI) 3 kinase [119]. These kinases allow further downstream signaling as described below.

Platelet-derived growth factors signal through a similar, though slightly distinct mechansim. PDGF is not a single growth factor molecule but exists in multiple isoforms, the most studied being the A and B monomers. These form dimers, i.e. PDGF-AA, -AB or –BB. PDGF-AB is the most prevalent dimer and most commonly used in PDGF studies. Both the PDGF-A and -B isoforms are capable of coupling to receptors, which allows a single PDGF molecule to induce PDGF receptor dimerization [120]. The PDGF receptor also exists in two monomeric forms: α and β. The α monomer binds both the PDGF A and B isoforms, whereas the β monomer is selective for PDGF-B [121;122]. This causes PDGF-AB and PDGF-BB to be more effective as mitogens for ASM, when compared to PDGF-AA [123].

Receptors binding insulin and insulin-like growth factors can exist in two disulfide bond-linked single membrane spanning proteins even in the inactive state. Signaling through these receptors is different from the other growth factors, requiring tyrosine phosphorylation of insulin receptor substrate (IRS) proteins [104;118]. Phosphorylated IRS proteins can act as docking sites for non-receptor tyrosine kinases and PI 3-kinase which are involved in downstream signaling events. Although these signaling events seem comparable to those induced by the other growth factor receptors mentioned, cellular signaling is usually growth factor-specific. For instance, insulin treatment increases actin and myosin expression in chick gizzard smooth muscle cells, whereas PDGF decreases their expression [107;124]. It is not clear whether such differential signaling is relevant to ASM.

Growth factors and signal transduction

As mentioned above, receptor tyrosine kinases can activate PI 3-kinase upon docking at tyrosine residues. This is an important signaling mechanism in smooth muscle as it is associated with cell proliferation, differentiation, migration and contraction [94;106;107;125-127]. In ASM, PI 3-kinase is less well studied and, surprisingly enough, associated with proliferation and migration only [94;125;128]. Further studies have indicated that different classes of PI 3-kinase are expressed by ASM (IA, II and III), of which class IA PI 3-kinase may be involved in ASM proliferation [129].

PI 3-kinase phosphorylates phospho-inositides at the 3-position of the inositol ring, which leads to the formation of PI3P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$. The $PI(3,4,5)P_3$ phospholipid appears to act as the most important of these second messengers [130]. These phospholipids can bind to and activate protein kinase B (PKB) either directly [131] or through activation of PIP_3 dependent protein kinase (PDK), which phosphorylates PKB [132]. PKB, in turn, is an upstream inhibitor of glycogen

synthase kinase 3 [133] and an activator of p70 S6 kinase [134]. Both activities are associated with transcriptional activation and protein synthesis leading to proliferation. PI 3-kinase activity may activate transcription and protein synthesis through other mechanisms as well: PI 3-kinase can activate the non-receptor tyrosine kinase Src directly [135], which in turn can activate other signaling pathways activated by receptor tyrosine kinases, such as the mitogen activated protein kinase (MAPK) pathway [136]. This PI 3-kinase mediated activation of the MAPK pathway has been shown to be important in the activation of MAPK by growth factors specifically at weakly mitogenic concentrations [137].

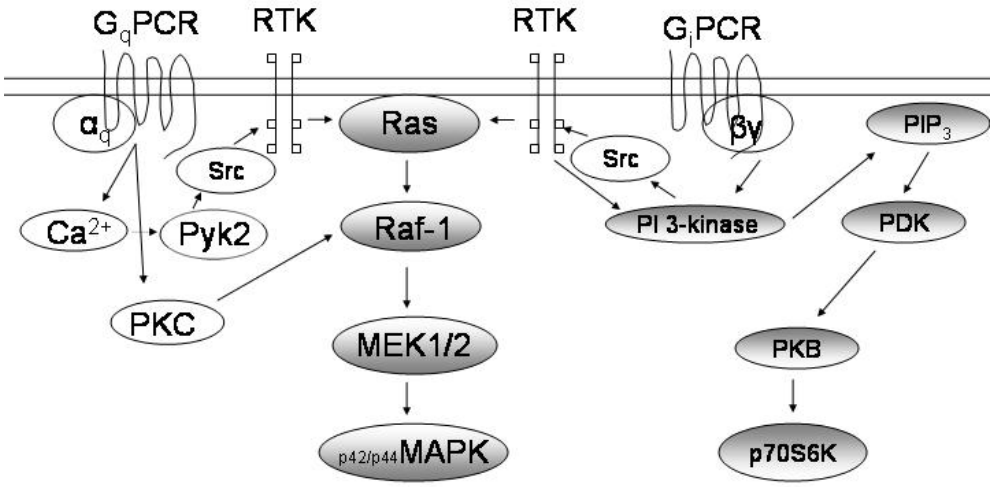

Figure 1.2 *Signal transduction pathways activated by growth factors and interactions with GPCRs. Both RTKs and GPCRs are involved in a variety of signaling cascades, which are linked through complex signaling networks. Shown are pro-mitogenic interactions at the level of p42/p44 MAP kinase, PI 3-kinase and PKB.*

Mitogen activated protein kinases are a superfamily of serine/threonine directed protein kinases involved in transcriptional regulation in response to a variety of extracellular stimuli, including growth factors [68]. Upon activation of receptor tyrosine kinases, an adaptor protein Shc binds phosphorylated tyrosine residues and recruits the nucleotide exchange factor Sos to the membrane which is involved in the activation of Ras, a monomeric G protein [119]. These events are followed by

the activation of a cascade of kinases that result in the activation of p42/p44 MAP kinase, also referred to as extracellular signalling regulated kinase (ERK) 1/2. p42/p44 MAP kinase is well studied and known to be involved in ASM proliferation, migration, cytokine and chemokine-production and contraction [95;138-140]. In addition, studies in vascular smooth muscle have shown its involvement in the regulation of smooth muscle phenotype [141]. Taken together, p42/p44 MAP kinase is considered a key signaling event in the regulation of smooth muscle function.

G protein coupled receptor agonists

Although receptor tyrosine kinases are potently and effectively coupled to signaling pathways involving PI 3-kinase and p42/p44 MAP kinase, G protein coupled receptors (GPCRs) are capable of regulating these pathways as well [142;143] (Figure 1.2). GPCRs are receptors with seven transmembrane spanning peptide chains and couple primarily to heterotrimeric G proteins. The subtype composition of the α, β and γ subunits which are associated to form the heterotrimeric G protein is critically important for the capacity of GPCRs to activate these mitogenic signaling pathways, as well as for the mechanisms involved.

G_s coupled receptors such as the β_2 adrenoceptor and the PGE_2 EP_2 receptor, activate adenylyl cyclase which increases the cytosolic cyclic AMP concentration [144;145]. By activating protein kinase A (PKA), cyclic AMP is capable of inhibiting p42/44 MAP kinase activity through inhibitory phosphorylation of Raf-1 [146]. Gs coupled receptors can also inhibit the expression of cell cycle regulatory proteins such as cyclin D_1, resulting in diminished progression through the cell cycle [147]. On the other hand, cell cycle inhibitory proteins such as $p21^{Cip1}$ and $p27^{Kip1}$ are induced by cAMP [148]. Not surprisingly therefore, β_2 agonists and PGE_2 are anti-mitogenic for ASM [149-151] and can inhibit ASM migration [83]. In addition, cytokine and chemokine production by ASM cells in culture can be inhibited by β_2 agonists and PGE_2 [152-154].

Agonists acting on Gi and Gq coupled receptors on the other hand can favour airway remodeling. Thrombin for instance, which activates both Gi and Gq through activation of protease activated receptors (PARs) is highly mitogenic for ASM, which is dependent on both PI 3-kinase and p42/p44 MAP kinase [94;155]. In addition, thrombin induces ASM cytokine and growth factor production [156;157]. In contrast, muscarinic receptor agonists acting on both G_i coupled M_2 and G_q coupled M_3 receptors are usually ineffective or nearly ineffective as mitogens [99;158]. This is not an exception since GPCR agonists such as histamine, LTD_4, endothelin-1, bradykinin and serotonin have all been described to be at most modestly mitogenic [14;92;159-163]. It is not yet fully clear what causes this discrepancy, since both G_i and G_q are capable of activating PI 3-kinase and p42/p44 MAP kinase. The βγ subunits released by the dissociation of G_i for instance are capable of directly activating PI 3-kinase, which in turn may activate p42/p44 MAP kinase through activation of receptor and non-receptor tyrosine kinases as described above [143] (Figure 1.2). G_q proteins which increase intracellular Ca^{2+} and activate protein kinase

C (PKC) may also activate these pathways either through transactivation of receptor tyrosine kinases by the Ca^{2+} dependent protein tyrosine kinase Pyk2 or through PKC mediated phosphorylation and activation of Raf-1 [164-167] (Figure 1.2).

One possible explanation for why GPCR agonists are generally not mitogenic by themselves is that GPCR-induced PI 3-kinase or p42/p44 MAP kinase signaling is often too weak or too short-lived to induce ASM proliferation [92;155]. In support of this hypothesis, GPCR agonists can be aided by growth factors that act on receptor tyrosine kinases. When combined with EGF for instance, LTD_4, endothelin-1 and histamine are potent mitogens [14;159;162]. The mechanisms involved in this cross-talk, however, are largely unknown. Nevertheless, insight in this cross-talk is warranted since it may have significant pathophysiological implications. When synergistically interacting with growth factors that are increased under inflammatory conditions, GPCR agonists may be important as modulators of the increase in ASM mass in airway remodeling.

In addition to effects on ASM cell number, GPCR-induced effects on ASM phenotype could be envisaged as well. Studies on this subject are not available, however, let alone those describing interactions with growth factors. Isolated components of ASM phenotypic regulation on the other hand have been described, such as the regulation of smooth muscle specific gene expression (e.g. actin, myosin). This regulation appears to be dependent on the activation of the small monomeric G protein Rho, which causes activation and translocation of serum response factor (SRF) to the nucleus [168-170]. SRF acts as a transcription factor for smooth muscle specific genes [171]. Since GPCR agonists are capable of activating Rho and Rho-kinase [171], GPCR agonists may be involved in the regulation of SRF. In addition, Rho and Rho-kinase are involved in contraction, predominantly through calcium-independent mechanisms. Therefore activation of Rho-kinase may be an important regulatory mechanism both in the acute and long-term regulation of contraction.

Aims of the studies

Based on the above mentioned observations and mechanisms, GPCR agonists and peptide growth factors are potentially important in the regulation of airway remodeling. The purpose of this thesis is to gain insight in the regulation of ASM phenotype and proliferation by growth factors, GPCR agonists and combinations hereof, and to investigate the potential contribution of this cross-talk in airway remodeling. For this purpose we used cell culture, organ culture, as well as *ex vivo* approaches. This broad methodological approach allows both the investigation of cellular and molecular biological mechanisms in cell culture as well as the relevance of these mechanisms to more intact physiological systems.

The majority of the studies was conducted on bovine tracheal smooth muscle (BTSM), based on the consideration that human ASM is available occasionally from resection material from patients undergoing surgery for lung carcinoma and only in

very limited amounts. The specific use of BTSM is based on its well characterised physiology, which is representative for human tissue as regards the parameters of interest. Thus, ASM obtained from both species proliferates in response to growth factors, including PDGF, EGF and IGF-1, with similar concentration dependencies and similar mechanisms involved (i.e. p42/p44 MAP kinase and PI 3-kinase; compare [92;94;138] with [99;125;155]), although the magnitude of the proliferative effects may differ between the species, possibly because of subtle signaling differences [92;172]. Also, BTSM expresses GPCRs relevant for human airway physiology, e.g. G_i coupled muscarinic M_2 and G_q coupled muscarinic M_3, histamine H_1 and bradykinin B_2 receptors [4;45;173-177].

The first part of the thesis focusses on the long-term regulation of ASM contractility through changes in smooth muscle phenotype. **Chapter 2** describes the occurrence of these phenotype changes at the level of contractility in intact BTSM. Organ cultured strips were used in this study instead of cultured cells, since cell to cell and cell to matrix interactions are preserved in intact tissue. This may be very relevant as extracellular matrix has been described to regulate ASM phenotype [17]. Moreover, extracellular matrix may influence or even determine the responses of ASM to therapeutic intervention [178]. The organ culture model was evaluated using fetal bovine serum and a panel of purified growth factors to establish a potential relationship between the mitogenic strength of the growth factor and the change in contractility. In **Chapter 3**, these studies are extended to the induction of hypercontractility. Cultured canine tracheal smooth muscle cells are known to become hypercontractile upon prolonged exposure to serum-free medium containing insulin. The induction of hypercontractility by serum deprivation and insulin exposure were therefore compared in our organ culture model.

In view of potential effects of GPCR agonists on ASM phenotype, establishing the role of Rho-kinase in the regulation of contraction, phenotype and proliferation is an important consideration, since phenotypic parameters like actin and myosin have very recently been described to be regulated by Rho/Rho-kinase-dependent pathways. **Chapter 4** addresses the involvement of Rho-kinase in proliferation and phenotype changes both in BTSM cells and organ cultured BTSM strips. In addition, the acute effects of Rho-kinase inhibition on both growth-factor and GPCR agonist induced contraction were measured in human bronchial smooth muscle, the results of which are described in **Chapter 5**. Finally, in **Chapter 6,** the involvement of GPCR agonists in the regulation of phenotype was investigated directly by long-term exposure of organ cultured BTSM strips to methacholine. Both contractility, proliferative capacity and contractile protein expression were used as parameters. The role of G_i coupled M_2 receptors, G_q coupled M_3 receptors and the signaling mechanisms involved (p42/p44 MAP kinase, PI 3-kinase, PKC) were determined using selective inhibitors. In addition, the possible interaction with PDGF-induced phenotype changes were studied.

The second part of the thesis involves the regulation of ASM proliferation by GPCR agonists and growth factors. The effects of the GPCR agonist methacholine and the

peptide growth factor PDGF on proliferation of BTSM cells were studied in **Chapter 7**, with particular focus on their interaction. This interaction was characterised and the role of G_i coupled M_2 receptors and G_q coupled M_3 receptors was determined in this study. The studies described in **Chapter 8** further explore the mechanisms involved in the interaction of GPCR agonists and growth factors, using the GPCR agonist bradykinin and the peptide growth factor EGF. The involvement of p42/p44 MAP kinase was measured using selective inhibitors and measurement of p42/p44 MAP kinase activation by Western analysis using phospho-specific antibodies. In addition, the role of PKC isozymes was studied using subtype selective inhibitors.

Finally, the functional implications of interactions between growth factors and GPCR agonists for ASM phenotype changes and proliferation *in vivo* were studied in **Chapter 9** using a guinea pig model for ongoing allergic asthma. Repeated allergen exposure-induced alterations in phenotype and ASM mass were studied by measurement of contractility, contractile protein expression, increases in cell number as well as morphometric analysis of total ASM mass. In addition, the involvement muscarinic receptors in the development of ASM remodeling was studied by treating the animals with the long-acting muscarinic antagonist tiotropium bromide.

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Chapter 2

Functional characterization of serum- and growth factor-induced phenotypic changes in intact bovine tracheal smooth muscle.

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Abstract
The present study aims to investigate whether phenotypic changes, reported to occur in cultured isolated airway smooth muscle (ASM) cells, are of relevance to intact ASM. Moreover, we aimed to gain insight into the signalling pathways involved. Culturing of bovine tracheal smooth muscle (BTSM) strips for up to 8 days in the presence of 10 % foetal bovine serum caused a time-dependent ($t_{½}$ = 2.8 days) decrease in maximal contraction (E_{max}) to methacholine compared to serum-deprived controls (E_{max} = 74 ± 4 % at day 8). A reduced E_{max} was also found using insulin-like growth factor-1 (30 ng/ml) and platelet-derived growth factor (30 ng/ml), but not using epidermal growth factor (10 ng/ml) (E_{max} = 83 ± 3 %, 67 ± 8 %, 100 ± 4 %, respectively). Similar serum and growth factor-induced changes in E_{max} were found for KCl-induced contraction (65 ± 9 %, 80 ± 7 %, 64 ± 11 % and 107 ± 2 %, respectively). Strong correlations were found between the growth factor-induced reductions in E_{max} and their proliferative responses, assessed by [^{3}H]thymidine-incorporation, in BTSM cells. (r = 0.97, *P* = 0.002 for methacholine and r = 0.93, *P* = 0.007 for KCl). The PDGF-induced reduction in E_{max} was inhibited completely by combined treatment with either PD98059 (30 µM) or LY294002 (10 µM). These results indicate that serum and growth factors may cause a functional shift towards a less contractile phenotype in intact BTSM, which is associated with their proliferative response and dependent on signalling pathways involving the mitogen-activated protein kinase pathway and the phosphatidylinositol 3-kinase pathway.

Introduction
Studies using primary cultures of airway smooth muscle (ASM) cells have revealed that airway myocytes develop an immature phenotype, also referred to as a synthetic phenotype, upon culturing in serum-rich culture media [1;2]. Characteristics of this synthetic phenotype include an increased proliferative and synthetic potency [3], but a decreased contractile responsiveness, due to a decreased expression of contractile proteins and cell surface receptors, such as the muscarinic M_3-receptor [1;4-6]. Changes toward the synthetic phenotype are, however, reversible: long-term treatment with serum-deprived media re-induces a contractile phenotype in canine ASM cells, characterized by its elongated morphology and re-expression of contractile proteins and muscarinic M_3-receptors [4;5]. Moreover, serum deprivation-induced transition towards a hypercontractile phenotype has been reported in canine ASM cells [7].

Isolated ASM preparations obtained from asthmatics may respond with an increased maximal contractility and/or sensitivity to contractile agonists [6;8;9]. Also, passively sensitized human airway smooth muscle exhibits an increased contractile responsiveness [10] and an increased myosin light chain kinase (MLCK) content [11]. Similar results have been obtained using preparations from ragweed sensitized dogs [6;12;13]. Although there is no evidence linking allergic sensitization or asthma-induced changes in contractility to phenotypic changes, it has been postulated that switching towards a synthetic airway smooth muscle phenotype may be involved in airway remodelling in patients with chronic asthma, characterized by increased

smooth muscle mass and irreversible airflow obstruction [14]. Transition from the synthetic to the (hyper)-contractile phenotype could be involved in enhanced airway hyperresponsiveness in these patients [15].

The mechanisms involved in smooth muscle phenotypic changes are poorly understood. Although multiple signalling pathways have been associated with phenotypic changes, the exact roles of these signalling pathways are not yet clear. [16;17] The mitogen-activated protein kinase pathway, also referred to as extracellular signal regulated kinase pathway (MAPK/ERK pathway) has been associated with smooth muscle cell de-differentiation towards the synthetic phenotype [18;19]. Less clear is the role of phosphatidylinositol 3-kinase (PI 3-kinase) activity in this process, because both maintenance of the contractile phenotype [20] and transition towards a synthetic phenotype [21;22] have been associated with PI 3-kinase activity. Also, activation of RhoA has been shown to be involved in the upregulation of smooth muscle specific gene expression through Rho-kinase-dependent translocation of serum response factor to the nucleus [16] and through RhoA-mediated actin polymerization [17].

A role for extracellular matrix components has also been implicated in regulating airway smooth muscle phenotype, showing that fibronectin and collagen type I favored progression towards a synthetic phenotype, whereas laminin and matrigel (a solubilized basement membrane matrix) strongly inhibited the progression towards a synthetic phenotype [23]. Knowing that cell to cell contacts and extracellular matrix components are preserved in intact smooth muscle, we were interested to learn about the occurrence of functional changes in intact bovine tracheal smooth muscle (BTSM). We evaluated the contractile properties of BTSM strips exposed to serum and to different growth factors (platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and epidermal growth facor (EGF)) in relation to their mitogenic potencies. In addition, we investigated the role of PI 3-kinase and p42/p44 MAPK signalling pathways in the phenotypic changes in contractility in response to stimulation with PDGF.

Methods

Tissue preparation and organ culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5 % CO_2 and 95 % O_2; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco's modification of Eagle's medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and

amphotericin B (1.5 µg/ml). Next, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml medium, with or without 10 % foetal bovine serum (FBS), was added per tissue strip. Strips were maintained in culture in an incubator shaker (37 ˚C, 55 rpm; to prevent tissue attachment and cellular outgrowth) for a maximum of 8 days, refreshing the medium on day 4. When applied, growth factors (PDGF, IGF-1, EGF) or kinase inhibitors (PD98059, LY294002, given 30 min prior to the growth factors) were added in a small volume (7.5 µl per tissue strip). Culture flasks containing kinase inhibitors were protected from light during the whole experiment. Occasionally, some strips were used for contraction experiments immediately after preparation.

Isometric tension measurements.
Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5 % CO_2 and 95 % O_2, pH 7.4 at 37 ˚C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37 ˚C, continuously gassed with 5 % CO_2 and 95 % O_2, pH 7.4. During a 90 min equilibration period, with washouts every 30 min, resting tension was graduallly adjusted to 3 g. Subsequently, muscle strips were precontracted with 20 mM and 30 mM isotonic KCl solutions. Following two wash-outs, basal smooth muscle tone was established by the addition of 0.1 µM isoprenaline and tension was re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min cumulative concentration response curves (CRCs) were constructed to stepwise increasing concentrations of isotonic KCl (5.6 – 50 mM) or methacholine (1 nM – 100 µM). When maximal KCl or methacholine-induced tension was obtained, the strips were washed several times and basal tone was established using isoprenaline (10 µM).

Isolation of bovine tracheal smooth muscle cells
After the removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 µm and three times at a setting of 100 µm. Tissue particles were washed two times with the medium mentioned above, supplemented with 0.5 % FBS. Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 ˚C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 µm gauze, cells were washed three times in medium supplemented with 10 % FBS.

[^{3}H]thymidine-incorporation
BTSM cells were plated in 24 well cluster plates at a density of 50,000 cells per well directly after isolation and were allowed to attach overnight in 10 % FBS containing medium. Cells were washed twice with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4)

and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg/ml), ascorbate (100 µM) and insulin (1 µM) for 72 h. Cells were then washed with PBS and stimulated with mitogens in FBS- and insulin-free medium for 28 h, the last 24 h in the presence of [^{3}H]thymidine (0.25 µCi/ml). After incubation the cells were washed twice with PBS at room temperature and once with ice-cold 5 % trichloroacetic acid (TCA). Cells were treated with this TCA-solution on ice for 30 min and subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

Data analysis
All data represent means ± s.e.mean from n separate experiments. The statistical significance of differences between data was determined by the Student's *t*-test for paired observations or one-way ANOVA, where appropiate. Differences were considered to be statistically significant when $P < 0.05$.

Materials
Dulbecco's modification of Eagle's Medium (DMEM) and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Foetal bovine serum, $NaHCO_3$ solution (7.5 %), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml / 5000 µg/ml) and amphotericin B solution (250 µg/ml) (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Platelet-derived growth factor-AB (PDGF-AB, human recombinant), epidermal growth factor (EGF, human recombinant), insulin-like growth factor-1 (IGF-1, human recombinant), insulin (from bovine pancreas), apotransferrin (human), soybean trypsin inhibitor and (-)isoprenaline hydrochloride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PD98059 and LY294002 were obtained from Tocris Cookson Ltd. (Bristol, UK.). L(+)ascorbic acid was from Merck (Darmstadt, Germany). [*methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.) Papain and Collagenase P were from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

Results

Effects of treatment with FBS-enriched or FBS-free media on methacholine-induced contraction.
Maximal methacholine-induced contractile force (E_{max}) of BTSM strips cultured for up to 8 days in the presence of 10 % FBS was significantly lower compared to strips cultured in FBS-free medium (E_{max} = 17.2 ± 0.8 g and 22.2 ± 2.2 g, respectively; $P < 0.05$; Figure 2.1). No difference in the sensitivity to methacholine was found (pEC_{50} = 6.99 ± 0.10 and 6.99 ± 0.05 for FBS-treated and FBS-deprived BTSM strips, respectively).

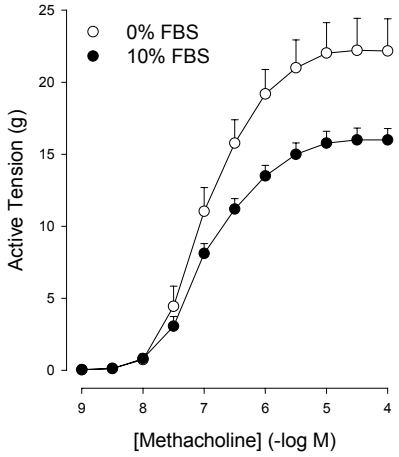

Figure 2.1 *Methacholine-induced contraction of BTSM strips, after exposure to 10 % FBS-enriched or FBS-free culture media for 8 days. Data represent the means ± s.e.mean from 6 experiments*

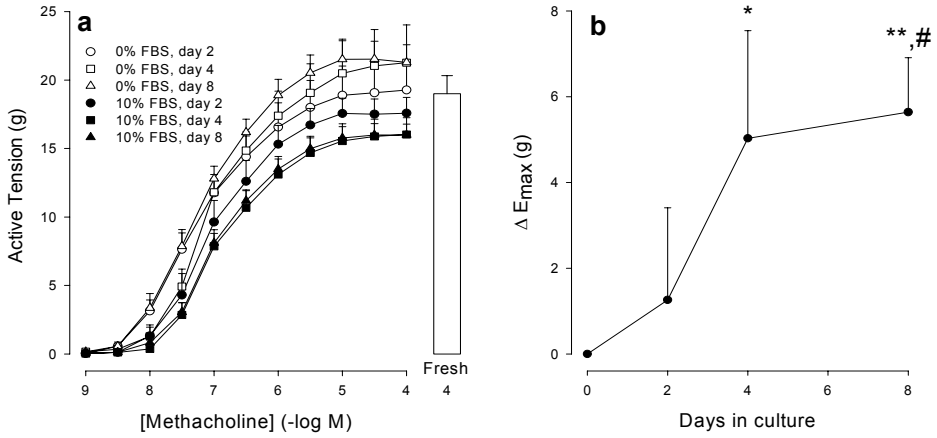

Figure 2.2 *Panel A: Cumulative concentration response curves of methacholine-induced contraction of BTSM strips, cultured in FBS-enriched (10 % FBS) or FBS-free (0 % FBS) medium for the different periods of time indicated. Data represent the means ± s.e.mean from 6 experiments. For comparison, E_{max} to methacholine (100 µM) in freshly used BTSM strips is included, depicted by the white bar. Panel B: Time-dependent differences in maximal methacholine-induced contraction (ΔE_{max}) between FBS-treated and FBS-deprived BTSM strips. Data are taken from panel A and represent means ± s.e.mean from 6 experiments. * P < 0.05, ** P < 0.001 compared to FBS-deprived strips, # P < 0.05 compared to day 2 in FBS-treated strips (Student's t-test for paired observations).*

The development of the differences in maximal methacholine-induced contraction between BTSM strips cultured in FBS-enriched and in FBS-free media was time-dependent (Figure 2.2A). The difference in E_{max} between FBS-treated and FBS-free conditions was maximal at day 8, with a half-maximal effect after 2.8 days (Figure 2.2B). Comparison with results obtained with freshly used strips shows that the E_{max} of strips maintained in FBS-free and FBS-enriched media diverged in time (Figure 2.2A).

Effects of treatment with growth factors on methacholine-induced contraction.
The ability to alter contractile behaviour was not limited to FBS treatment. Treatment for 8 days with IGF-1 (30 ng/ml) and PDGF (30 ng/ml) were also effective in inducing a statistically significant depression of the E_{max} for methacholine-induced contraction compared to vehicle-treated BTSM strips (Figure 2.3). No effect was observed for EGF (10 ng/ml), however. The PDGF-induced decrease in E_{max} was concentration-dependent (Table 2.1). As with FBS-treated BTSM strips, the pEC_{50} of methacholine-induced contraction was unaltered upon treatment with the above mentioned growth factors (Table 2.1).

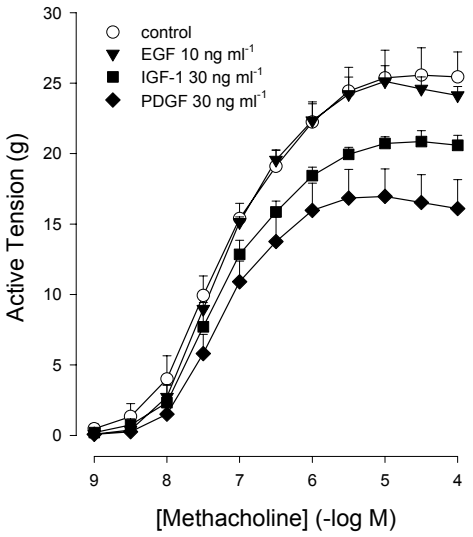

Figure 2.3 *Methacholine-induced contraction of BTSM strips, cultured for 8 days in FBS-free medium containing EGF (10 ng/ml), IGF-1 (30 ng/ml), or PDGF (30 ng/ml). Vehicle-treated strips served as controls. Data represent means ± s.e.mean from 5 experiments.*

Effects on KCl-induced contraction
To establish whether the growth factor-induced effects on BTSM contractility were limited to methacholine-induced contraction, contraction to KCl was also determined in BTSM strips pre-treated with growth factors for 8 days. As observed for methacholine, maximal KCl-induced contraction was sensitive to pretreatment with IGF-1 (30 ng/ml) and PDGF (30 ng/ml), but not EGF (10 ng/ml). Moreover, changes in maximal KCl-induced contraction depended on the concentration of PDGF used. The potency (EC_{50}) of KCl was unchanged after treatment with growth-factors,

irrespective of the growth factor applied. Interestingly, changes in KCl and methacholine-induced contraction were similar, both qualitatively and quantitatively (Table 2.1).

Table 2.1 *Contractile properties to methacholine and KCl of BTSM strips, cultured for 8 days in the presence of growth factors and the proliferative responses of these growth factors in BTSM cells.*

	Contraction				DNA-synthesis
	Methacholine		KCl		
	E_{max} *(% vehicle)*	pEC_{50} *(-log M)*	E_{max} *(% vehicle)*	EC_{50} *(mM)*	*(% vehicle)*
Vehicle	100	7.3 ± 0.2	100	20.6 ± 1.0	100
EGF 10 ng/ml	99.6 ± 3.9	7.2 ± 0.0	107.1 ± 2.4	20.6 ± 1.7	103 ± 18
IGF-1 30 ng/ml	82.7 ± 2.8*	7.2 ± 0.1	80.0 ± 6.5*	21.9 ± 0.8	166 ± 30*
PDGF 3 ng/ml	91.7 ± 3.5	7.2 ± 0.2	99.4 ± 7.7	19.4 ± 1.9	149 ± 13*
PDGF 10 ng/ml	80.7 ± 7.5*	7.2 ± 0.1	76.9 ± 8.3*	20.8 ± 2.4	191 ± 20**
PDGF 30 ng/ml	67.3 ± 8.3**	7.3 ± 0.1	63.3 ± 11.2**	20.2 ± 2.7	207 ± 19***

*Data represent the means ± s.e.mean from 4-6 experiments. * P<0.05, ** P<0.01, *** P<0.001 compared to vehicle-treatment (Student's t-test for paired observations).*

Relationship between growth factor-induced proliferation and inhibition of maximal contraction.

As indicated in Figure 2.3 and Table 2.1, the effects of growth factors on maximal contraction of BTSM strips to methacholine or KCl depended on the nature and the concentration of the growth factor used. The proliferative responses of isolated BTSM cells, measured as [^{3}H]thymidine incorporation, were also dependent on the nature and concentration of the growth factors under investigation, with a reciprocal rank order of potency (Table 2.1). A strong correlation was observed between the efficacy of growth factors to induce depression of maximal methacholine or KCl-induced contraction of BTSM strips and the proliferative potency of these factors in BTSM cells (r = 0.97, *P* = 0.002 and r = 0.93, *P* = 0.007 for methacholine and KCl-induced contraction, respectively; Figure 2.4)

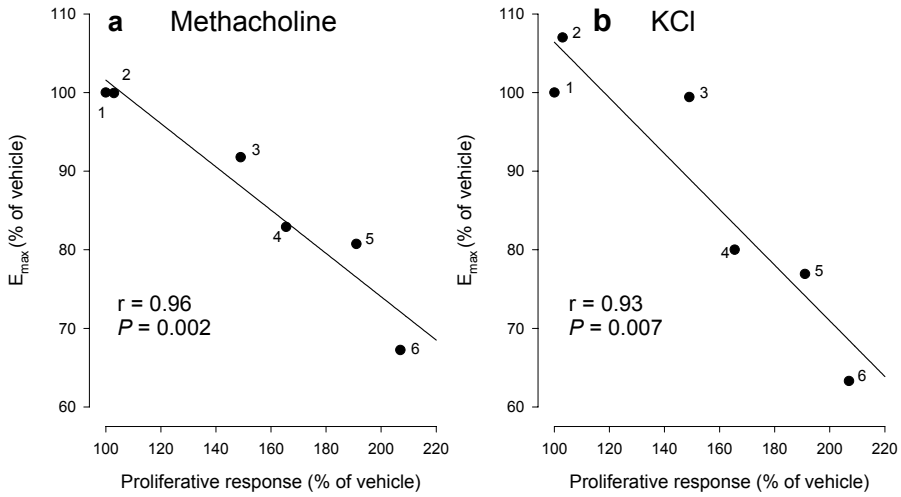

Figure 2.4 *Relationships between depression of maximal methacholine (panel A) and KCl (panel B) induced contraction of BTSM strips and proliferative responses of BTSM cells induced by treatment with vehicle (1), EGF 10 ng/ml (2), PDGF 3 ng/ml (3), IGF-1 30 ng/ml (4), PDGF 10 ng/ml (5) or PDGF 30 ng/ml (6). Data represent means from 4-6 experiments. Significance level was obtained using one-way analysis of variance (ANOVA).*

Effects of kinase inhibitors on PDGF-induced depression of maximal methacholine-evoked contraction.

Since the growth factor-induced depression of BTSM contraction depended on the proliferative effect of the growth factors used, we explored the involvement of proliferation-associated signalling pathways in growth factor-induced depression of maximal contractility to methacholine. For this purpose, PD98059 (30 μM), an inhibitor of the p42/p44 MAPK pathway or LY294002 (10 μM), an inhibitor of PI 3-kinase were added to the culture medium 30 min before the addition of PDGF (10 ng/ml). In agreement with the above observations, PDGF alone reduced the maximum methacholine-induced contraction (Figure 2.5A). However, in the presence of PD98059, no measurable change in contractility was induced by PDGF (Figure 2.5B). PD98059 by itself did not significantly affect maximal methacholine-induced contraction or sensitivity (E_{max} = 20.5 ± 3.0 and 22.1 ± 2.1 g and pEC_{50} = 7.29 ± 0.08 and 7.15 ± 0.10 for PD98059 and vehicle treated strips, respectively). Also in the presence of LY294002, PDGF did not induce a measurable change in methacholine-evoked contraction (Figure 2.5C). However, pretreatment with LY294002 by itself induced a significant depression of maximum contraction compared to vehicle-treated strips (E_{max} = 18.9 ± 2.0 and 22.1 ± 2.1 g for LY294002 and vehicle treated strips, respectively, $P < 0.05$). The potency of methacholine-induced contraction remained unaltered (pEC_{50} = 7.29 ± 0.07 and 7.15 ± 0.10 for LY294002 and vehicle treated strips, respectively).

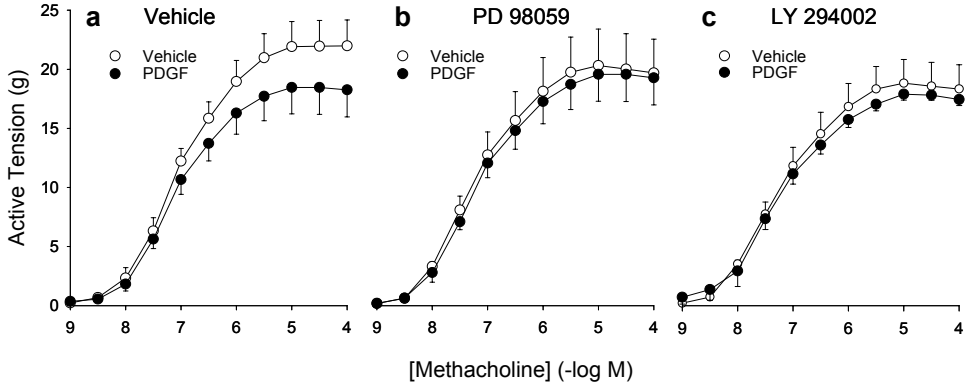

Figure 2.5 *Methacholine-induced contraction of BTSM strips, cultured for 8 days in FBS-free medium. Strips were treated with vehicle (Panel A), 30 µM PD98059 (Panel B) or 10 µM LY294002 (Panel C) in the absence or presence of PDGF 10 ng/ml. Data represent means ± s.e.mean from 5-6 simultaneously performed experiments.*

Discussion

FBS treatment-induced decrease of contractile function has been demonstrated recently in organ-cultured vascular tissue. In cultured rat tail arterial smooth muscle, contractile responses to both noradrenaline and 60 mM K^+ were diminished upon FBS-treatment for 4 days [24]. A similar effect has also been observed in guinea-pig ileum smooth muscle, which showed a decreased contractility to carbachol and 60 mM K^+ after treatment with FBS-rich medium [25]. This loss of contractile function was explained by a continuously elevated intracellular $[Ca^{2+}]_i$ upon culturing in FBS, thus leading to a decreased function of voltage-operated calcium channels (VOC) and/or a decreased sensitivity of the contractile apparatus for Ca^{2+}. Effects of long-term exposure to FBS on contraction of organ cultured airway smooth muscle have not yet been described. FBS contains a variety of mitogenic stimuli and it causes contraction of canine ASM [26]. Therefore, long-term effects of FBS are not necessarily the consequence of proliferative stimulation, but may also be caused by continuously elevated $[Ca^{2+}]_i$ levels or stimulation of the contractile apparatus.

Our data show that pretreatment with both 10 % FBS and the growth factors PDGF and IGF-1, followed by a washout period of over 120 min, induce a depression in maximal response to methacholine. The observed effects for 10 % FBS are time-dependent. The diverging change of E_{max} in time (Figure 2.2) implies that the differences in E_{max} on day 8 are the consequence of both deprivation-induced increases in E_{max} and FBS-induced decreases in E_{max}. It is crucial for the interpretation of our results, that KCl-induced contraction and methacholine-induced contraction were influenced similarly by long-term exposure to growth factors, both quantitatively and qualitatively. These results imply that KCl and methacholine-induced contraction were affected by a common mechanism. However,

methacholine induces contraction through stimulation of PI-turnover and subsequent release of Ca^{2+} from intracellular stores and influx through receptor operated Ca^{2+}-channels [27]. In contrast, KCl-induced contraction is fully dependent on VOC mediated Ca^{2+}-influx and does not require receptor mediated stimulation of PI-turnover. Of note, KCl-induced contraction is independent of acetylcholine release from nerve terminals as it is insensitive to atropine (Boterman, Schaafsma et al., unpublished observations). Therefore, quantitative similarities between KCl and methacholine-induced contraction can be achieved only by affecting contraction downstream intracellular Ca^{2+}-increases. Further evidence that changes in $[Ca^{2+}]_i$ or in the sensitivity of the contractile apparatus to Ca^{2+} cannot explain the 10 % FBS or growth factor induced effects, comes from the observation that the potency (pEC_{50}) of methacholine is not affected by culturing in the presence of 10 % FBS or growth factors. Methacholine requires only fractional stimulation of PI-turnover and subsequent elevations of $[Ca^{2+}]_i$ for BTSM contraction [28]. Altering events *upstream* of changes in $[Ca^{2+}]_i$ or in the sensitivity of the contractile apparatus for Ca^{2+}, should therefore be accompanied by a shift in the concentration-response curve to methacholine.

Modulation towards a less contractile phenotype is associated with a decrease in contractile protein expression [4], although changes in cytoskeletal organization should also be considered as an explanation for the altered maximal contraction [5]. However, agonist-induced cytoskeletal reorganization occurs rapidly (within 5 min) in human ASM cells [29]. Considering the long-term nature of the 10 % FBS-induced decline in maximal contractile response, growth-related changes towards a less contractile phenotype become the most likely explanation for the observed effects. Western analysis of proteins harvested from canine ASM cells in primary culture shows a decline in the expression of contractile proteins (e.g. smooth muscle myosin heavy chain, smooth muscle α-actin) in the period prior to proliferation [1]. We therefore hypothesized that the degree of decline in contractile response should be reciprocally related to the degree by which proliferation is stimulated as a consequence of phenotypic modulation. Indeed, a strong correlation exists between the proliferative responses of the applied growth factors on BTSM cells and the degree of depression in contractile response, both for KCl and methacholine. Since our proliferation data are consistent with earlier findings in BTSM cells [30], a similar relationship could be constructed using our contraction data and the proliferative data published by Kelleher *et al*. ($r = 0.98$ and $r = 0.97$ for methacholine and KCl, respectively). The relative inactivity of EGF, applied in its maximally mitogenic concentration in BTSM cells, is in agreement with the low proliferative efficacy as obtained by us and others [30].

The p42/p44 MAPK pathway and the PI 3-kinase pathway were examined in order to gain insight in the mechanisms involved in the growth factor-induced change in phenotype. Inhibition of the p42/p44 MAPK pathway using PD98059 inhibited the development of contractile depression at a concentration that has been shown to fully inhibit PDGF-induced proliferation in BTSM cells [31]. This supports the hypothesis, that the development of a decreased contractile function is the

consequence of proliferation-associated phenotypic modulation to a less contractile state. Interestingly, modulation of arterial smooth muscle cells towards a synthetic phenotype could also be inhibited by PD98059, though only partially [18].

Inhibition of the PI 3-kinase pathway by LY294002, applied during organ culture at a concentration known to reduce PDGF-mediated proliferation of BTSM cells to basal levels [32], inhibited the PDGF induced decrease of contractile response, suggestive for inhibition of modulation to a less contractile phenotype. In the absence of PDGF, LY294002 treatment itself lowered maximal contraction to methacholine. This effect might be attributed to a diminished PI 3-kinase mediated maintenance of the cytoskeleton [33;34]. In addition, the effects of PI 3-kinase inhibition may also suggest an increase in apoptosis. This would imply apoptosis to be a significant regulatory mechanism in the intact BTSM strip. However, growth factors inhibit apoptosis, and this would induce enhancement rather than a depression of contractility. Although we do not know the exact nature of the LY294002-induced effect, the inhibitory effect on PDGF-induced contractile depression is in agreement with the concept that proliferation and modulation to a less contractile state are stimulated simultaneously. Also, it implies a role for PI 3-kinase in functional modulation of BTSM.

Activation of the p42/p44 MAPK cascade is achieved *via* the PI 3-kinase pathway, but a redundant pathway stimulates p42/p44 MAPK independent of PI 3-kinase when a large number of receptors are activated [35]. Since LY294002 and PD98059 both totally inhibited the PDGF-induced change in phenotype, one might conclude that the pathway stimulated through PI 3-kinase, rather than the redundant p42/p44 MAPK-pathway, is responsible for the observed effects in phenotypic switching. Separate, parallel functioning pathways opposed to serially functioning pathways can, however, not be excluded: proliferation of BTSM cells induced by high concentrations of PDGF-BB has been reported to be inhibited completely by either PD98059 or LY294002, whereas PD98059 does not inhibit PDGF-stimulated PI 3-kinase activity, nor does LY294002 inhibit PDGF-stimulated p42/p44 MAPK activity [32].

Changes in phenotype may be relevant in asthma. Growth factors can be released in asthmatic airways from epithelial cells, inflammatory cells and ASM cells, or leaked into the airways as a consequence of extravasation, contributing to the formation of phenotypically altered myocytes with increased proliferative and synthetic capabilities. When extravasation ceases and growth factor concentrations return to basal levels redifferentiation to the contractile phenotype or even to a hypercontractile phenotype may occur as has been shown in cultured canine ASM cells [2]. Induction of synthetic smooth muscle phenotypes as postulated to occur in asthma [14] is therefore not favorable and may contribute to typical features of asthmatic airways, such as hyperreactivity, irreversible airflow obstruction and the progressive increase in the severity of the disease.

In conclusion, BTSM contractility is sensitive to treatment with FBS or growth factors *in vitro*. Both receptor-dependent (methacholine) and receptor-independent (KCl) stimulation of BTSM, treated with FBS or growth factors, led to a decrease in maximal contraction to these agonists. This implies that changes occurred at the level of the contractile machinery. Moreover, the decline in contractile response correlates with the proliferative conditions of the culture medium and can be totally inhibited by PD98059 and LY294002. As a consequence, it seems that intact BTSM is sensitive to phenotypic changes and that these changes are regulated through signaling pathways involving both the p42/p44 MAPK and the PI 3-kinase-pathway.

Acknowledgement

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Chapter 3

Insulin induces a hypercontractile airway smooth muscle phenotype.

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Abstract
This study aims to investigate the effects of insulin on bovine tracheal smooth muscle (BTSM) phenotype *in vitro*. Contractility of muscle strips and DNA-synthesis ([^{3}H]thymidine incorporation) of isolated cells were used as parameters for smooth muscle phenotyping. Insulin (1 μM) was mitogenic for BTSM and potentiated DNA-synthesis induced by other growth factors. In contrast, after pretreatment of unpassaged BTSM cells in culture, the mitogenic response induced by growth factors was strongly diminished, with no difference in the basal incorporation. Pretreatment of BTSM strips in organ culture with insulin increased maximal contraction to methacholine and KCl. These results show that insulin acutely augments DNA-synthesis in the presence of other growth factors. In contrast, insulin pretreatment induces a hypercontractile phenotype with a decreased mitogenic capacity. This mechanism may be involved in the putative negative association between asthma and type I diabetes. In addition, these findings may have implications for the use of aerosolized insulin in diabetes mellitus.

Introduction
Cultured airway smooth muscle (ASM) cells are known to develop a less contractile phenotype when exposed to serum-rich culture media and growth factors, characterized by a decreased shortening capacity and contractile protein expression, while the proliferative and synthetic capabilities of these cells are enhanced [1-3]. Phenotype switching is known to be regulated by extracellular matrix proteins that either promote (e.g. collagen type I, fibronectin) or inhibit (e.g. laminin) progression toward the less contractile and more proliferative state [4]. In Chapter 2, we have demonstrated that intact ASM, embedded in its own extracellular matrix, is also sensitive to phenotype changes induced by exogenously applied growth factors [5]. Progression to the less contractile state can be induced by serum, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF), which is linearly related to the mitogenic response of the growth factor applied [5].

It is unknown whether the observed relationship between proliferation and phenotypic modulation is shared by all growth factors that stimulate receptors with intrinsic tyrosine kinase activity, including insulin. Insulin is known to be mitogenic for cultured human ASM cells and to potentiate ASM mitogenesis induced by other receptor tyrosine kinase agonists, such as EGF, and by G-protein coupled receptor agonists, such as thrombin [6]. Consequently, one would expect insulin to promote progression toward the less contractile state. However, induction of functionally hypercontractile myocytes has been reported after treatment with serum-free media containing insulin [3]. Hence, it is of great interest to solve this discrepancy. Moreover, insight in the long-term effects of insulin on ASM phenotype is warranted in view of recent publications on the application of aerosolized insulin in diabetes mellitus [7-9]. Inducing a phenotype switch by this mode of administration could limit its use, especially in patients suffering from airway diseases. Moreover, long-term

effects of insulin could also explain the repeatedly reported negative association between type I diabetes and asthma [10;11].

Therefore, we investigated the effects of insulin on bovine tracheal smooth muscle (BTSM) phenotype in vitro, using both intact tissue and isolated cells, in which we measured contractility and proliferative responsiveness, respectively, as parameters for smooth muscle phenotype. Insulin was acutely mitogenic for BTSM cells and synergistically potentiated mitogenesis induced by PDGF, IGF-1 and EGF. However, *pre*-treatment with insulin induced a hypercontractile and hypoproliferative phenotype of these cells.

Methods

Tissue preparation and organ culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5 % CO_2 and 95% O_2; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco's modification of Eagle's medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (1.5 µg/ml). Next, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml medium was added per tissue strip. Strips were maintained in culture in an incubator shaker (37 ˚C, 55 rpm) for 8 days, refreshing the medium on day 4. Either fetal bovine serum (FBS) or insulin were present during the entire incubation period, when applied.

Isometric tension measurements.

Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5 % CO_2 and 95 % O_2, pH 7.4 at 37 ˚C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37 ˚C, continuously gassed with 5 % CO_2 and 95 % O_2, pH 7.4. During a 90 min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. In separate experiments it was established that strips stretched to 3 g passive tension responded optimally. Subsequently, muscle strips were precontracted with 20 mM and 30 mM isotonic KCl solutions. Following two wash-outs, basal smooth muscle tone was established by the addition of 0.1 µM (-)-isoprenaline and tension was re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. Following another equilibration period of 30 min, cumulative concentration response curves were constructed to stepwise increasing concentrations of isotonic KCl (5.6 – 50 mM) or methacholine (1 nM – 100 µM). The

increase in KCl concentration was compensated for by substitution with NaCl to maintain iso-osmolarity. When maximal KCl or methacholine-induced tension was obtained, the strips were washed several times and basal tone was re-established using (-)-isoprenaline (10 μM).

Isolation of bovine tracheal smooth muscle cells
Tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 μm and three times at a setting of 100 μm. Tissue particles were washed two times with the medium mentioned above, supplemented with 0.5 % FBS. Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 ˚C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 μm gauze, cells were washed three times in medium supplemented with 10 % FBS.

[^{3}H]Thymidine-incorporation
BTSM cells were plated in 24 well cluster plates at a density of 30,000 cells per well in 10 % FBS containing medium at 37 ˚C in a humidified 5 % CO_2-incubator. After attachment overnight, cells were washed two times with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4). Subsequently cells were made quiescent by incubation for 72 h in serum-free medium supplemented with 0.1 % FBS, apo-transferrin (5 μg/ml) and ascorbate (100 μM). When pretreatment effects of insulin were studied, 0.1 % FBS was replaced for insulin (1 μM).

After quiescence, cells were washed with PBS and stimulated with mitogens in serum-free medium for 28 h, the last 24 h in the presence of [^{3}H]thymidine (0.25 μCi/ml), followed by two washes with PBS at room temperature and one with ice-cold 5 % trichloroacetic acid. Cells were treated with this trichloroacetic acid-solution on ice for 30 min; subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting.

Data analysis
All data represent means ± s.e.mean from *n* separate experiments and EC_{50} was expressed as the concentration required to induce half the maximal effect (E_{max}). pD_2 values were calculated as $-\log EC_{50}$. The statistical significance of differences between data was determined by the Student's *t*-test for paired observations (two-tailed). Differences were considered to be statistically significant when $P < 0.05$.

Materials
DMEM and methacholine chloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Fetal bovine serum, $NaHCO_3$ solution (7.5 %), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin

solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml / 5000 µg/ml), amphotericin B solution (250 µg/ml) (Fungizone) and trypsin were obtained from Gibco BRL Life Technologies (Paisley, U.K.). EGF (human recombinant), IGF-1 (human recombinant), PDGF (human recombinant), insulin (from bovine pancreas), apotransferrin (human) and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). [*Methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.). Papain and collagenase P were from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

Results

Acute effects of insulin on bovine tracheal smooth muscle DNA-synthesis

Acute effects of insulin on DNA-synthesis were assessed, using cells that were made quiescent in serum-free medium for a period of 3 days. Insulin (1 µM) increased [^{3}H]thymidine incorporation to 159 ± 11 % of basal (Figure 3.1A, P <0.01). In combination with other growth factors (PDGF, IGF-1, EGF), insulin induced a synergistic enhancement of the responses. Synergism was expressed as the difference between the sum of individual responses and the measured combined response. Interestingly, as compared to IGF-1 (10 ng/ml), PDGF (10 ng/ml)-induced and EGF (10 ng/ml)-induced incorporation were potentiated to a larger extent (Figure 3.1).

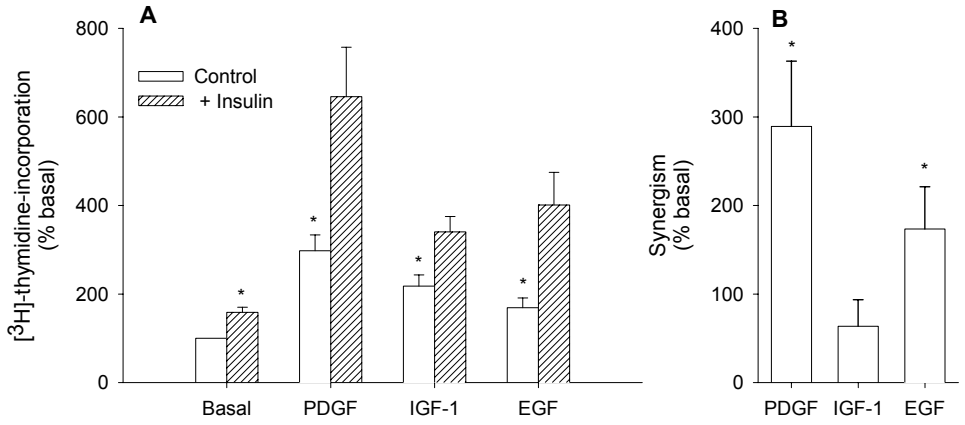

Figure 3.1 *A: [^{3}H]Thymidine incorporation of unpassaged BTSM cells. Basal responses and those in response to EGF (10 ng/ml), IGF-1 (10 ng/ml) and PDGF (10 ng/ml) were measured, both in the absence and presence of insulin (1 µM). B: Calculated synergism of DNA-synthesis of the applied growth factors due to the presence of insulin. Data represent means ± s.e.mean. of 4-5 experiments each performed in triplicate. * P < 0.05 compared to control basal; † P < 0.05 compared to absence of insulin.*

Effects of pretreatment with insulin on bovine tracheal smooth muscle DNA-synthesis

In order to investigate the effect of pretreatment with insulin on BTSM DNA-synthesis, cells were made quiescent in media with and without insulin (1 µM) for a period of 3 days. After this period, cells were washed and stimulated with growth factors. No difference in basal [^{3}H]thymidine incorporation could be observed for pretreatment without and with insulin that averaged 3,661 ± 803 and 3,459 ± 740 dpm/well (n=15), respectively. However, the mitogenic effect induced by PDGF (10 ng/ml), was significantly reduced after pretreatment with insulin. Similarly, a reduction in incorporated [^{3}H]thymidine was observed for IGF-1 (10 ng/ml), whereas the response to EGF (10 ng/ml) was suppressed completely (Figure 3.2). Analysis of the concentration-response relationship for PDGF showed that the observed decrease manifested itself both as a decrease in maximal effect and as a rightward shift, indicating a decreased sensitivity (E_{max} = 338 ± 26 and 207 ± 20 % of basal (*P*<0.001) and EC_{50} = 2.1 ± 0.7 and 4.0 ± 1.4 ng/ml (*P*<0.05) for pretreatment in medium without and with insulin, respectively, Figure 3.3).

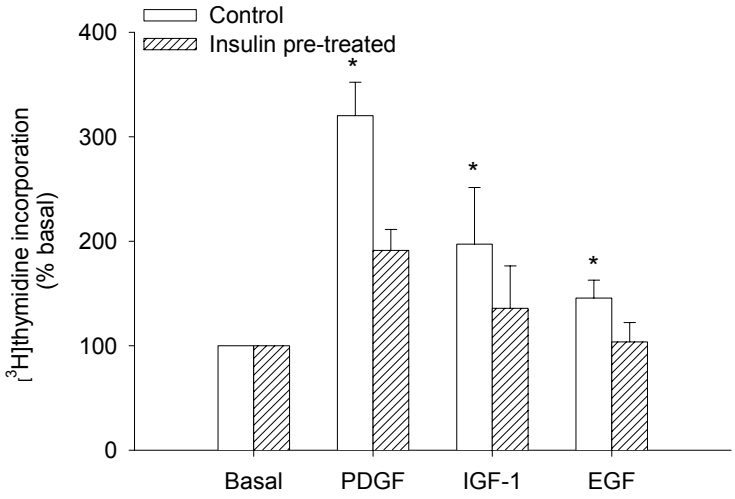

Figure 3.2 *[^{3}H]Thymidine incorporation of unpassaged BTSM cells, pre-treated with serum-free medium with or without insulin (1 µM) for a period of 3 days. Basal responses and those in response to EGF (10 ng/ml), IGF-1 (10 ng/ml) and PDGF (10 ng/ml) were measured. Data represent means ± s.e.mean. of 6 experiments each performed in triplicate. * P < 0.05 compared to basal; † P < 0.05 compared to serum-free pre-treatment.*

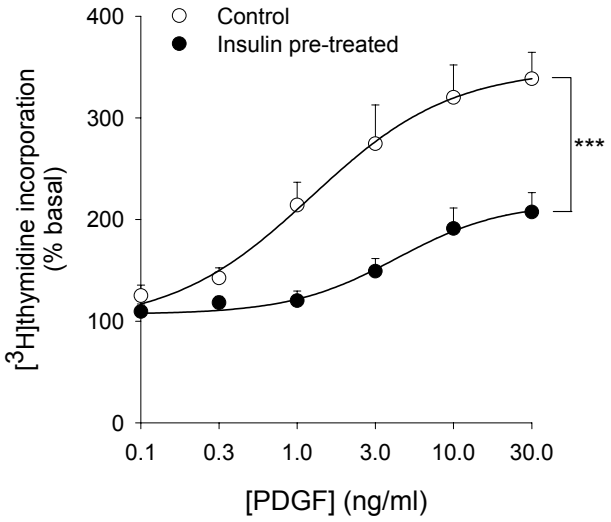

Figure 3.3 *PDGF-induced [^{3}H]thymidine incorporation of unpassaged BTSM cells, pre-treated with serum-free medium with or without insulin (1 μM) for a period of 3 days. Data represent means ± s.e.mean. of 6 experiments each performed in triplicate. *** P < 0.001.*

Effects of pretreatment with insulin on bovine tracheal smooth muscle contractility

The effects of insulin (1 μM) on BTSM phenotype were investigated using intact organ-cultured smooth muscle strips as described in Chapter 2. In view of the time course of the phenotypic switch in intact tissue (t½ = 2.8 days), strips were pretreated with insulin for a period of 8 days. As positive controls, some preparations were treated with 10 % FBS, known to switch to a less contractile phenotype. As expected, strips treated with 10 % FBS responded with a decrease in E_{max} for methacholine. No change in sensitivity (pD_2) was observed after treatment with 10 % FBS (Figure 3.4). In contrast, strips treated with insulin responded with an increase in maximal contraction for methacholine when compared to serum-free medium pre-treated strips. This increase was quantitatively similar to the decrease in E_{max} induced by 10 % FBS. In addition, a small but significant leftward shift could be observed in the dose-response relationship for methacholine after pretreatment with insulin (pD_2 = 7.0 ± 0.1 and 7.2 ± 0.1 for pretreatment with and without insulin, P <0.01). Almost similar results were found for KCl-induced contraction, both quantitatively and qualitatively. However, no shift in sensitivity (EC_{50}) after pretreatment with insulin was observed for KCl (Figure 3.4).

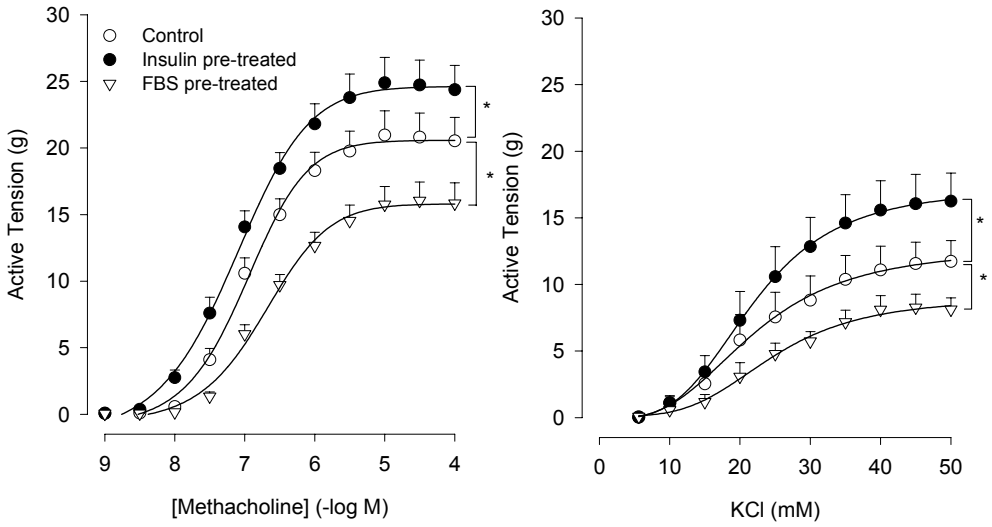

Figure 3.4 *Methacholine-(left panel) and KCl (right panel)-induced contraction of BTSM strips, pre-treated with serum-free medium (control), medium containg 10 % FBS or medium containing insulin (1 µM) for a period of 8 days. Data represent means ± s.e.mean of 8 experiments each performed in duplicate.*
** P<0.05.*

Discussion

As shown in this study, the acute effects of insulin on BTSM cells are dependent on the presence of other growth factors. Insulin, applied in a concentration generally used in ASM cell culture media was mitogenic by itself and augmented the proliferative effects induced by submaximally effective concentrations of PDGF, IGF-1 and EGF [12]. This augmentation was more profound for PDGF and EGF when compared to IGF-1, which is in line with results obtained by others using human ASM cells [6]. Probably structural similarities between insulin and IGF-1 cause these two growth factors to act to some extent through the same receptors [13].

In contrast to the acute effects of insulin, *pretreatment* with insulin induced a decrease in proliferative responsiveness. The presence of insulin during the quiescence period may have stimulated the cells to proliferate to a small extent, resulting in fewer cells that are available for stimulation by other growth factors. However, if this were the explanation for the decreased proliferative responses seen after pretreatment with insulin, basal thymidine incorporation should have been decreased as well. Moreover, proliferative responses to all growth factors should have been equally decreased. However, basal incorporated activity was similar for

control and insulin pretreated cells, demonstrating that insulin-induced differences occurred selectively at the level of growth factor-induced thymidine incorporation. Furthermore, the decrease in proliferative responsiveness was dependent on the growth factor applied: the PDGF response was diminished by approximately 50 %, whereas the EGF response was abolished. Since insulin pretreatment also increased contractility, the results indicate that insulin *pretreatment* induced a phenotypic shift towards a hypercontractile and less proliferative phenotype.

One could argue that the smooth muscle cells in strip preparations maintained in insulin are simply more viable due to the very presence of insulin and therefore respond more efficiently after 8 days in organ culture. However, serum-free maintained strips exhibit increased rather than decreased contractile responses as compared to freshly isolated strips. Moreover, growth factors which would stimulate rather than inhibit the number of viable cells, decrease contractility of BTSM strips as demonstrated in Chapter 2.

It is important to note that the increase in contractility after pretreatment with insulin and the decreased contractility after treatment with FBS and other growth factors are observed for both methacholine and KCl-induced contraction. Methacholine requires receptor-induced stimulation of phosphoinositide turnover to induce calcium release, whereas KCl uses voltage dependent calcium channels to induce calcium influx [14]. Therefore, qualitative and quantitative similarities between KCl and methacholine-induced contraction can be achieved only by modulating contraction downstream of intracellular Ca^{2+}-increases. Considering the long-term nature of the change in contractility (c.f. Chapter 2), changes at the level of the contractile machinery are the most likely explanation for the observed effects.

The hypercontractile phenotype is somewhat unexpected, since the results in Chapter 2 show that regulation of contractility by growth factors, including IGF-1, is reciprocally related to their mitogenic responses. Differences in the balance of activation of distinct kinase-isoforms may underlie this discrepancy: *e.g.* Akt1 and Akt2 are known to have opposite effects on skeletal muscle differentiation induced by insulin [15]. These kinases both act downstream of phosphoinositide 3- kinase (PI 3-kinase). It should be noted that PI 3-kinase is involved, at least in part, in the growth factor-induced phenotype shift (Chapter 2).

Previous studies concerning a role for insulin in ASM phenotype switching are not available. However, insulin is often used as a substituent in serum-free media, in which others have succeeded in inducing a hypercontractile canine ASM phenotype [3]. However, this was attributed to serum deprivation rather than to the presence of insulin [16]. Indeed, in chick gizzard smooth muscle cells, insulin has been shown to be involved in phenotypic switching to a hypercontractile phenotype [17]. In addition, prolonged treatment of PAC1 cells with insulin induces a switch from a vascular smooth muscle phenotype to a skeletal muscle phenotype as demonstrated by the expression of skeletal muscle specific proteins. Interestingly, RT-PCR analysis in these cells showed that this smooth muscle to skeletal muscle differentiation is

accompanied by increases in smooth muscle specific protein expression, such as myosin-light chain kinase (sm-MLCK), smooth muscle heavy chain (sm-MHC) and sm-calponin [18]. These findings suggest that insulin-induced changes toward a (hyper)contractile phenotype may not be confined to smooth muscle of bovine tracheal origin.

A lower prevalence of asthma and atopy symptoms in patients with type I diabetes mellitus has been reported in a number of epidemiological studies [11]; [10;19], although this is also debated [20]. The mechanism of this putative association is still unclear. Based on the present study, low plasma levels of insulin might be protective towards symptoms of asthma, since insulin may extend the range of airway smooth muscle phenotypic shifting either toward a proliferative or a hypercontractile phenotype, conditional on the presence of other growth factors. This could also contribute to the controversy with respect to the negative association of asthma and diabetes mellitus, since diabetics that are under well-controlled insulin treatment would be equally subjective to asthma as non-diabetic individuals. In line with this hypothesis, an increased function of inhibitory prejunctional muscarinic M_2-receptors and a decreased antigen challenge-induced influx of inflammatory cells in the airways have been demonstrated in rat model of streptozotocin-induced type I diabetes which could be reversed by the administration of insulin [21;22]. Using the same model, a diminished tracheal contractility was observed in long-term (8 week) diabetic rats [23], but not in 1 week diabetic rats [21;24]. A similar time-dependency has been observed for calmodulin expression [25]. Phenotype switching *in vivo* may be a slower process than *in vitro*, since it is still continuing 35 days after the last challenge in repeatedly allergen-challenged rats [26], whereas growth factor induced phenotype switching in intact BTSM *in vitro* is characterized by a t½ of 2.8 days (Chapter 2).

The long-term effects of insulin on ASM phenotype switching may also be important in view of recent human studies on the effectiveness of aerosolized insulin in diabetes management [7-9]. If used for diabetes treatment, lung concentrations of insulin will be chronically elevated as compared to other ways of administration. In diabetics suffering from airway diseases such as asthma as well, such treatment may worsen ASM hyperplasia and contractility by extending the phenotype switching capacity.

In conclusion, insulin is mitogenic and potentiates mitogenesis induced by other growth factors. In contrast, *pre*-treatment with insulin induces a hypercontractile and hypoproliferative BTSM phenotype. Therefore, insulin may enhance either contractility or proliferation of ASM, dependent on the duration of exposure to insulin. This may provide an explanation for the putative negative association between asthma and type I diabetes. In addition, this shows that aerosolized administration of insulin may result in adverse effects on airway smooth muscle mass and function.

Acknowlegdement
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Chapter 4

Role of Rho-kinase in maintaining airway smooth muscle contractile phenotype.

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Abstract

This study aims to investigate the role of Rho-kinase in phenotype switching and proliferation of bovine tracheal smooth muscle (BTSM). To induce different phenotypic states, BTSM strips were cultured (8 days) in 10% foetal bovine serum (FBS, less contractile phenotype) or insulin (1 μM, hypercontractile phenotype) and compared to strips cultured in serum-free medium. In contraction experiments, the Rho-kinase inhibitor (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y27632) (1 μM) decreased sensitivity to methacholine and KCl and lowered maximal responsiveness to KCl in all strips irrespective of the phenotype present. To investigate effects of Rho-kinase BTSM phenotypic regulation, strips were pretreated with Y27632 (1 μM) for 8 days. This resulted in a decreased maximal contractility to both methacholine and KCl, quantitatively comparable to the decrease in contractility induced by platelet-derived growth factor (PDGF, 10 ng/ml). The combination of Y27632 and PDGF responded additively. Y27632 did not affect basal or PDGF-induced BTSM cell proliferation, determined both as increases in [^{3}H]thymidine incorporation and cell number. Inhibitors of the p42/p44 mitogen activated protein kinase (MAPK)-pathway, the p38 MAPK-pathway and the phosphatidyl inositol 3-kinase (PI 3-kinase) pathway all inhibited PDGF-induced proliferation and phenotype changes. These results show that the functional contribution of Rho-kinase to BTSM contraction is not dependent on phenotypic state. In addition, Rho-kinase is not involved in phenotypic modulation or proliferation induced by PDGF, whereas p42/p44-, p38-MAPK and PI 3-kinase are. Rho-kinase is, however, a major regulator involved in the basal maintenance of contractility in BTSM.

Introduction

Modulation of airway smooth muscle (ASM) cell phenotype affects contractile, synthetic and/or proliferative characteristics [1]. Reduction of smooth muscle specific protein expression (e.g. smooth muscle-α-actin, smooth muscle-myosin heavy chain and smooth muscle-myosin light chain kinase) can be induced in response to serum-rich media [2]. Serum withdrawal leads to reconstitution of the contractile phenotype indicating the reversible nature of phenotype switching [3]. Chapter 2 of this thesis describes the occurrence of phenotype switching in organ-cultured intact bovine tracheal smooth muscle (BTSM) [4], showing that serum and growth factors are capable of shifting BTSM phenotype toward a less contractile phenotype, which is linearly related to their mitogenic response. Insulin on the other hand has been shown to induce hypercontractility in ASM cells, as described in Chapter 3 [5].

Since inflammatory cells as well as plasma are potential sources of growth factors, phenotype switching may occur as a result of recurrent periods of allergen exposure in asthmatic airways. Repeated allergen challenge indeed has been shown to increase ASM mass, together with reductions in smooth muscle specific protein expression and contractility, in a Brown-Norway rat model of allergic asthma [6]. As such, phenotype switching has been postulated to contribute to remodelling of the

ASM layer in asthma and therefore to the chronic increase in severity of the disease [7].

It can be envisaged that the Rho/Rho-kinase pathway is able to oppose ASM phenotypic modulation induced by growth factors as this pathway is reported to control smooth muscle specific gene expression by mediating the nuclear localisation of serum-response factor (SRF) [8;9]. In addition, the organisation state of the contractile apparatus could be directly linked to transcriptional regulation through Rho-kinase dependent regulation of actin polymerisation [10]. Furthermore, high Rho protein expression has been observed in the contractile phenotype of aortic smooth muscle cells [11]. Taken together, this suggests that the Rho/Rho-kinase pathway is involved in maintaining the contractile smooth muscle phenotype, with a relatively more profound contribution to contraction in the more contractile state of the smooth muscle.

Paradoxically, the Rho/Rho-kinase pathway has been shown to be involved in thrombin-induced rat aortic smooth muscle cell proliferation and in serum-induced rat hepatic stellate cell growth [12;13]. Mechanistically this may be explained by Rho-kinase dependent activation of p42/p44 mitogen activated protein kinase (MAPK) [12]. Since stimulation of proliferation and modulation to the less contractile phenotype coincide and since p42/p44 MAPK is associated with phenotypic modulation in BTSM (Chapter 2), the Rho/Rho-kinase pathway might thus be related to a shift to the less contractile phenotype. Therefore, to clarify this role of Rho-kinase in phenotype switching, we used both organ cultured BTSM strips and cultured BTSM cells in which contractility and proliferation were measured, respectively.

Methods

Tissue preparation and organ culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5 % CO_2 and 95 % O_2; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco's modification of Eagle's medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (1.5 µg/ml). Organ culture was performed as described in Chapter 2. In brief, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml medium was added per tissue strip. Strips were maintained in culture in an incubator shaker (37 ˚C, 55 rpm) for 8 days, refreshing the medium on day 4. When applied, insulin, PDGF and/or kinase inhibitors (given 30 min prior to growth factors)

were added in a small volume (7.5 µl per tissue strip). Culture flasks containing kinase inhibitors were protected from light during the whole experiment.

Isometric tension measurements.
Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5 % CO_2 and 95 % O_2, pH 7.4 at 37 ˚C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37 ˚C, continuously gassed with 5 % CO_2 and 95 % O_2, pH 7.4. During a 90 min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, muscle strips were precontracted with 20 mM and 30 mM isotonic KCl solutions. Following two wash-outs, maximal relaxation was established by the addition of 0.1 µM (-)-isoprenaline. In > 95 % of experiments no basal myogenic tone was detected. Tension was now re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min cumulative concentration response curves were constructed to stepwise increasing concentrations of isotonic KCl (5.6 – 50 mM) or methacholine (1 nM – 100 µM). Occasionally, Y27632 (1 µM) was added 30 min prior to the construction of methacholine concentration response curves. When maximal KCl or methacholine-induced tension was obtained, the strips were washed several times and maximal relaxation was established using (-)-isoprenaline (10 µM).

Isolation of bovine tracheal smooth muscle cells
After the removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 300 µm and three times at a setting of 100 µm. Tissue particles were washed two times with the medium mentioned above, supplemented with 0.5 % foetal bovine serum (FBS). Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 ˚C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 µm gauze, cells were washed three times in medium supplemented with 10 % FBS.

[^{3}H]thymidine-incorporation
BTSM cells were plated in 24 well cluster plates at a density of 50,000 cells per well directly after isolation and were allowed to attach overnight in 10 % FBS containing medium. Cells were washed twice with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4) and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg/ml), ascorbate (100 µM) and insulin (1 µM) for 72h. Cells were then washed with PBS and stimulated with mitogens in FBS- and insulin-free medium for 28 h, the last 24 h in the presence of [^{3}H]thymidine (0.25 µCi/ml). After incubation the cells were washed twice with PBS at room temperature and once with ice-cold 5 % trichloroacetic acid. Cells were treated with this trichloro acetic acid-solution on

ice for 30 min and subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

MTT assay
BTSM cells were treated similarly as described above. Subsequently, cells were stimulated with mitogens for 7 days, after which cell number was estimated using the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. Briefly, cells were washed twice with PBS and 200 µl medium containing 0.5 mg/ml MTT was added to each well. After five hours, 200 µl solubilisation solution (composition: 10 % sodium dodecylsulphate in 0.01 N HCl) was added and the cells were solubilised overnight at 37 ˚C. The amount of formazan in the obtained solution was estimated by measuring optical density at a test wavelength of 550 and a reference wavelength of 650 nm.

Data analysis
All data represent means ± s.e.mean from *n* separate experiments. Concentration response curves of contractile responses were analyzed by measuring myogenic tension only. No corrections were made for basal tone. Maximal tension (E_{max}) and EC_{50} were calculated from the concentration response curves. Curves were fitted using the logistic 4-parameter model (Sigmaplot 8.0, SPSS Inc.). The statistical significance of differences between data was determined by the Student's *t*-test for paired observations or one-way analysis of variance, where appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Materials
Dulbecco's modification of Eagle's Medium (DMEM) and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Foetal bovine serum, $NaHCO_3$ solution (7.5%), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml ; 5000 µg/ml) and amphotericin B solution (250 µg/ml) (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Platelet-derived growth factor AB (PDGF-AB, human recombinant), insulin (from bovine pancreas), MTT, sodium-dodecyl sulphate, apo-transferrin (human), soybean trypsin inhibitor and (-)-isoprenaline hydrochloride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine (SB203580), (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y27632), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) were obtained from Tocris Cookson Ltd. (Bristol, UK.). L(+)ascorbic acid was from Merck (Darmstadt, Germany). [*methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.) Papain and Collagenase P were from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

Results

Effects of Y27632 on contraction in different phenotypic states

To obtain different phenotypic states, BTSM strips were cultured in serum-free medium (vehicle treatment), serum-free medium containing insulin (1 μM, hypercontractile state) or in serum-rich medium containing 10 % FBS (less contractile state). Both methacholine- and KCl-induced contractions were susceptible to this phenotypic shift: E_{max} increased upon insulin pretreatment to 128 ± 11% and 134 ± 18% of vehicle pretreated preparations and decreased upon 10 % FBS treatment to 73 ± 10% and 54 ± 9% of vehicle pretreated preparations, for methacholine and KCl, respectively. The role of Rho-kinase in methacholine-induced contraction in these different phenotypic states was evaluated by treatment with Y27632 (1 μM) for 30 min. In 8 days vehicle pretreated strips, Y27632 induced a slight decrease (ΔpD_2 = 0.33 ± 0.05, P<0.01) in sensitivity to methacholine with no effect on maximal contraction (Figure 4.1A). Comparable shifts in sensitivity were obtained in insulin pretreated (ΔpD_2 = 0.33 ± 0.07, P<0.05; Figure 4.1B) and FBS pretreated BTSM strips (ΔpD_2 = 0.37 ± 0.08, P<0.001; Figure 4.1C). treated, respectively, Figure 4.2)

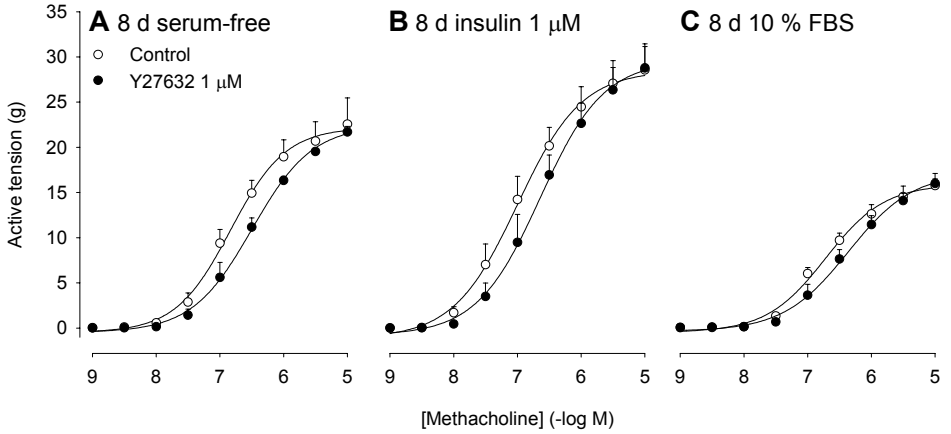

Figure 4.1 *Methacholine-induced contraction of organ-cultured BTSM strips, pretreated for 8 days with (A) serum-free medium; (B) insulin (1 μM) or (C) 10 % FBS. Cumulative concentration-response curves to methacholine were constructed both in the absence (open symbols) and presence (closed symbols) of 1 μM Y27632. Data represent means ± s.e.mean of 4 experiments each performed in duplicate.*

Furthermore, maximal contraction was not affected by Y27632 in either state. Interestingly, KCl-induced contraction was more sensitive to Y27632 when compared to methacholine (Figure 4.2). Treatment with Y27632 induced a rightward

shift in the KCl concentration-response curve of vehicle-treated preparations (ΔEC_{50} = 7.6 ± 1.4 mM, P<0.05). In addition, maximal contraction was significantly decreased (E_{max} = 69 ± 11 % of control, P<0.05). After 8d pretreatment of BTSM strips with insulin or FBS, the relative effects of Y27632 on KCl-induced contraction were not altered (ΔEC_{50} = 7.2 ± 0.7 mM and 7.6 ± 0.8 mM and E_{max} = 75 ± 6 % and 66 ± 12 % for insulin and FBS.

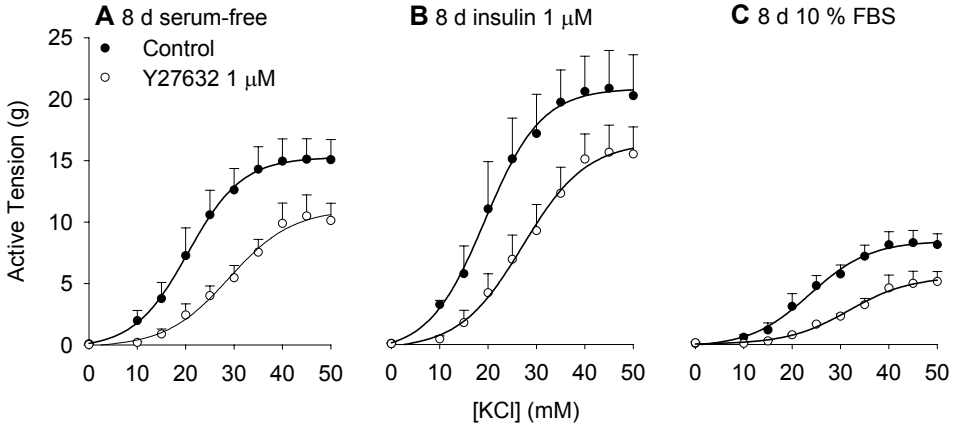

Figure 4.2 *KCl-induced contraction of organ-cultured BTSM strips, pretreated for 8 days with (A) serum-free medium; (B) insulin (1 μM) or (C) 10 % FBS. Cumulative concentration-response curves to KCl were constructed both in the absence (open symbols) and presence (closed symbols) of 1 μM Y27632. Data represent means ± s.e.mean of 4 experiments each performed in duplicate.*

Effects of Y27632 on bovine tracheal smooth muscle contractile phenotype

The role of Rho-kinase in long-term regulation of BTSM contractility was determined by culturing BTSM strips for 8 days in serum-free medium containing Y27632 (1 μM). Y27632 pre-treated strips responded with a decreased maximal contraction to 78 ± 7% and 86 ± 4% for methacholine and KCl, respectively (Figure 4.3). No shift in sensitivity was observed for methacholine or for KCl after 8 days pre-treatment with Y27632. The effects of Y27632 were both quantitatively and qualitatively similar to the effects induced by 8 days treatment with 10 ng/ml PDGF, known to shift BTSM phenotype to a less contractile state (Figure 4.3). However, 8 days pretreatment with the combination of PDGF and Y27632 further decreased maximal contraction in an additive fashion to 54 ± 9% and 59 ± 11% for methacholine and KCl, respectively.

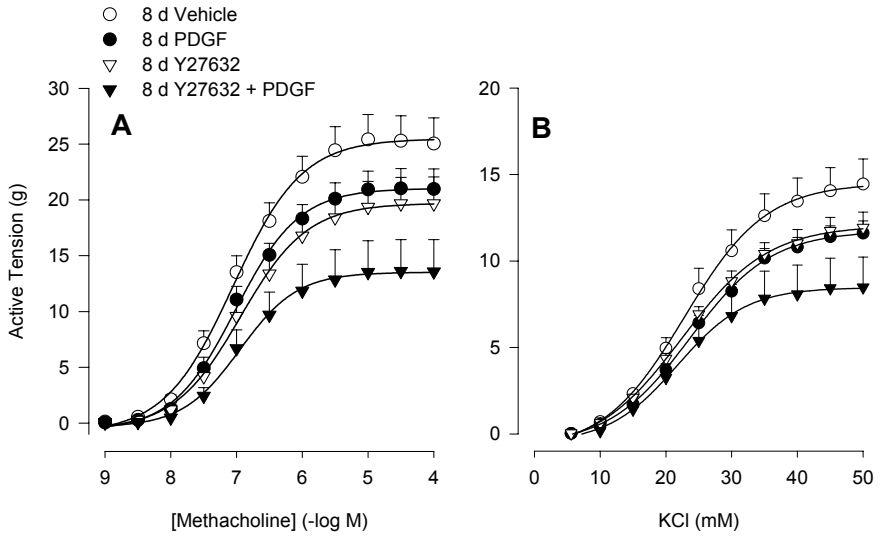

Figure 4.3 *(A) Methacholine- and (B) KCl-induced contractions of organ-cultured BTSM strips, pretreated for 8 days with serum-free medium containing Y27632 (1 μM), PDGF (10 ng/ml) or both. Data represent means ± s.e.mean of 5 experiments each performed in duplicate.*

In contrast to inhibition of Rho-kinase, combined pretreatment (8 days) with inhibitors of either p42/p44 MAPK (PD98059, 30 μM), phosphatidyl inositol 3-kinase (PI 3-kinase) (LY294002, 10 μM) or p38 MAPK (SB203580, 10 μM) prevented PDGF-induced depression of methacholine-induced contraction (Figure 4.4). Similar results were obtained using KCl as a contractile stimulus (data not shown). Note that maximal methacholine-induced contraction was not affected by acute treatment (30 min) with any of the kinase inhibitors mentioned. Rather, they induced a slight rightward shift in the concentration response curve to methacholine (Table 4.1).

Table 4.1 *Acute effects of the kinase inhibitors used on methacholine-induced contraction of BTSM strips.*

	E_{max} (%)	pD_2 (-log M)
Control	100	6.92 ± 0.10
LY294002 10 μM	101 ± 8	6.45 ± 0.13 [a]
PD98059 30 μM	101 ± 9	6.62 ± 0.11 [a]
SB203580 10 μM	106 ± 12	6.59 ± 0.12 [a]
Y27632 1 μM	103 ± 10	6.49 ± 0.15 [a]

Data represent means ± s.e.mean of 4 experiments each performed in duplicate. [a] *$P<0.05$ compared to control.*

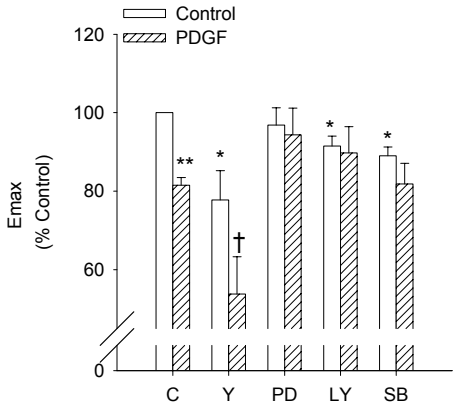

Figure 4.4 *Maximal methacholine-induced contraction (E_{max}) of organ-cultured BTSM strips after 8 days pretreatment with Y27632 (Y, 1 µM), PD98059 (PD, 30 µM), LY294002 (LY, 10 µM) or SB203580 (SB, 10 µM). Vehicle-treated strips served as controls (C). BTSM strips were organ-cultured both in the absence (open bars) and presence (hatched bars) of PDGF (10 ng/ml). Data represent means ± s.e.mean of 5-6 experiments each performed in duplicate. * P<0.05; ** P<0.01 compared to control, † compared to Y27632.*

Effects of Y27632 on bovine tracheal smooth muscle DNA-synthesis and proliferation

Since stimulation of proliferation and phenotypic modulation are tightly correlated in BTSM (Chapter 2) [^{3}H]thymidine incorporation measurements were also performed in response to the above mentioned inhibitors, both in the absence and presence of 10 ng/ml PDGF. Y27632 (1 µM) did not affect basal [^{3}H]thymidine incorporation (96 ± 2%). The response induced by 10 ng/ml PDGF was not affected significantly by Y27632 as well (Figure 4.5). In contrast, PD98059, LY294002 and SB203580 all strongly inhibited basal incorporation to 64 ± 4%, 50 ± 4% and 20 ± 1% of basal, respectively. PDGF-induced DNA-synthesis was strongly reduced by these inhibitors as well (Figure 4.5). Cell proliferation as measured by cell number again was not affected by Y-27632. As expected, PD98059, SB203580 and LY294002 reduced PDGF-induced increases in cell number (Figure 4.6). Interestingly, basal cell number was reduced only after treatment with LY294002 and SB230580 to 70 ± 7% and 70 ± 3% of serum-free treated cell number, respectively.

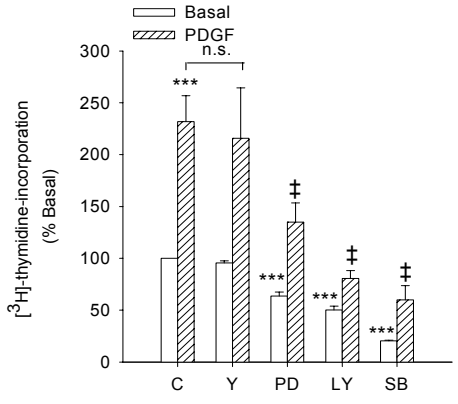

Figure 4.5 *[^{3}H]thymidine incorporation of BTSM cells. Basal responses (open bars) and those in response to PDGF (10 ng/ml, hatched bars) were measured in the presence of vehicle (C), Y27632 (Y, 1 µM), PD98059 (PD, 30 µM), LY294002 (LY, 10 µM) or SB203580 (SB, 10 µM) . Data represent means ± s.e.mean of 6 experiments each performed in triplicate. *** P < 0.001 compared to basal; ‡ P < 0.01 compared to control PDGF response.*

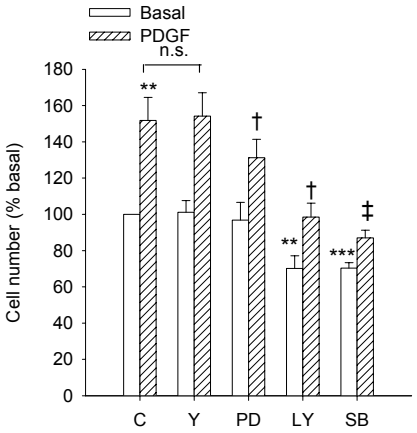

Figure 4.6 *MTT assay of BTSM cells. Basal responses (open bars) and those in response to PDGF (10 ng/ml, hatched bars) were measured in the presence of vehicle (C), Y27632 (Y, 1 μM), PD98059 (PD, 30 μM), LY294002 (LY, 10 μM) or SB203580 (SB, 10 μM) . Data represent means ± s.e.mean of 6 experiments each performed in triplicate. ** P<0.01; *** P < 0.001 compared to basal; † P<0.05; ‡ P < 0.01 compared to control PDGF response.*

Discussion

Rho-kinase has been shown to be involved in ASM contraction through a number of mechanisms. It is known to affect the phosphorylation state of myosin, either by direct phosphorylation [14] or by inhibition of myosin light chain phosphatase [15;16]. In addition, a role for Rho-kinase in non-capacitative calcium entry has been observed in guinea pig ASM [17;18]. Therefore, Rho-kinase may be involved in both calcium-dependent and independent regulation of contraction. In the present study, we show that Y27632 at a Rho-kinase selective concentration [19] inhibits contraction induced by both methacholine and KCl. The relatively large inhibition of KCl-induced contraction was unexpected as Rho-kinase has been described so far to be activated only through receptor-dependent mechanisms [20]. This suggests that potassium depolarization-induced contraction, mediated by opening L-type Ca^{2+}-channels, relies more on Rho-kinase activation than muscarinic agonist-induced contraction which acts mainly through inositol 1,4,5-triphosphate-induced Ca^{2+}-mobilization [21].

We further investigated if Rho-kinase might contribute to phenotype switching or long-term maintenance of contractility. Indeed, prolonged (8 days) pretreatment of BTSM strips with Y27632 induced a significant reduction in contractility. This reduction is comparable to that induced by high concentrations of growth factor (PDGF 10 ng/ml or IGF-1 30 ng/ml (Chapter 2), indicating that Rho-kinase may be a major signalling pathway in the maintenance of contractility. As observed after pretreatment with growth factors, E_{max} to KCl and methacholine were influenced similarly, both quantitatively and qualitatively. These two contractile agonists use totally distinct mechanisms to achieve elevated $[Ca^{2+}]_i$. Therefore changes at the contractile machinery are likely to explain these effects. The observed depression with no effect on sensitivity for methacholine after pretreatment with Y27632 outrules the possibility that the observed effects are the consequence of remaining Y27632 in the tissue during measurements of contraction, since acute treatment with Y27632 induces a rightward shift with no effect on E_{max} at all (Table 4.1). The similarities

between KCl and methacholine after 8 days pretreatment with Y27632 provide additional evidence, since acute effects of Y27632 are more eminent for KCl when compared to methacholine (cf. Figures 4.1 and 4.2).

Since the effects of pretreatment with Y27632 are similar to the effects induced by PDGF, we hypothesised that these effects could be mediated through a common mechanism. However, combined pretreatment with Y27632 and PDGF induced additive depression of both KCl and methacholine-induced contraction, suggesting distinct mechanisms of action. In addition, PDGF is mitogenic, whereas Y27632 is not. The strong relationship (r =0.97) between growth factor-induced effects on contractility and proliferation (Chapter 2) therefore exlcudes the same mechanism to be involved.

In contrast to Rho-kinase, p42/p44 MAPK (PD98059), p38 MAPK (SB203580) and PI 3-kinase (LY294002) all appeared to be involved in phenotypic modulation induced by PDGF, which results in depression of contraction both for KCl and methacholine. As for Y27632, the effects of PD98059, SB203580 and LY294002 cannot be explained by remaining kinase inhibitor in the tissue during the contraction experiment, as neither inhibitor showed acute effects on maximal methacholine-induced contraction (Table 4.1).

In addition to phenotypic modulation, p42/p44 MAPK, p38 MAPK and PI 3-kinase were involved in PDGF-induced DNA-synthesis and proliferation in BTSM cells as well, whereas no such role for Rho-kinase was found. It is noteworthy that pretreatment with SB203580 and LY294002 slightly lowered both basal contractility and basal proliferation, whereas PD98059 did not. These effects may be explained by a role for p38 MAPK and PI 3-kinase, but not for p42/p44 MAPK in preserving cell number. Effects on cell number will, however, not explain the pretreatment effects of Y27632 on contractility in view of the lack of effect on basal cell number. Studies using other smooth muscle cell types have revealed similar effects on phenotypic modulation for p42/p44 MAPK [22;23] and p38 MAPK [23]. The role for PI 3-kinase remains controversial, as it has both been associated with maintenance of the contractile [5] and modulation to the less contractile phenotype [24]. Isoform-specific effects of PI 3-kinase or of downstream targets may explain these opposite effects, as observed for Akt1 and Akt2 in insulin induced differentiation in C2C12 cells [25].

The results presented in this study show that Rho-kinase is involved in maintaining contractility, but has no effect on the induction of the less contractile phenotype by growth factors, which corresponds to the observation that Rho-kinase is not involved in proliferation or DNA synthesis. The effects of Y27632 on contractility may be explained by Rho-kinase dependent effects on the localisation of the transcription factor SRF or on actin remodelling, both of which regulate smooth muscle specific gene expression [9;10]. Lack of effect of Rho-kinase inhibition on mitogenesis as observed by us for BTSM, was also shown for human saphenous vein smooth muscle cell proliferation induced by PDGF-AB [26]. In contrast, Rho-kinase dependent proliferation of rat aortic smooth muscle cells induced by thrombin [13]

and of hepatic stellate cells induced by serum [12] were reported. These differences are likely the result of cell type- or stimulus-specific effects.

In conclusion, the functional contribution of Rho-kinase to contraction is not dependent on the phenotypic state of intact smooth muscle. In addition, Rho-kinase is not involved in phenotypic modulation or proliferation induced by PDGF. However, Rho-kinase is a major regulator involved in the basal maintenance of contractility in BTSM. Apart from beneficial acute effects on contraction, long-term effects of Rho-kinase inhibition may also be beneficial for treatment of airway diseases, as this will induce a less contractile airway smooth muscle state. Since the effect is quantitatively comparable to that induced by growth factors, without the induction of a proliferative phenotype, treatment with Rho-kinase inhibitors do not necessarily contribute to disadvantageous airway remodelling.

Acknowledgements

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Chapter 5

Growth factor-induced contraction of human bronchial smooth muscle is Rho-kinase dependent.

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European Journal of Pharmacology (2004) 494: 73-76.

Abstract
Growth factors have been implicated in the pathophysiology of asthma. However, the putative effects of these growth factors on human airway smooth muscle tone are still largely unknown. We performed contraction experiments using human bronchial smooth muscle ring preparations.The growth factor insulin-like growth factor-1 (IGF-1) induced a slowly developing sustained contraction, which was dependent on Rho-kinase, since contraction was almost completely inhibited by (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y27632; 1 μM). Angiotensin II, a G_q-coupled receptor agonist which can act as a growth factor as well, induced a biphasic contraction, the sustained phase of which was also almost completely inhibited by Y27632. We conclude that angiotensin II and IGF-1 induce a Rho-kinase dependent sustained contraction of human bronchial smooth muscle. Since growth factors are associated with pathophysiologiocal conditions such as asthma, inhibition of Rho-kinase could be effective under these conditions.

Introduction
Growth factors are involved in proliferation and differentiation of smooth muscle cells originating from a variety of tissues, including the vasculature and the airways [1;2]. They are potential contributors to the increased airway smooth muscle (ASM) mass, as found in patients suffering from persistent severe asthma, by stimulating airway smooth muscle proliferation [2].

However, in vascular smooth muscle, several growth factors have been shown to induce a concentration-dependent contraction and to be potential inducers of contractile mediator release [3;4]. The mechanism by which growth factors induce contraction has only been partly elucidated. Recent evidence shows that growth factor receptors, such as the insulin-like growth factor-1 (IGF-1)-receptor, can activate the Rho/Rho-kinase pathway directly [5] and may be involved in smooth muscle contraction via Rho-kinase [6].

As regards ASM, growth factors are known to have long-term effects on bovine tracheal smooth muscle (BTSM) contractile phenotype (Chapter 2) [7] and to have acute contractile effects on guinea-pig tracheal smooth muscle [8]. Thus far however, the acute contractile effects of growth factors on human bronchial smooth muscle and the potential contribution of Rho-kinase has not been described. Therefore, we investigated human bronchial smooth muscle contraction induced by the growth factor IGF-1 and by angiotensin II which can transactivate different growth factor receptors [9] and was shown to be a hypertrophic growth factor for human ASM cells [10]. In addition, we evaluated the possible involvement of Rho-kinase in growth factor-induced contraction.

Methods
Macroscopically normal sections of human lungs, obtained from patients undergoing surgery as a consequence of lung carcinoma, were transported to our laboratory in Krebs-Henseleit (KH) buffer (composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18,

$CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with O_2:CO_2 95:5 % v/v, pH = 7.4). Human bronchi, typically 3-4 mm in diameter, were dissected free from connective tissue and cut into rings of approximately 3 mm in width. Bronchial rings were transferred into Dulbecco's modification of Eagle's medium, supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) and maintained overnight at 37 ˚C in a humidified atmosphere containing 5 % CO_2. The next day, bronchial rings were transferred into organ baths containing KH-buffer (37 ˚C), continuously gassed with O_2:CO_2 95:5 % v/v. Rings were connected to an isometric force displacement transducer, using surgical wire. After equilibration for 90 min with 3 washings, rings were contracted using iso-osmotic KCl-solutions (20 and 40 mM) followed by washing. Finally, after re-equilibration in KH-buffer, rings were contracted with 40 mM KCl as a reference. After two additional washouts, rings were preincubated for 30 min with (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y27632; 1 µM), a selective inhibitor of the Rho-kinase pathway [11] or vehicle, followed by the administration of growth factors or histamine.

Data analysis

All data represent means ± s.e.mean from *n* separate experiments. The statistical significance of differences between data was determined by one-way analysis of variance. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Dulbecco's modification of Eagle's Medium was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Penicillin/streptomycin solution (5000 U/ml ; 5000 µg/ml) was obtained from Gibco BRL Life Technologies (Paisley, U.K.). Insulin-like growth factor (IGF-1, human recombinant) and angiotensin II were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). Y-27632 was from Tocris Cookson, Ltd. (Bristol, U.K.). All other chemicals were of analytical grade.

Results

IGF-1 (10 ng/ml) induced a slow rise of myogenic tone, reaching 17.3 ± 5.7 % of the 40 mM KCl response at t = 30 min (Figure 5.1A). This sustained contraction induced by IGF-1 was prevented almost completely in the presence of Y27632 (1 µM, $P<0.001$; Figure 5.1A). In contrast to IGF-1, angiotensin II (1 µM) induced a biphasic contraction in time, typically reaching its first transient peak approximately at 2 min after administration, which averaged 18.9 ± 6.0 % of the KCl reference, followed by a slower and more sustained rise in myogenic tone, reaching a maximum of 24.5 ± 8.3 % at t = 10 min (Figure 5.1B). Interestingly, in the presence of the Rho-kinase inhibitor Y27632 (1 µM), which lowered basal tone by itself, angiotensin II still induced the transient early-phase rise in contraction at t = 1 min (8.6 ± 3.0 %). The sustained rise, however, was no longer present ($P<0.001$; Figure 5.1B). Contractions induced by the G_q-coupled receptor agonist histamine were not affected at all by treatment with Y27632 (Figure 5.2).

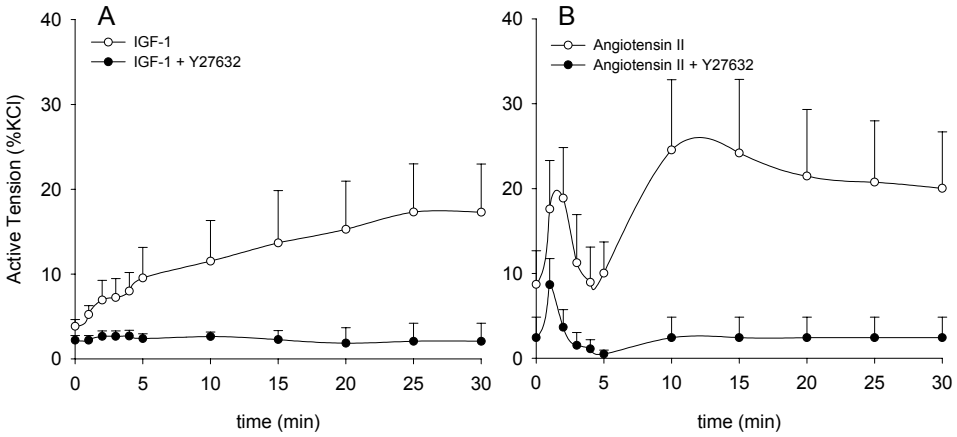

Figure 5.1 *Time-dependent IGF-1 (A) and angiotensin II (B)-induced contraction of isolated human bronchi. Contractile responses were measured both in the absence (open symbols) and presence (closed symbols) of 1 μM Y27632. Shown are the means ± s.e.mean of 3 to 4 experiments each performed in duplicate.*

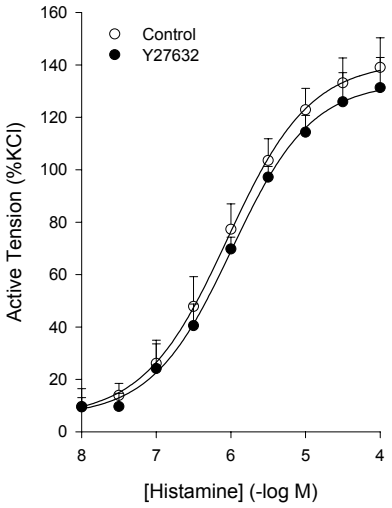

Figure 5.2 *Histamine-induced contraction of isolated human bronchi. Contractile responses were measured both in the absence (open symbols) and presence (closed symbols) of 1 μM Y27632. Shown are the means ± s.e.mean of 4 experiments each performed in duplicate.*

Discussion

The results presented in this study demonstrate that the growth factors IGF-1 and angiotensin II induce Rho-kinase-dependent contractions of human bronchial smooth muscle. The concentrations of growth factor applied in this study are commonly used and represent submaximal to maximal concentrations with respect to other effects such as mitogenesis [12;13]. Quantitatively, these contractions

amounted approximately 20-25 % of 40 mM KCl-induced contraction. These limited contractile effects may clearly have physiological implications as small changes in internal diameter may result in significant limitation of airflow, since flow is proportional to internal radius to the fourth power (Poiseuille's law).

Interestingly, histamine-induced contraction was not affected by this concentration of Y27632, which indicates receptor-specificity for the contribution of Rho-kinase to contraction in the human bronchus. Similarly, methacholine- and KCl-induced contractions are differentially senstitive to Y27632 as also observed in BTSM (Chapter 4; [14]). Since Y27632 predominantly affected the sustained angiotensin II-induced contraction, it seems likely that angiotensin II receptors couple to the Rho/Rho-kinase-pathway selectively for this phase of contraction. In addition to the G_q-coupled consequences of angiotensin AT_1-receptor activation, the angiotensin AT_1-receptor has been shown to be able of transactivating different growth factor receptors [9]. Such a transactivation leads to activation of downstream signaling molecules also used by growth factors themselves. Our results show that Rho-kinase is not involved in the transient contraction by angiotensin II, but only in the slowly developing sustained phase, indicating possible transactivation of growth factor receptors by angiotensin II. In accordance with our observation that IGF-1 induced responses are fully Rho-kinase dependent, this phase is completely abolished by Y27632.

Since growth factors are involved in tissue repair processes, growth factor-induced contraction may protect damaged areas in the airways from the outside air throughout the repair process. In asthma, however, the repair process is usually not restricted to a single segment of the airways and growth factors may then contribute to airflow obstruction. The growth factors used in the present study may be relevant for asthma for a number of reasons. The growth factor IGF-1 is known to be secreted in the airways from various inflammatory cells and ASM [15]. In addition, plasma exudation may increase their tissue concentration during allergen exposure. Also, increased levels of the IGF-binding protein protease matrix metalloproteinase-1 (MMP-1) have been demonstrated in human asthmatic airways, which could increase the bio-availability of IGF-1 [16]. Angiotensin II has also been implicated in asthma as its serum levels are increased in patients with acute severe asthma [17]. Moreover, intravenous administration of angiotensin II in concentrations similar to those endogenously observed in severe asthmatics causes acute bronchoconstriction in mild asthmatics [18]. In addition, antigen-induced airway hyperresponsiveness in guinea pigs has been found in part angiotensin AT_1-receptor dependent [19].

Another Rho-kinase inhibitor, fasudil, is being tested in clinical trials and has been shown to be very effective in inhibiting symptoms associated with hypertension [20] and coronary artery spasm [21]. Rho-kinase inhibitors may surpass other drugs considering their selectivity for the pathophysiological condition. Such a

pathophysiology-primed role may also be relevant in the airways, as repeated allergen challenge is known to increase the role of Rho-mediated Ca^{2+}-sensitization in antigen-induced airway hyperresponsive rats [22]. In addition, Y27632 is known to suppress airway hyperresponsiveness induced by ovalbumin and respiratory syncytial virus in mice [23]. Therefore, the contraction induced by growth factors, which is completely dependent on Rho-kinase for IGF-1 and angiotensin II, may be more pronounced in inflamed areas and Rho-kinase inhibitors may relieve airflow limitations more pronouncedly in these areas when compared to control segments.

In conclusion, this study showed that the growth factors angiotensin II and IGF-1 induce a sustained contraction of human bronchial smooth muscle, which is completely dependent on Rho-kinase, in contrast to the histamine-induced contraction. Since growth factors are associated with pathophysiological conditions such as asthma, growth factor-induced contraction may be pathophysiologically relevant. Under these conditions, inhibition of Rho-kinase may be effective.

Acknowledgements

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Chapter 6

Muscarinic M_3 receptor dependent regulation of airway smooth muscle contractile phenotype

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Abstract

Airway smooth muscle (ASM) cells are known to switch from a contractile to a proliferative and synthetic phenotype in culture in response to serum and growth factors. Phenotype switching in response to contractile agonists, however, is poorly characterised, despite of the possible relationship between ASM phenotype and airway remodelling in asthma. To investigate the effects of muscarinic receptor stimulation on ASM phenotype, we used organ-cultured bovine tracheal smooth muscle (BTSM) strips, in which contractile responsiveness, contractile protein expression and proliferation were measured after pretreatment with methacholine. Long-term methacholine pretreatment (8 days) decreased maximal contraction and sensitivity to methacholine as well as to histamine and KCl. This decrease was dose-dependent (pEC_{50} = 5.2 ± 0.1). Pretreatment with the highest concentration of methacholine applied (100 μM) could suppress maximal histamine-induced contraction to 8 ± 1 % of control. In addition, contractile protein expression (myosin, actin) was downregulated twofold. No concomitant increase in proliferative capacity was observed. The M_3/M_2 muscarinic receptor antagonist DAU 5884 (0.1 μM) completely inhibited the observed decrease in contractility. In contrast, the M_2/M_3 muscarinic receptor antagonist gallamine (10 μM) was ineffective, demonstrating that M_2 receptors were not involved. Pretreatment (8 days) with 60 mM KCl could mimick the strong decreases in contractility. This was completely prevented by pretreatment with verapamil (1 μM). Regulation of contractility was not affected by protein kinase C inhibition, whereas inhibitors of phosphatidyl inositol 3-kinase and p42/p44 mitogen activated protein kinase were partially effective. These results show that long-term methacholine pretreatment (8 days) induces a M_3 receptor-dependent decrease in BTSM contractility without increased proliferative capacity.

Introduction

Serum and growth factors are known to induce a switch in airway smooth muscle (ASM) phenotype, which is associated with a decreased contractile responsiveness and an increased proliferative capacity [1]. In a previous study, described in Chapter 2, we showed that long-term pretreatment up to 8 days of organ-cultured bovine tracheal smooth muscle (BTSM) strips with growth factors decreases contractility in a time-dependent fashion. In addition, it was shown that the extent to which growth factors modulate contractility is linearly related to their proliferative response, which is in agreement with the concept that phenotypic changes occur in intact smooth muscle [2]. The mechanisms involved in this phenotypic shift are still not fully clear, although considerable evidence exists to support the hypothesis that the p42/p44 MAPK-pathway is involved in modulation toward a less contractile phenotype [2-4]. PI 3-kinase activity, however, has both been associated with a decrease in contractility [2] and an increase in contractility in smooth muscle cells [5].

Muscarinic receptor stimulation may also play a role in phenotypic modulation. Both the G_i-coupled M_2 receptor and the G_q-coupled M_3 receptor are known to activate p42/p44 MAP kinase [6] and PI 3-kinase [7] and may as such modulate ASM phenotype. ASM proliferation is dependent on the same pathways [8], yet

muscarinic receptor agonists are poor mitogens [9]. Since proliferative response and modulation of contractility are linearly correlated, this would imply a relatively small role for non-mitogenic GPCR agonists in phenotypic modulation. However, proliferation synergy between growth factors and GPCR agonists has been reported on multiple occasions [9-11]. Hence, it can be imagined that muscarinic receptor agonists may augment growth factor-induced phenotypic modulation to a proliferative, less contractile state without large effects on phenotype by themselves. It has been reported, however, that the Rho/Rho-kinase pathway is involved in the activation of smooth muscle specific gene transcription through regulation of the nuclear localisation of serum response factor (SRF) [12]. By activating RhoA, muscarinic receptor agonists might increase contractility or reduce the growth factor-induced decrease in contractility.

It has been postulated that ASM phenotype switching may be involved in airway remodelling in patients with chronic asthma, characterised by increased smooth muscle mass and irreversible airflow obstruction [13]. Since exaggerated cholinergic reflex mechanisms contribute to bronchial hyperreactivity in asthmatics [14] and in allergen challenged guinea pigs [15] and since dysfunctional inhibitory M_2 autoreceptors contribute to increased vagal acetylcholine in asthmatics [14] and in guinea pigs [16], the long-term effects of muscarinic receptor agonists on ASM phenotype may be relevant for asthma. Since extracellular matrix proteins may influence ASM phenotype [2;17;18], we used organ cultured BTSM strips with intact endogenous matrix. It was found that pretreatment with the muscarinic receptor agonist methacholine induces a less contractile phenotype with no concomitant increase in proliferative capacity, that was mediated by muscarinic M_3 receptors.

Methods

Tissue preparation and organ culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5 % CO_2 and 95 % O_2; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco's modification of Eagle's medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (1.5 µg/ml). Next, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml serum-free medium was added per tissue strip. Drugs were added in a small volume (7.5 µl). Strips were maintained in culture in an incubator shaker (37 ˚C, 55 rpm) for 8 days, refreshing the medium on day 4.

Isometric tension measurements

Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5 % CO_2 and 95 % O_2, pH 7.4 at 37 ˚C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37 ˚C, continuously gassed with 5 % CO_2 and 95 % O_2, pH 7.4. During a 90 min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, muscle strips were precontracted with 20 mM and 30 mM isotonic KCl solutions. Following two wash-outs, basal smooth muscle tone was established by the addition of 0.1 µM isoprenaline and tension was re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min cumulative concentration response curves (CRCs) were constructed to stepwise increasing concentrations of histamine (10 nM – 100 µM), isotonic KCl (5.6 – 50 mM) or methacholine (1 nM – 100 µM). Only one CRC was constructed per tissue strip. When maximal tension was obtained, the strips were washed several times and basal tone was established using isoprenaline (10 µM).

[^{3}H]Thymidine-incorporation

To obtain a proper comparison with functional responses of tissue-cultured strips (with intact extracellular matrix), we used tissue slices instead of cells for [^{3}H]thymidine-incorporation experiments, following essentially the same procedure as in BTSM cells [10]. After dissection of the smooth muscle layer, the muscle was chopped three times at a setting of 500 µm and three times at a setting of 100 µm (McIlwain tissue chopper). Tissue slices were then transferred to suspension culture flasks containing medium, supplemented as mentioned above. Methacholine (10 µM) was included in the medium for 8 days, refreshing the medium on day 4. Tissue slices were then washed extensively by centrifugation with phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4) and transferred into 6-well cluster plates at 50 mg wet weight/ml per well. Slices were stimulated with medium with or without PDGF (10 ng/ml) for 48 hours in the presence of [^{3}H]thymidine. After incubation the slices were washed twice with PBS at room temperature and once with ice-cold 5 % trichloroacetic acid (TCA). Slices were treated with this TCA-solution on ice for 30 min and subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

Western analysis of contractile protein expression

After pretreatment of tissue strips as described under organ culture procedures, homogenates were prepared by pulverizing tissue under liquid nitrogen and subsequent sonification in homogenization buffer (composition in mM: NaCl 150.0, Tris HCl 10.0, 2-glycerophosphoric acid 5.0, EGTA 2.0, DTT 2.0, PMSF 1.0, Na_3VO4 1.0, NaF 1.0, pH 7.5, supplemented with 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 % Triton X-100). Homogenates were stored at –80 ˚C until further use. Protein content was determined according to Bradford [19]. 20 µg of protein per lane was separated by SDS/PAGE on 6 % (for sm-myosin heavy chain, sm-MHC) or 10 % (for

sm-α-actin) polyacrylamide gels. Proteins in the gel were then transferred onto nitrocellulose membranes, which were blocked overnight in blocking buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; Tween 20 0.1 %, dried milk powder 5 %). After two washes in wash buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; Tween 20 0.1 %), membranes were incubated for 1 h at room temperature in primary antibodies (anti-sm-MHC or anti-sm-α-actin, both diluted 1:200 in blocking buffer). After three washes, membranes were incubated in horseradish peroxidase labelled secondary antibodies (dilution 1:1000 in blocking buffer) at room temperature for 1 h, followed by another three washes. Antibodies were then visualised by enhanced chemiluminescence. Blots were analyzed by densitometry (Totallab ^{tm}).

Data analysis

All data represent means ± s.e.mean from *n* separate experiments. The statistical significance of differences between data was determined by the Student's *t*-test for paired observations or one-way ANOVA, where appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Dulbecco's modification of Eagle's Medium (DMEM) and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Foetal bovine serum, $NaHCO_3$ solution (7.5 %), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml / 5000 µg/ml) and amphotericin B solution (250 µg/ml) (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Mouse monoclonal anti sm-MHC was from Neomarkers (Fremont, CA, USA). Platelet-derived growth factor AB (PDGF-AB, human recombinant), insulin (from bovine pancreas), mouse monoclonal anti sm-α-actin, rabbit anti-mouse IgG (peroxidase conjugate), sodium-dodecyl sulphate, aprotinin, leupeptin, apotransferrin (human), soybean trypsin inhibitor, gallamine triethiodide, histamine dihydrochloride and (-)isoprenaline hydrochloride were from Sigma (St. Louis, MO, U.S.A.). Enhanced chemiluminescence reagents were from Pierce (Rockford, IL, USA). PD98059 and LY294002 were obtained from Tocris Cookson Ltd. (Bristol, UK.). DAU5884 was a kind gift of Dr. H.N. Doods (Dr. Karl Thomae GmbH, Biberach, Germany). L(+)ascorbic acid was from Merck (Darmstadt, Germany). [*methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.) All other chemicals were of analytical grade.

Results

Effects of pretreatment with methacholine on bovine tracheal smooth muscle phenotype.

Long-term pretreatment (8 days) of BTSM strips with 10 µM methacholine resulted in a decline in contractility, as determined using both KCl, histamine and methacholine-induced contraction (Figure 6.1, Table 6.1). Apart from a diminished maximal

contraction (E_{max}), sensitivity (pEC_{50}) to all agonists used was decreased as well. This decrease in sensitivity was the most profound for methacholine itself (Table 6.1).

Table 6.1 *Maximal contraction (E_{max}) and sensitivity of BTSM strips for KCl, histamine and methacholine after 8 d pre-treatment in the absence or presence of 10 µM methacholine.*

	Control		*Methacholine (10 µM) pre-treated*	
	sensitivity	E_{max} (%)	sensitivity	E_{max} (%)
KCl	20.8 ± 1.1	100	25.7 ± 0.9*	37 ± 5***
Histamine	5.9 ± 0.1	100	5.7 ± 0.1*	46 ± 3***
Methacholine	7.3 ± 0.1	100	6.5 ± 0.1*	48 ± 6*

*Sensitivity to KCl expressed as EC_{50} (mM); sensitivity to histamine and methacholine expressed as pEC_{50} (-log M). Data represent means ± s.e.mean from 4 (methacholine) to 8 (KCl, histamine) experiments, each performed in duplicate. *P<0.05; ***P<0.001 compared to control.*

Pretreatment with 10 ng/ml PDGF also induced a decline in E_{max} of histamine, though considerably smaller as compared to methacholine pretreatment, but no effect on sensitivity was observed (Figure 6.2). Remarkably, combined pretreatment with 10 µM methacholine and 10 ng/ml PDGF depressed maximal contraction in an additive rather than synergistic fashion (Figure 6.2).

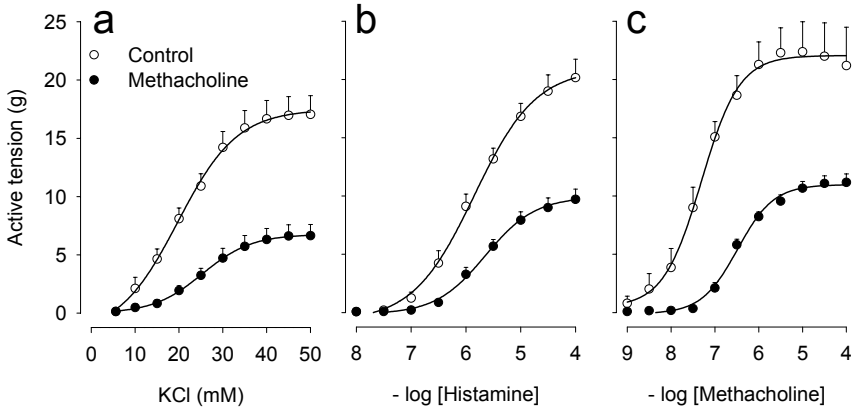

Figure 6.1 *Effect of 8 d methacholine pretreatment on BTSM contractility. BTSM strips were organ cultured for 8 days in the absence (open symbols) or presence (closed symbols) of 10 µM methacholine, after which contraction to (A) KCl, (B) histamine and (C) methacholine-induced was determined. Data represent means ± s.e.mean from 4 (methacholine) to 8 (KCl, histamine) experiments, each performed in duplicate.*

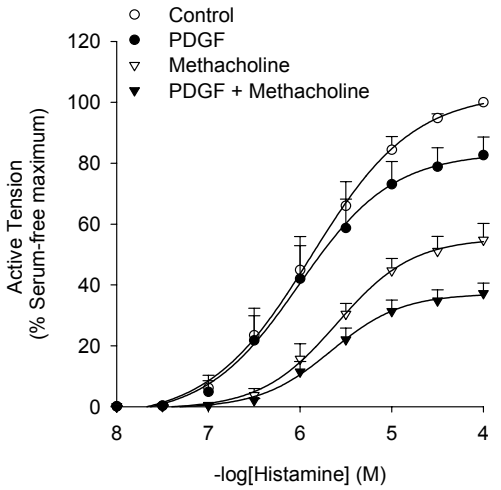

Figure 6.2 *Interaction of methacholine and PDGF pretreatment on histamine-induced BTSM contraction. BTSM strips were organ cultured for 8 days in the absence or presence of methacholine (10 μM), PDGF (10 ng/ml) and the combination hereof. Data represent means ± s.e.mean from 5 experiments, each performed in duplicate.*

The decrease in contractile capacity after pretreatment with methacholine was not accompanied by an increase in proliferative capacity. Basal incorporation of [^{3}H]thymidine was not changed after pretreatment with methacholine. Interestingly, stimulated (PDGF-induced) incorporation of [^{3}H]thymidine was decreased rather than increased (P<0.05, Figure 6.3). It should be noted that methacholine was present during pretreatment only and was washed away thoroughly before the start of the [^{3}H]thymidine-incorporation experiment, to prevent a functional interaction with PDGF.

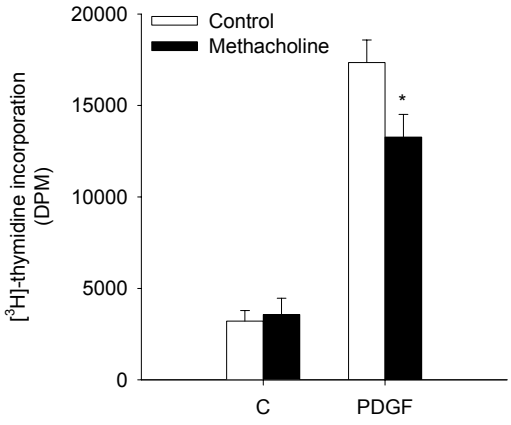

Figure 6.3 *Basal and PDGF-stimulated (10 ng/ml) [^{3}H]thymidine incorporation in BTSM tissue slices, organ cultured for 8 days in the absence or presence of 10 μM methacholine. After the cultue period, tissue slices were thoroughly washed and stimulated thereafter. Data represent means ± s.e.mean from 4 experiments, each performed in duplicate. *P<0.05 compared to control.*

A concentration response curve could be constructed for the inhibitory effect of methacholine pretreatment on maximal histamine-induced contraction, that was characterized by a pEC_{50} of 5.2 ± 0.1. Maximal contraction to histamine was almost abolished by pretreatment with the highest concentration of methacholine tested (Figure 6.4). A similar concentration dependency was observed for contractile protein expression in BTSM strips, pretreated with methacholine (Figure 6.5). Both sm-α-actin and sm-MHC were considerably downregulated to 41 ± 12 % and 50 ± 13 % of control, respectively at the highest concentration of methacholine applied (100 μM).

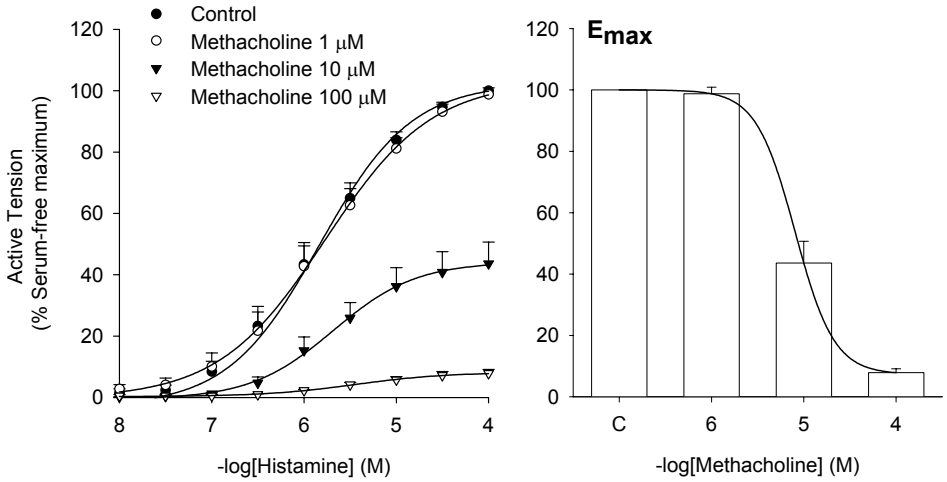

Figure 6.4 *Concentration dependency of the methacholine-induced pretreatment effects. (A) Histamine-induced contraction of BTSM strips, organ cultured for 8 days in the absence or presence of increasing concentrations of methacholine. (B) Maximal contraction (E_{max}) of histamine of BTSM strips, organ cultured with increasing concentrations of methacholine. Data in (B) represent maximal contractions measured under (A). Data represent means ± s.e.mean from 4 experiments, each performed in duplicate.*

Role of M_2 and M_3 receptors

To establish the muscarinic receptor subtype(s) involved in these responses to methacholine, we measured the inhibitory effects of selective receptor antagonists DAU5884 and gallamine. Since tracheal smooth muscle expresses M_3 and M_2 muscarinic receptors only [20], DAU5884 was applied in a concentration selective for M_3 over M_2 receptors and gallamine in a concentration selective for M_2 over M_3 receptors [21]. The antagonists were applied during the entire pretreatment period. Interestingly, combined pretreatment with DAU5884 could completely prevent the

strong methacholine (10 µM)-induced decline in E_{max} of histamine. In contrast, gallamine was fully ineffective (Figure 6.6).

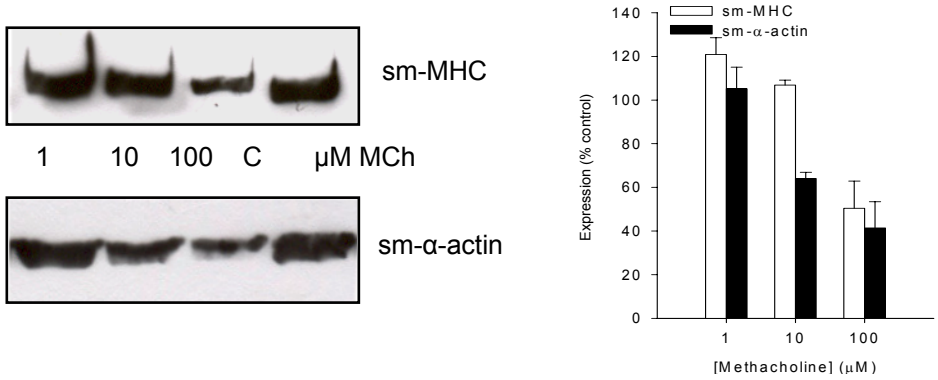

Figure 6.5 *Western analysis of contractile protein expression (sm-MHC, sm-α-actin) in BTSM strips, organ cultured for 8 days in the absence (C) or presence of increasing concentrations of methacholine. Blots shown are representative for 4 expriments. Graph shows the means ± s.e.mean obtained after densitometry scans of the blots.*

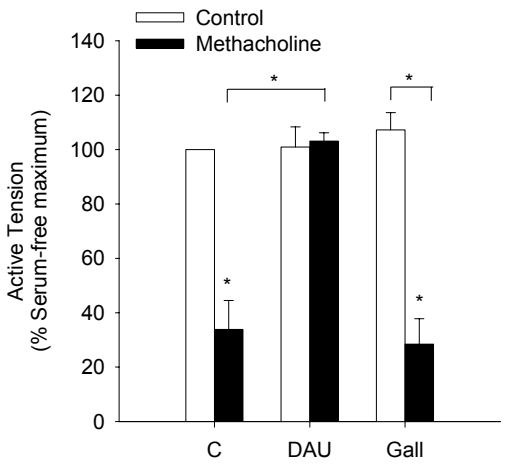

Figure 6.6 *Role of M_2 and M_3 receptors in the methacholine induced effects on BTSM contractility. BTSM strips were organ cultured for 8 days in the absence (open bars) or presence (closed bars) of 10 µM methacholine. During the entire culture period, BTSM strips were also incubated with DAU5884 (0.1 µM, DAU) or gallamine (10 µM, Gall) where indicated. Untreated preparations served as controls (C). Data shown represent maximal contractile responses to histamine and are means ± s.e.mean from 4 experiments, each performed in duplicate. *P<0.05.*

Effect of prolonged $[Ca^{2+}]_i$-increases

Long-term pretreatment with methacholine may induce a prolonged rise in intracellular $[Ca^{2+}]$. To investigate a possible role for Ca^{2+} in long-term regulation of contractility, strips were pretreated for 8 days with medium suppplemented with $CaCl_2$ (2.5 mM) and KCl (60 mM) to induce a prolonged increase in intracellular $[Ca^{2+}]$ by opening of L-type Ca^{2+}-channels. This pretreatment strongly decreased histamine-induced contraction when compared to control strips. Combined pretreatment with the Ca^{2+}-entry blocker verapamil (1 µM) completely prevented this 60 mM K^+-induced decrease (Figure 6.7).

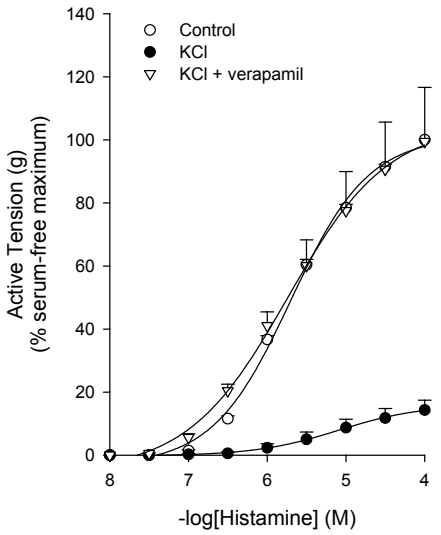

Figure 6.7 *Effects of prolonged increases in intracellular $[Ca^{2+}]$ on BTSM contractility. BTSM strips were organ cultured for 8 days in serum-free medium (open circles), in medium supplemented with $CaCl_2$ and KCl to produce final concentrations of 2.5 mM Ca^{2+} and 60 mM K^+, respectively (closed circles), or in medium supplemented with verapamil (1 µM) and $CaCl_2$ and KCl (open triangles). Data shown represent contractile responses to histamine and are means ± s.e.mean from 3 experiments, each performed in duplicate. * P<0.05; ** P<0.01.*

Role of protein kinase C, PI 3-kinase and p42/p44 MAP kinase

To investigate the contribution of protein kinase C (PKC), PI 3-kinase and p42/p44 MAP kinase to the methacholine-induced decline in contractility, selective inhibitors were used: GF109203X (10 µM) for PKC, LY294002 (10 µM) for PI 3-kinase and PD 98059 (30 µM) for p42/p44 MAP kinase. All kinase inhibitors were dissolved in dimethylsulfoxide (DMSO) at a final concentration of 0.1 % (vehicle), which did not affect the effect of methacholine pretreatment (E_{max} = 34 ± 11 % and 39 ± 11 % in the absence and presence of DMSO, respectively). With GF109203X present during the culture period, no reduction in the methacholine-induced effects was observed. In contrast, both PD98059 and LY294002 were partially but significantly effective (Figure 6.8).

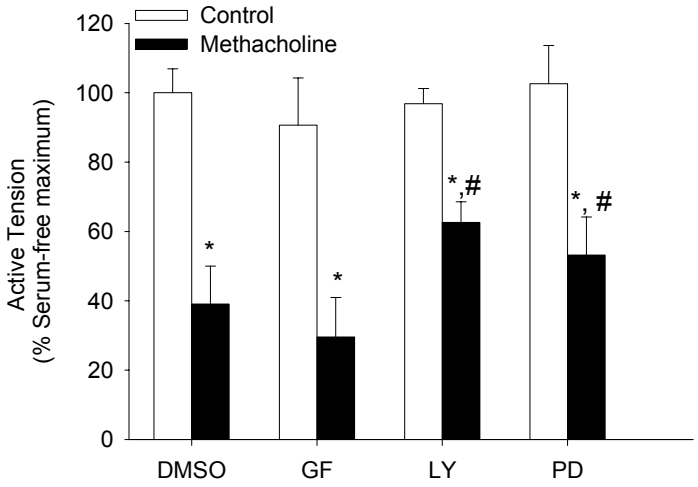

Figure 6.8 *Role of PKC, p42/p44 MAP kinase and PI 3-kinase in the methacholine induced effects on BTSM contractility. BTSM strips were organ cultured for 8 days in the absence (open bars) or presence (closed bars) of 10 μM methacholine. During the entire culture period, GF 109203X (10 μM, GF) PD 98059 (30 μM, PD) or LY 294002 (10 μM, LY) were also present where indicated. Vehicle treated (0.1% DMSO) preparations served as controls. Data shown represent maximal contractile responses to histamine and are means ± s.e.mean from 4 experiments, each performed in duplicate. * P<0.05 compared to controls, # P<0.05 compared to methacholine (10 μM) and DMSO (0.1 %)-pretreated.*

Discussion

In the present study we found that pretreatment with methacholine dramatically decreases contractile responsiveness of organ-cultured BSTM strips towards both receptor- and nonreceptor-mediated stimuli. Since contractility of 8 days serum-free pretreated strips is only slightly higher compared to fresh tissue (Chapter 2), it can be concluded that the methacholine-induced phenotype is also less contractile when compared to fresh tissue. This less contractile state induced by methacholine appeared to be different of nature from that induced by growth factors and serum: pretreatment with methacholine could almost completely abrogate contractile responses, whereas maximal modulation of contractility induced by the highly mitogenic growth factor PDGF amounts only 33-37 % (Chapter 2).

Furthermore, sensitivities (EC_{50} / pEC_{50}) to KCl, histamine and methacholine were all decreased after pretreatment with methacholine, which is not observed after pretreatment with serum and growth factors. Not surprisingly, this shift in sensitivity was the largest for methacholine itself, indicating homologous desensitisation of

muscarinic M_3 receptors as well. Hypothetically, histamine H_1 receptors and voltage dependent Ca^{2+} channels may also have been downregulated or desensitised, since G_q coupled receptors may induce both homologous and heterologous desensitisation [22]. Nevertheless, it is unlikely that changes in receptor density or coupling efficiency account for the observed decreases in maximal contraction. First, maximal KCl, histamine and methacholine-induced contraction were influenced similarly by methacholine pretreatment, both qualitatively and quantitatively. This implies the involvement of a common mechanism. However, methacholine and histamine induce contraction through stimulation of PI-turnover and subsequent release of Ca^{2+} from intracellular stores followed by capacitative Ca^{2+}-entry [20]. In contrast, KCl-induced contraction is fully dependent on voltage dependent Ca^{2+}-influx and does not require GPCRs. Therefore, quantitative similarities between KCl, histamine and methacholine can be achieved only by affecting contraction downstream of intracellular Ca^{2+}-increases. Moreover, contractile protein expression (sm-α-actin, sm-MHC) was dramatically decreased after methacholine pretreatment with a similar concentration dependency as observed for the decrease in contractility. Taken together, this clearly indicates that changes in contractile protein expression rather than changes in receptor density are responsible for the observed decrease in maximal contraction.

Basal incorporation of [^{3}H]thymidine was not changed after pretreatment with methacholine, whereas stimulated (PDGF) incorporation of [^{3}H]thymidine was decreased, which might indicate desensitisation of receptor tyrosine kinases. Note that the present experiments were performed in tissue slices rather than in cells to avoid loss of cell culture-induced muscarinic receptor expression [23]. However, PDGF-induced incorporation of [^{3}H]thymidine produced quantitatively similar results in tissue slices and cells. The observation that the decrease in contractility induced by methacholine was not accompanied by an increase in proliferative capacity further indicates that the phenotype change induced by methacholine is different from the classical switch induced by serum and growth factors. The decline in contractility induced by growth factors is accompanied by an increase in proliferation, which represents a shift in smooth muscle function for optimal adaptation to a mitogenic environment.

Given the inability of methacholine to induce ASM proliferation by itself [9], and the strong correlations found for loss of contractility and proliferative responses induced by growth factors (Chapter 2), methacholine and growth factors are not likely to rely on the same mechanism to induce phenotype changes. Indeed, although PI 3-kinase and p42/p44 MAP kinase are involved, at least in part, in the growth factor-induced phenotype shift (Chapters 2, 4), inhibitors of the p42/p44 MAP kinase pathway and PI 3-kinase only modestly inhibited the methacholine-induced decline in contractility. Inhibition of PKC using GF109203X was even completely ineffective. It is noteworthy that the applied concentrations of GF109203X and LY294002 are known to almost completely inhibit PKC and PI 3-kinase, respectively [24], whereas the applied concentration of PD98059 is known to fully inhibit p42/p44 MAPK activation in BTSM cells [25]). Therefore, it appears that p42/p44 MAPK, PI 3-kinase

and PKC do not play a key role in the methacholine -induced effects, although p42/p44 MAPK and PI 3-kinase may contribute to some extent.

The observed effects of methacholine pretreatment are dependent on muscarinic M_3 receptors as determined using DAU5884. In contrast, M_2 receptors are not relevant in view of the lack of effect of gallamine. Importantly, BTSM contraction is fully dependent on M_3 receptors [21]. Therefore it could be envisaged that the presented regulation of contractility by methacholine is dependent on the contractile state of the muscle during the pretreatment period. Pre-treatment with 1 μM methacholine has no effect at all, however, on any of the tested phenotype parameters (c.f. Figures 6.4 and 6.5), whereas BTSM strips acutely exposed to this concentration of methacholine contract almost maximally (88 ± 6 % of maximum). More specifically, the sensitivities of methacholine for the long-term effects on contractility (pEC_{50} = 5.2) and acute effects on BTSM contraction (pEC_{50} = 7.0) are too deviant. It could be envisaged that methacholine may have underwent some degree of metabolism during the incubation period, which would (in part) explain the difference in pEC_{50} values for contraction and for contractility changes. However, even the strips exposed to 1 μM methacholine were still contracted on day 4 when refreshing the medium and on day 8 when preparing for the experiment, demonstrating that a causal relationship between contractile status and regulation of contractility can be excluded. A better match is observed with the potency of methacholine on phosphoinositide metabolism (pEC_{50} = 5.6, [26]. Moreover, prolonged increases in $[Ca^{2+}]_i$ are known to attenuate contractility in the organ cultured rat tail artery [27;28] and of the organ cultured guinea pig ileum [30]. In addition, induction of a sustained increase in $[Ca^{2+}]_i$ using medium containing 60 mM K^+ and 2.5 mM Ca^{2+} strongly decreased contractility of BTSM, which was fully dependent on Ca^{2+}-entry in view of the effect of combined pretreatment with verapamil. Therefore, it seems reasonable to assume that the prolonged increases in $[Ca^{2+}]_i$ caused by long-term M_3 receptor stimulation is the cause of the observed decrease of contractility. The mechanisms behind these effects of $[Ca^{2+}]_i$ remain to be established, however.

A regulatory mechanism in smooth muscle cells to adjust for prolonged increases in $[Ca^{2+}]_i$ may be physiologically relevant. However, activation of the Rho/Rho-kinase/SRF-pathway may act as an opposing mechanism to increase contractility [12;30]. It is not clear how the balance in these mechanisms relates to long-term regulation of contractility. The phenotypic starting-point may be of critical relevance: the highest SRF-mediated smooth muscle specific gene transcription is observed in synthetic, not contractile smooth muscle cells [31]. Since intact BTSM was used in our present study, which consists of contractile cells, studies using cultured synthetic cells may observe other effects of GPCR stimulation. The phenotypic status may therefore be very relevant for the effects of long-term stimulation with GPCR agonists and ultimately determining the effect on contractility *in vivo*. In addition, load applied to the muscle during culture may also interact with GPCR-induced effects on smooth muscle contractility or phenotype since mechanical strain is known to increase RhoA activation and ASM contractile protein expression [32;33]. Since

ASM is subjected to (some) load under physiological conditions, the *in vivo* response to prolonged GPCR agonist exposure may therefore be somewhat more complex.

In conclusion, prolonged treatment with methacholine strongly decreases BTSM contractility without a concomitant increase in proliferative capacity. The decreased contractility is explained by diminished contractile protein expression and depends on M_3 receptor stimulation and subsequent increases in intracellular [Ca^{2+}] . Since muscarinic M_3 receptor stimulation is non-mitogenic by itself, this indicates that the phenotype change induced by methacholine is different from the classical switch induced by serum and growth factors.

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Chapter 7

Muscarinic M_3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle

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Abstract
Muscarinic receptor agonists have been considered to act synergistically in combination with growth factors on airway smooth muscle (ASM) growth. Characterization of the proliferative responses and of the receptor subtype(s) involved has not yet been studied. Therefore, we investigated mitogenesis induced by stimulation of muscarinic receptors, alone and in combination with stimulation by platelet-derived growth factor (PDGF). For this purpose, [^{3}H]thymidine-incorporation was measured at different culture stages in bovine tracheal smooth muscle (BTSM) cells. Functional muscarinic M_3-receptors, as measured by formation of inositol phosphates, were present in unpassaged cells, but were lacking in passage 2 cells. Methacholine (10 μM) by itself was not able to induce a proliferative response in both cell culture stages. However, methacholine interacted synergistically with PDGF in a dose-dependent fashion (0.1-10 μM), but only in cells having functional muscarinic M_3-receptors. This synergism could be suppressed significantly by the selective M_3-receptor antagonists DAU5884 (0.1 μM) and 4-DAMP (10 nM), but not at all by the M_2-subtype selective antagonist gallamine (10 μM). These results show that methacholine potentiates mitogenesis induced by PDGF solely through stimulation of muscarinic M_3-receptors in BTSM cells.

Introduction
Airway smooth muscle (ASM) expresses muscarinic receptors of the M_2- and the M_3-subtype. The M_3-subtype is responsible for contraction, largely through G_q-mediated activation of phosphoinositide turnover and subsequent Ca^{2+} mobilization [1]. The role of the majority of M_2-receptors in ASM is still unclear, however. In bovine tracheal smooth muscle (BTSM), a role for the M_2-receptor in functional antagonism of β-adrenergic responses through inhibition of adenylyl cyclase has been suggested. This was observed only after substantial 4-DAMP mustard-induced alkylation of the M_3-receptor population [2]; under normal conditions, M_2-receptors are not interfering with the isoprenaline-induced relaxation of cholinergic tone as demonstrated by the lack of effect of selective M_2-receptor blockade [3]. Recently, however, a role for M_2-receptors in Ca^{2+}-sensitization and cytoskeletal reorganization has been proposed [4-6]. Furthermore, M_2-receptors may stimulate non-selective cation channels through $G_i/G_{(o)}$-proteins, resulting in a rise in $[Ca^{2+}]_i$ [7].

Muscarinic receptor agonists have been reported to be mitogenic for human ASM cells, though at most modestly, and to respond synergistically in combination with growth factors [8;9]. Although carbachol-induced mitogenesis has been reported to be pertussis toxin (PTX)-sensitive [8;10], suggesting a role for the G_i-protein-coupled muscarinic M_2-receptor, measurements were carried out using human ASM cells in culture, which are known to have a relatively small functional M_3-receptor population compared to non-cultured cells. This loss in receptor function is far less profound for the M_2-receptor subtype [11]. A role for the M_3-subtype in proliferation can therefore not be ruled out, nor can the putative relevance of the M_2-subtype in ASM proliferation be properly estimated because of the diminished presence of muscarinic M_3-receptors. Moreover, although PTX was found to decrease the

carbachol-induced mitogenic response, proliferation to all applied stimuli was reduced to a similar extent by treatment with PTX [8]. Therefore, it may not be appropriate to conclude that muscarinic receptor stimulation-induced mitogenic responses are M_2-receptor mediated.

Theoretically, both M_2 and M_3-receptors could account for the mitogenic contribution of muscarinic receptor stimulation. The p42/p44 mitogen activated protein kinase (MAPK)-pathway is generally associated with proliferation and is known to be involved in the proliferative responses to various mitogens in BTSM [12-15]. Muscarinic M_3-receptor-stimulation induces a considerable rise in $[Ca^{2+}]_i$, which may lead to the activation of p42/p44 MAPK through Pyk2 and Ras-dependent mechanisms [16]. Moreover, the M_3-receptor subtype activates protein kinase C (PKC), which may lead to p42/p44 MAPK-activity through PKCα-mediated phosphorylation of Raf-1 [17]. In support of this hypothesis, CHO-cells transfected with the wild-type M_3-receptorsubtype, demonstrated activation of the p42/p44 MAPK pathway induced by carbachol [18]. This M_3-receptor mediated p42/p44 MAPK-activity was inhibited almost completely using the PKC-inhibitor Ro-318220. Moreover, stimulation with the PKC-activator PDBu could partially mimick this response, suggestive of a significant role for PKC in the response [18;19].

G_i-proteins, however, activated by muscarinic M_2-receptors, may also activate the p42/p44 MAPK-pathway through either $G_{i\alpha}$ [20] or $G_{i\beta\gamma}$-dependent [21] mechanisms. In canine colonic smooth muscle, it has been demonstrated that M_2- rather than M_3-receptors are responsible for p42/p44 MAPK-activation [22]. Moreover, it has been demonstrated that M_2-receptors activate the non-receptor tyrosine kinase Src in the same tissue [23], which acts as a key intermediate in tyrosine kinase signaling. Also in CHO-cells, transfected with the wild-type M_2-receptor, methacholine-induced p42/p44 MAPK-activation has been reported [24].

Proliferative responses following selective M_2- or M_3-muscarinic receptor stimulation in airway smooth muscle have not yet been described. In the present study, we investigated their putative involvement in BTSM cells. Since muscarinic M_3-receptors have been reported to lose their function rapidly in culture [11], we used both passaged and unpassaged BTSM cells. In order to gain insight in the receptor subtype(s) involved in methacholine-induced mitogenic responses, we studied the effects of subtype-selective receptor blockade. It was demonstrated that mitogenic responses to muscarinic receptor stimulation alone were absent. However, the mitogenic responses to platelet-derived growth factor (PDGF) were augmented by methacholine, which was solely mediated by the M_3-receptor subtype.

Methods

Isolation of bovine tracheal smooth muscle cells

Bovine tracheae were obtained from local slaughterhouses and transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and

glucose 5.50, pregassed with 5 % CO_2 and 95 % O_2; pH 7.4. After dissection of the smooth muscle layer and removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 300 µm and three times at a setting of 100 µm. Tissue particles were washed two times with Dulbecco's Modification of Eagle's Medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml) and 0.5 % Fetal Bovine Serum (FBS). Enzymatic digestion was performed using the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and Soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 ˚C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 µm gauze, cells were washed three times in DMEM, supplemented as above, containing 10 % FBS.

Cell culture

After isolation, BTSM cells were either used directly for experiments (unpassaged cells) or seeded in 25 cm^2 culture flasks at a density of $1x10^6$ cells/ml for further culturing. Cultured cells were kept viable in medium containing 10 % FBS at 37˚C in a humidified 5 % CO_2-incubator. Medium was refreshed every 48-72 h. Cell cultures were allowed to grow and, upon confluency, were passaged further at a 1:2 split ratio, by means of trypsinization. Cultured cells were used for experiments in passage 2.

[^{3}H]Thymidine-incorporation

BTSM cells were plated in 24 well cluster plates at a density of 30,000 cells per well and were allowed to attach overnight in 10 % FBS containing medium at 37˚C in a humidified 5 % CO_2-incubator. Cells were washed two times with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4) and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg/ml), ascorbate (100 µM) and insulin (1 µM) for 72 h. Cells were then washed with PBS and stimulated with mitogens in FBS-free medium for 28 h, the last 24 h in the presence of [^{3}H]thymidine (0.25 µCi/ml), followed by two washes with PBS at room temperature and one with ice-cold 5 % trichloroacetic acid (TCA). Cells were treated with this TCA-solution on ice for 30 min; subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting.

Accumulation of [^{3}H]-inositol phosphates

BTSM cells were plated after isolation or after passage 2 in 6 well cluster plates at a density of $1x10^6$ cells/well. After attachment overnight in medium containing 10 % FBS at 37˚C in a humidified 5 % CO_2-incubator, cells were washed twice in sterile PBS and treated with serum-free medium for 72 h containing apo-transferrin (5 µg/ml), ascorbate (100 µM), insulin (1 µM) and [^{3}H]-inositol (2 µCi/ml). Next, cells were washed twice with Ringer buffer containing (in mM) NaCl 125.0, KCl 6.0, $MgCl_2$ 2.5, $CaCl_2$ 1.2, NaH_2PO_4 1.2, HEPES 25.0 and glucose 11.0, pH 7.4. After a 15 min

incubation period in the same buffer, supplemented with 5 mM LiCl, cells were stimulated with methacholine in varying concentrations for another 30 min. Reactions were terminated by replacing the Ringer buffer for 1 ml of a methanol:0.12 mM HCl mixture (1:1 v/v), which had been previously kept at –20 ˚C and cells were allowed to lyse for another 30 min at –20 ˚C. 800 µl of the lysate was neutralized to pH = 7 using 3 ml buffer (composition 25 mM Tris / 0.5 M NaOH / H_2O 10:1:30 v/v/v) and [^{3}H]inositol phosphates were finally separated form free [^{3}H]inositol using Dowex-AG 1X8 anion exchange chromatography as described by Hoiting et al. [25].

Data analysis

All data represent means ± s.e.mean from *n* separate experiments. The statistical significance of differences between data was determined by the Student's *t*-test for paired observations or one-way ANOVA where appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Dulbecco's modification of Eagle's Medium (DMEM) and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Fetal bovine serum, $NaHCO_3$ solution (7.5 %), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml / 5000 µg/ml), amphotericin B solution (250 µg/ml) (Fungizone) and trypsin were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Platelet-derived growth factor AB (PDGF-AB, human recombinant), insulin (from bovine pancreas), apotransferrin (human), soybean trypsin inhibitor and gallamine triethiodide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). DAU5884 was a kind gift of Dr H. N. Doods (Dr Karl Thomae GmbH, Biberach, Germany) and 4-DAMP methobromide was kindly provided by Dr R. B. Barlow, Bristol, UK. [*Methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.). [^{3}H]myo-inositol (specific activity 59.9 Ci/mmol) was obtained from NEN Life Sciences Products (Boston, MA, USA). Papain and collagenase P were from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

Results

Methacholine-induced accumulation of inositol phosphates in unpassaged and passage 2 bovine tracheal smooth muscle cells.

BTSM cells treated for 1 day with 10 % FBS, followed by 3 days with serum-deprived medium (unpassaged BTSM cells) responded to methacholine with an increase in the accumulation of inositol phosphates (maximal effect (E_{max}) = 348 ± 43 % of basal, sensitivity (pEC_{50}) = 5.61 ± 0.21). In contrast, in cells cultured up to passage 2 no dose-dependent effects of methacholine could be determined. In this condition, basal formation of inositol phosphates remained unchanged for all concentrations of methacholine applied (average 98 ± 6 %, Figure 7.1).

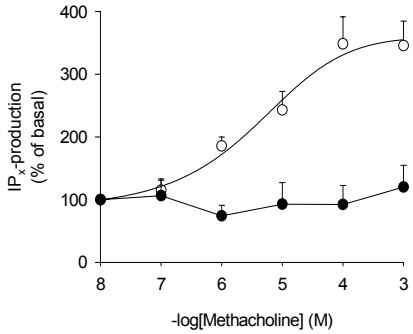

Figure 7.1 *Methacholine-induced inositol phosphates (IP_x)-accumulation in unpassaged (open circles) and passage 2 (closed circles) BTSM cells. Data represent means ± s.e.mean from 3 experiments each performed in triplicate.*

Effects of methacholine, alone and in combination with PDGF on [^{3}H]thymidine-incorporation in unpassaged and passage 2 bovine tracheal smooth muscle cells.

Stimulation of passage 2 BTSM cells with PDGF (10 ng/ml) induced an increase in [^{3}H]thymidine-incorporation to 255 ± 46 % of basal. Methacholine (10 µM) did not induce any proliferative response (91 ± 14 % of basal) and did not potentiate the proliferative response to PDGF either (295 ± 59 %, Figure 7.2A). In unpassaged BTSM cells, methacholine (10 µM) was unable to produce a proliferative response by itself (114 ± 14 %). Interestingly, the PDGF-induced increase in incorporated thymidine was significantly potentiated in these cells (246 ± 33 % and 371 ± 59 % for PDGF and PDGF + methacholine-treated cells, respectively, $P < 0.05$. Note that passage number did not influence the PDGF-response (Figure 7.2B).

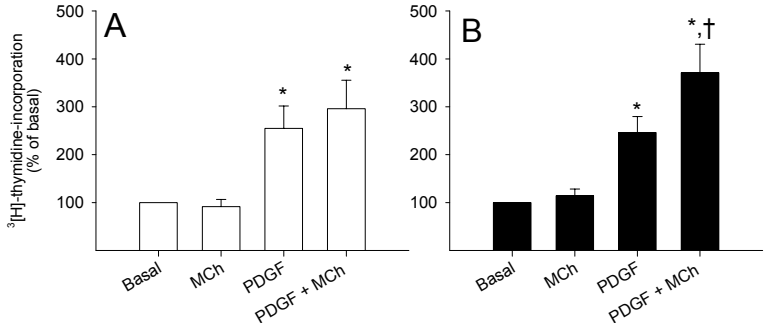

Figure 7.2 *[^{3}H]thymidine-incorporation induced by 10 µM methacholine (MCh), 10 ng/ml PDGF and the combination in both passaged (passage 2) BTSM cells (panel A) and unpassaged BTSM cells (panel B). Data represent means ± s.e.mean from 4 (passaged) and 6 (unpassaged) experiments each performed in triplicate. * P < 0.05 compared to basal; † P < 0.05 compared to PDGF.*

The effects of methacholine appeared to be concentration-dependent. Although at concentrations of 0.1-10 µM methacholine was unable to induce a proliferative response by itself, the agonist raised the E_{max} and reduced the EC_{50} of PDGF in a concentration-dependent fashion, which was most pronounced at 10 µM methacholine (E_{max} = 326 ± 45 % and 421 ± 46 %, $P < 0.001$ and EC_{50} = 4.8 ± 0.8 and 3.0 ± 0.5 ng/ml, $P < 0.05$ for PDGF and PDGF + 10 µM methacholine, respectively, Figure 7.3).

Effects of subtype-selective antagonists on methacholine and PDGF-induced synergism in [^{3}H]thymidine-incorporation

To establish the muscarinic receptor subtype(s) involved in the mitogenic responses to methacholine, we measured the inhibitory effects of selective receptor antagonists (DAU5884, 4-DAMP and gallamine) on the methacholine-induced potentiation of the proliferative response to PDGF.

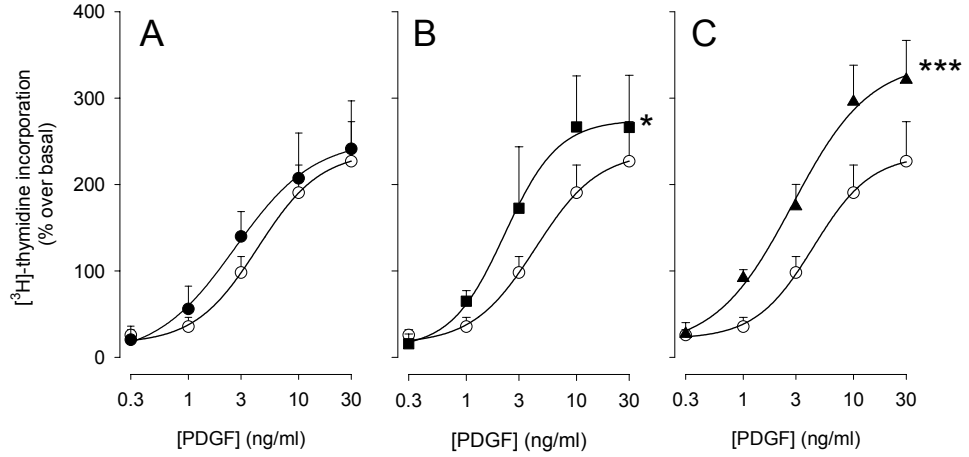

Figure 7.3 *Dose-dependent [^{3}H]thymidine-incorporation in response to PDGF in unpassaged BTSM cells in the absence (open circles) or presence of 0.1 µM (filled circles, panel A), 1 µM (filled squares, panel B) and 10 µM methacholine (filled triangles, panel C). Data represent means ± s.e.mean from 6 (0.1 and 1 µM methacholine) and 12 (10 µM methacholine) experiments, each performed in triplicate. * P < 0.05; *** P < 0.001 compared to control.*

Synergism was calculated as the methacholine-induced increase in the PDGF response and expressed relative to a control response (10 ng/ml PDGF). As shown in Figure 7.4A, synergism was dependent on the dose of methacholine applied. Maximal methacholine-induced synergism averaged 8.9 ± 10.6, 36.0 ± 15.9 and 55.3 ± 11.9 % for 0.1, 1 and 10 µM methacholine, respectively.

The potentiation induced by 10 µM methacholine was almost completely suppressed by DAU5884 (0.1 µM) (Figure 7.4B). The concentration of DAU 5884 applied results in an almost complete blockade of the M_3-receptor, with minor effects on the M_2-receptor (Table 7.1). Another M_3-receptor selective antagonist, 4-DAMP (10 nM), with a lower fractional M_3-receptor occupancy (Table 7.1) also inhibited the methacholine-induced synergism, though not completely (Figure 7.4B). In contrast, the M_2-receptor selective antagonist gallamine (10 µM, Table 7.1) did not alter the synergistic response (Figure 4B), which was virtually identical to control (compare with Figure 7.4A, open symbols).

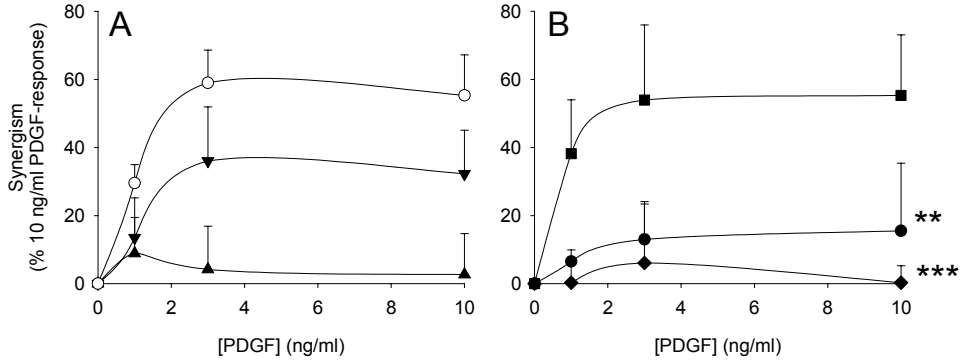

Figure 7.4 *Panel A: Synergism in [^{3}H]thymidine-incorporation in unpassaged BTSM cells, induced by 0.1 µM (triangles), 1 µM (inverted triangles) and 10 µM (open circles) methacholine in combination with PDGF. Data represent means ± s.e.mean from 6-12 experiments each performed in triplicate. Panel B: : Synergism in [^{3}H]thymidine-incorporation in unpassaged BTSM cells, induced by 10 µM methacholine in combination with PDGF in the presence of DAU5884 0.1 µM (diamonds), 10 nM 4-DAMP (filled circles) or 10 µM gallamine (squares). Data represent means ± s.e.mean from 4-6 experiments each performed in triplicate. * P < 0.05 compared to absence of antagonist.*

Table 7.1 *Fractional occupancy of muscarine M_2- and M_3-receptors by gallamine, DAU 5884 and 4-DAMP.*

Antagonist	Fractional M_2-receptor occupancy (%)	Fractional M_3-receptor occupancy (%)
gallamine, 10 µM	98.8	8.4
DAU 5884, 0.1 µM	26.2	99.1
4-DAMP, 10 nM	35.0	95.4

Estimated values for fractional occupancy were obtained using affinity data reported by Roffel et al. [26;27]

Discussion

Cultured airway smooth muscle cells undergo a variety of changes in protein expression and function as a consequence of modulation towards a synthetic phenotype. This phenotypic modulation is known to diminish the contractile properties of the airway smooth muscle cell; in contrast, their synthetic and proliferative capacities increase [28]. Plasticity in receptor-protein expression is one of the consequences of phenotypic modulation, which may both lead to down-regulation (e.g. muscarinic M_3-receptors in human ASM) and up-regulation of receptor proteins (e.g. PDGF-β receptors in porcine uterus) [29;30]. As regards muscarinic receptors in cultured ASM cells, the M_3-subtype is known to be downregulated rapidly upon culturing, whereas the M_2-receptor function is not markedly altered for up to passage 10 in human ASM cells [11]. Therefore it is important to characterize the functional presence of the M_3-receptor under different cell culture conditions, particularly since our aim was to study the relative involvement of M_2 and M_3-receptors in mitogenic responses.

In the unpassaged condition, M_3-receptor function, assessed by methacholine-induced increase in inositol phosphates formation, was comparable to earlier observations in freshly isolated BTSM cells [25]. It is of importance to note that the M_3-receptor is the only receptor mediating inositol phosphates formation in BTSM, as established using selective muscarinic receptor antagonists [31]. In contrast to the unpassaged condition, the M_3-receptor was no longer functional in cells that were passaged twice. It has been shown that passaged cultured canine ASM cells do not functionally express M_3-receptors either, whereas subsequent prolonged deprivation of serum re-induces functional coupling of the M_3-receptor, selectively for a subset of elongated contractile cells [32]. These elongated cells shorten by > 70 % of their original length in response to acetylcholine and express M_3-receptors on the outer membrane, whereas serum-fed cells demonstrate a perinuclear distribution of M_3-receptors that are not functionally coupled to inositol phosphates production [33]. This shows that cell surface coupled M_3-receptors are reversibly lost upon transition to the synthetic phenotype. Most likely, this plastic behaviour in M_3-expression is the basis behind the differences in functional M_3-responses in the different cell culture stages. Recently, it was shown that AP-2α may act as an important transcriptional regulator in this process: the M_3-receptor gene contains 8 AP-2 consensus binding motifs and AP-2α is known to be upregulated upon serum withdrawal [34].

In passaged human ASM cells, it has been found that the inositol phosphates response to carbachol is still present, although decreased. The loss of functional M_3-receptors in human ASM appeared to be largely determined by post-transcriptional regulation, not by decreased mRNA expression [11]. This process might be even more active in BTSM cells in view of the total lack of response.

Interestingly, the same culture condition that maintained functional M_3-expression, ensured a synergistic mitogenic response for methacholine and PDGF. In passaged (passage 2) cells, however, this synergism in mitogenesis was absent, as was

functional M_3-receptor expression. These differences suggest a role for the M_3-receptor in the proliferative potentiation induced by methacholine. Indeed, selective blockade of the M_3-receptor by DAU5884 or 4-DAMP resulted in suppression of this synergistic response. The suppression was not as profound for 4-DAMP as it was for DAU5884. However, DAU5884 was used in a concentration that results in 0.9 % of the M_3-receptor fraction available for stimulation by methacholine, whereas in case of 4-DAMP, a higher fraction (4.6 %) of M_3-receptors remained unoccupied. Remarkably, selective blockade (98.8 %) of the M_2-receptor by gallamine was totally ineffective. Hence, the M_3-receptor apparently is the only subtype involved in the regulation of the mitogenic responses by methacholine in unpassaged BTSM cells. Since M_3-expression in cultured airway myocytes appears not to be homogeneous [32;33], this could indicate that muscarinic agonist-induced growth synergy is mediated by a particular subset of these cells. In 1321N1 human astrocytoma cells acetylcholine did also induce proliferation via M_3-receptors, despite the presence of M_2 and M_5-subtypes [35].

These findings raise the question why this synergistic response is absent in passaged BTSM cells, but present in passaged human ASM cells, as described previously [9]. This difference may be the result of species-differences in M_3-receptor expression: although diminished, the presence of a functional M_3-receptor population in cultured human ASM has been reported on several occasions, having a response of approximately 35 % of that induced by histamine, as determined by the formation of inositol phosphates [11;36]. Furthermore, a small, but not absent population of functional M_3-receptors is consistent with the finding that carbachol is relatively weak in inducing proliferation synergy in human ASM cells when compared to other G protein coupled agonists [8;9].

Methacholine did not produce a proliferative response by itself, which is in line with observations by others, showing no [9] or a relatively small [8] increase in proliferation of ASM induced by muscarinic agonists. However, muscarinic receptor stimulation may play an important modulatory role. Muscarinic receptor stimulation was mitogenic only in combination with other mitogens, like growth factors. The combined response induced by methacholine (10 μM) and a concentration of PDGF (1 ng/ml), unable to induce proliferation by themselves, resulted in approximately 45 % of the maximal PDGF-induced response under control conditions. This would imply a threshold either in the activation of transduction cascades or in the response of the cell to transductional activation.

The modulatory role may become of physiological relevance particularly in an environment, in which growth factors are abundant, for instance due to secretion by inflammatory cells. Therefore, endogenous acetylcholine may contribute to the pathophysiology of inflammatory airway diseases, in which an increase in smooth muscle mass leads to airflow obstruction such as chronic asthma [37].

On a molecular level, M_3-mediated mitogenic responses are in agreement with biochemical studies, showing that G_q-coupled muscarinic receptors may couple to

pathways known to be involved in transcriptional regulation [38], such as the p42/p44 MAPK-pathway [24], the PI-3-kinase/PKB-pathway [39], and stress-activated members of the MAPK-superfamily, like the c-Jun N-terminal kinase (JNK)-pathway [24] and the p38-MAPK-pathway [40]. In addition, coupling to $G_{12/13}$-proteins may be responsible for the observed effects. Although less well studied, $G_{\alpha 12/13}$ subunits are known to be involved in cellular growth and to potently activate JNK, whereas ERK-activity may both be inhibited or stimulated, dependent on the cell type [41].

The more challenging question is why M_2-receptors do not seem to be relevant, since in theory, M_2-receptors may couple to the same pathways [24;42;43]. Moreover, other G_i coupled-stimuli have been shown to respond synergistically in combination with growth factors in human ASM cells [8]. Perhaps other transductional pathways are involved in the muscarinic receptor-mediated mitogenic responses. Though, differences in the kinetics of activation may also account for the unexpected observation. In CHO-cells, transfected with either human wild-type M_2- or M_3-receptors, it was found that both the M_2- and the M_3-transfected cells mediated p42/p44 MAPK- and JNK-activation; however, M_2-receptor mediated responses were transient, whereas M_3-receptor responses were sustained [24]. This difference may be of critical importance, since sustained p42/p44 MAPK/ERK-activity determines the proliferative responses in human [44] and bovine ASM cells [14]. Since little is known about the quantitative contribution of the pathways mentioned in regulating cross-talk between G-protein coupled receptors and tyrosine kinase coupled receptors, the absence of a M_2-mediated mitogenic response might perhaps not be generalized.

In conclusion, in BTSM cells methacholine does not induce mitogenesis by itself, but potentiates PDGF induced proliferation. This was dependent on the presence of functional M_3-receptors as controlled by the cell culture conditions applied. This synergism could be abolished by selective M_3-receptor antagonists, like DAU5884 and 4-DAMP, but not by the M_2-subtype selective antagonist gallamine. These results show that methacholine potentiates mitogenesis induced by PDGF through stimulation of M_3-receptors in BTSM cells.

Acknowledgement

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Chapter 8

Bradykinin augments growth factor-induced airway smooth muscle mitogenesis by activation of conventional PKC iso-enzymes

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Submitted

Abstract

This study aims to investigate the effects of bradykinin, alone and in combination with epidermal growth factor (EGF) on DNA-synthesis ([^{3}H]thymidine incorporation) in cultured bovine tracheal smooth muscle (BTSM) cells. Bradykinin did not induce DNA-synthesis by itself, but concentration-dependently augmented EGF-induced [^{3}H]thymidine incorporation. This was mediated by B_2 receptors as determined using the selective B_2 receptor antagonist HOE140 and not dependent on cyclo-oxygenase as determined using indomethacin. Bradykinin-induced synergism with EGF could be suppressed by the protein kinase C (PKC) inhibitors GF109203X (specific for conventional and novel PKCs) and Gö6976 (specific for conventional PKCs). In addition, sole activation of PKC using the phorbol ester PMA was sufficient for a synergistic interaction with EGF. PMA was mitogenic by itself which was not at all affected by Gö6976, but abolished by GF109203X. Western analysis of activated p42/p44 MAP kinase showed transient activation by bradykinin, which was abolished by both GF109203X and Gö6976. In contrast, PMA-induced activation of p42/p44 MAP kinase was sustained and could be inhibited only by GF109203X. Neither the combination of bradykinin and EGF or that of PMA and EGF induced synergistic activation of p42/p44 MAP kinase. These results show that bradykinin B_2 receptor-stimulation is not mitogenic by itself but augments growth factor-induced DNA-synthesis through parallel rather than consecutive activation of conventional PKC isozymes and p42/p44 MAP kinase. In addition, the results show that PKC isozyme-specificity underlies stimulus-specific differences in mitogenic capacity for bradykinin and PMA.

Introduction

Bradykinin is a nonapeptide generated by kallikrein-mediated breakdown of kininogens during inflammatory responses. It is involved in a variety of (patho)physiological responses in the airways, including microvascular leakage, bronchoconstriction, mucus secretion and pain perception [1]. Evidence exists to assume the involvement of bradykinin in asthma, since asthmatics display exaggerated bronchoconstrictor responses to bradykinin when compared to healthy controls [2]. Moreover, kallikrein levels are increased in the bronchoalveolar lavage fluid of asthmatic subjects after allergen challenge [3]. Furthermore, the bradykinin B_2 receptor antagonist HOE140 has been shown to improve airway function of asthmatics [4] and is known to attenuate allergen-induced microvascular leakage and bronchoconstriction in guinea pigs [5-7].

Apart from acute inflammation and bronchoconstriction, asthma is also characterised by structural alterations in the airways. Thus, cross-sections of pulmonary airways of asthmatics reveal a thickened airway smooth muscle (ASM) layer in comparison to age-matched controls. The increase in ASM mass worsens with age, which may consequently lead to a further increase in airway narrowing [8]. Growth factors may in part be responsible for this process by increasing cell number (hyperplasia) or size (hypertrophy) through the activation of receptor tyrosine kinases. In addition, G protein coupled receptor (GPCR) agonists may contribute to this increase in ASM

mass as they can be mitogenic for cultured ASM cells and/or can augment growth factor induced proliferation [9]. For example, cholinergic signalling (as described in Chapter 7) [10], tachykinins [11], inflammatory mediators such as histamine [12] and leukotriene D_4 [13] have been reported to be pro-mitogenic, alone or in combination with growth factors.

Bradykinin may signal through B_1 and B_2 receptor subtypes, but in the airways no evidence exists for the functional presence of B_1 receptors [1]. Moreover, effects on ASM such as contraction [14], intracellular Ca^{2+}-increases [15] and cytokine release [16] are B_2 receptor-mediated. Since activation of pro-mitogenic signaling pathways such as the p42/p44 mitogen activated protein kinase (MAPK) pathway by the B_2 receptor has been described in ASM [16], it could be envisaged that bradykinin induces ASM proliferation or proliferation synergy in combination with growth factors.

Studies using human [17] and bovine [18] ASM cells have revealed no mitogenic responses of bradykinin by itself, presumably because of the inability to induce sustained p42/p44 MAP kinase activation. However, weakly mitogenic or even non-mitogenic GPCR activation can be sufficient to potentiate receptor tyrosine kinase-induced growth [9;10]. Therefore we investigated the effects of bradykinin on bovine tracheal smooth muscle (BTSM) in combination with a peptide growth factor. We found that, while bradykinin is not mitogenic by itself, it augments epidermal growth factor (EGF)-induced mitogenic responses through activation of B_2 receptors and with the involvement of conventional protein kinase C (PKC) isozymes.

Methods

Isolation of bovine tracheal smooth muscle cells

Bovine tracheae were obtained from local slaughterhouses and transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5 % CO_2 and 95 % O_2; pH 7.4. After dissection of the smooth muscle layer and removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 300 µm and three times at a setting of 100 µm. Tissue particles were washed two times with Dulbecco's Modification of Eagle's Medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml) and 0.5 % Foetal Bovine Serum (FBS). Enzymatic digestion was performed using the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 ˚C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 µm gauze, cells were washed three times in DMEM, supplemented as above, containing 10 % FBS.

Cell culture
After isolation, BTSM cells were seeded in culture flasks at a density of $1x10^6$ cells/ml for further culturing. Cultured cells were kept viable in medium containing 10 % FBS at 37 ˚C in a humidified 5 % CO_2-incubator. Medium was refreshed every 48-72 h. Cell cultures were allowed to grow and, upon confluency, were passaged further at a 1 : 2 split ratio, by means of trypsinization. Cultured cells were used for experiments in passage 1-3.

$[Ca^{2+}]_i$-measurements
Measurements of $[Ca^{2+}]_i$ were carried out according to Hoiting *et al*. [19]. Briefly, cells were detached from the flask bottom by trypsinization and washed three times in Krebs-Ringer-Henseleit buffer (composition in mM: NaCl 125.0, KCl 6.0, $MgCl_2$ 2.5, $CaCl_2$ 1.2, NaH_2PO_4 1.2, HEPES 25.0 and glucose 11.0, pH 7.4), supplemented with 2 % BSA. Next, cells were loaded with the fluorescent dye Fura-2/AM (3 µM) for 30 min at 20 ˚C. Fura-2/AM loaded cells were washed, diluted to a concentration of 1.10^6 cells/ml and were used for experiments within 2-4 h following the loading procedure. Measurements were carried out at 37 ˚C, during which Fura-2 emitted fluorescence was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm with a Perkin-Elmer Spectrometer (LS-50B). $[Ca^{2+}]_i$ was calculated every 0.2 s according to Grynkiewicz [20].

$[^3H]$Thymidine-incorporation
BTSM cells were plated in 24 well cluster plates at a density of 30,000 cells per well in 10 % FBS containing medium at 37 ˚C in a humidified 5 % CO_2-incubator. After 48 h, cells were washed two times with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4) and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg/ml), ascorbate (100 µM) and insulin (1 µM) for 72 h. Cells were then washed with PBS and stimulated with mitogens in FBS-free medium for 28 h, the last 24 h in the presence of $[^3H]$thymidine (0.25 µCi/ml), followed by two washes with PBS at room temperature and one with ice-cold 5 % trichloroacetic acid (TCA). Cells were treated with this TCA-solution on ice for 30 min; subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated $[^3H]$thymidine was quantified by liquid-scintillation counting. When applied, inhibitors (HOE140, GF109203X, Gö6976, PD98059, U0126) were pre-incubated with 30 min before stimulation.

Activation of p42/p44 MAPK
BTSM cells were plated in 6-well cluster plates at a density of 120,000 cells.well^{-1} in medium, containing 10 % FBS. After 48 h, cells were washed two times with sterile PBS and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg.ml^{-1}), ascorbate (100 µM) and insulin (1 µM) for 72 h. Cells were then washed with PBS and stimulated with bradykinin, EGF and their combination in FBS-free medium. When applied, cells were pre-incubated with inhibitors (GF109203X, Gö6976) 30 min before stimulation. At different time-points, cells were washed twice in ice-cold PBS and lysed in 0.5 ml of homogenisation

buffer (composition in mM: NaCl 150.0, Tris HCl 10.0, 2-glycerophosphoric acid 5.0, EGTA 2.0, DTT 2.0, PMSF 1.0, Na_3VO4 1.0, NaF 1.0, pH 7.5), containing 0.5 µg/ml leupeptin, 2 µg/ml aprotinin and 1 % w/v Triton X-100. Cell lysates were stored at –80 ˚C until further use. Protein content was determined according to Bradford [21]. Homogenates containing equal amounts of protein per lane were then subjected to immunoblot analysis using antibodies that recognise the phosphorylated forms of p42/p44 MAPK (Thr^{202}/Tyr^{204}), as described previously [22].

Data analysis

All data represent means ± s.e.mean from *n* separate animals. The statistical significance of differences between data was determined by the Student's *t*-test for paired observations or one-way ANOVA where appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Dulbecco's modification of Eagle's Medium (DMEM) was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Foetal bovine serum (FBS), $NaHCO_3$ solution (7.5 %), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml / 5000 µg/ml), amphotericin B solution (250 µg/ml) (Fungizone) and trypsin/EDTA were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Epidermal growth factor (EGF, human recombinant), insulin (from bovine pancreas), PMA, apo-transferrin (human), bradykinin, aprotinin, leupeptin, soybean trypsin inhibitor and fura-2/AM were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). [*Methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.). Papain and collagenase P were from Roche Diagnostics (Mannheim, Germany). Anti-phospho-p42/p44 MAPK (rabbit polyclonal IgG) and HRP-linked goat anti-rabbit IgG were from Cell Signaling Technology (Beverly, MA, U.S.A.) All other chemicals were of analytical grade.

Results

Bradykinin responsiveness in cultured BTSM cells

Since cell culture may affect expression of G protein coupled receptors in ASM [23], the functional presence of G_q-coupled bradykinin receptors was confirmed by measurements of bradykinin-induced changes in intracellular [Ca^{2+}] in cultured (passage 1-3) BTSM cells, using the fluorescent dye Fura-2AM as described previously [19]. In all cell cultures tested, bradykinin (10 µM) induced a strong transient increase in intracellular [Ca^{2+}], that averaged 3.0 ± 0.4-fold of basal [Ca^{2+}] (basal value: 132 ± 20 nM; $P<0.01$).

Effects of bradykinin on [^{3}H]thymidine incorporation

Bradykinin did not increase [^{3}H]thymidine incorporation in cultured BTSM cells, when applied in concentrations ranging from 1 nM to 10 µM (Figure 8.1a). The mitogenic response induced by epidermal growth factor (EGF, 10 ng/ml), however, was concentration-dependently augmented by bradykinin. At the highest concentration of

bradykinin applied (10 µM), the EGF-induced response was augmented 1.8 ± 0.1-fold (P<0.01; Figure 8.1A). In the presence of a fixed concentration of bradykinin (10 µM), the responses to increasing concentrations of EGF were consistently augmented as well, with no difference in sensitivity to EGF (Figure 8.1B).

Since cyclo-oxygenase products have been reported to be involved in bradykinin-induced effects, such as cytokine production from smooth muscle cells [16;24], we studied the effects of indomethacin (3 µM) in an additional set of experiments. The synergistic mitogenic response of bradykinin and EGF was not affected, however, by treatment with indomethacin (1.8 ± 0.1-fold and 1.9 ± 0.4-fold of EGF response in the absence and presence of indomethacin, respectively).

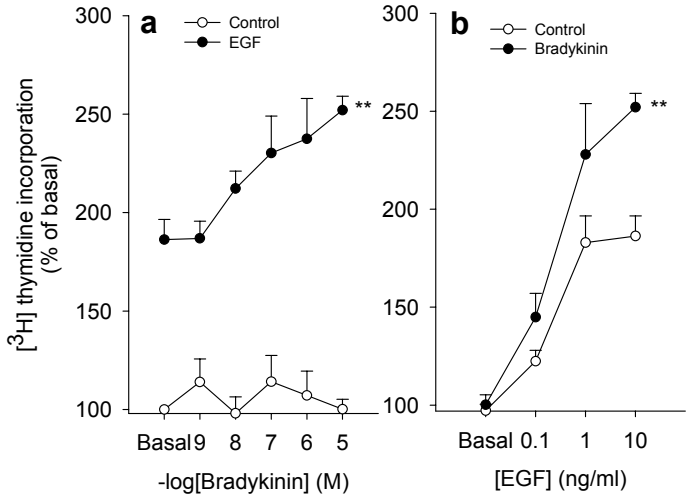

***Figure 8.1**: Effects of bradykinin on [^{3}H]thymidine incorporation in cultured BTSM cells. Panel (A): Dose-response relationship to bradykinin in the absence and presence of 10 ng/ml EGF. Panel (B): dose-response relationship to EGF in the absence and presence of a fixed concentration of bradykinin (10 µM). Data shown represent the means ± s.e.mean of 5-6 experiments each performed in triplicate. ** P< 0.01 compared to absence of bradykinin.*

Role of bradykinin B_2 receptors

To establish the B_2 receptor nature of the observed effects of bradykinin, cells were treated with the potent B_2 receptor antagonist HOE 140 (1 µM). At this concentration, B_2 receptor occupancy by HOE 140 is >99.9 % (pK_i = 9.1, [25]). The presence of HOE 140 completely prevented the synergistic mitogenic response induced by bradykinin (10 µM) and EGF (10 ng/ml, Figure 8.2).

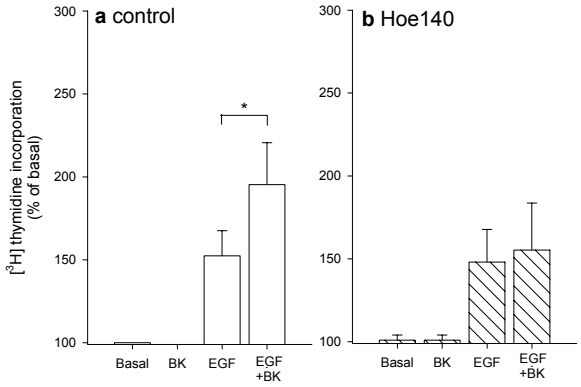

***Figure 8.2**: Role of B_2 receptors in synergism between bradykinin and EGF. [^{3}H]thymidine incorporation in cultured BTSM cells was measured to a fixed concentration of bradykinin (10 μM), EGF (10 ng/ml) and their combination in the (A) absence and (B) presence of HOE140 (1 μM). Data shown represent the means ± s.e.mean of 4 experiments each performed in triplicate. * P< 0.05 compared to absence of bradykinin.*

Role of Protein kinase C

To test the involvement of PKC, two specific PKC-inhibitors were used: GF109203X (10 μM) and Gö6976 (300 nM). At these concentrations, GF109203X inhibits both conventional (in BTSM: α, βI and βII) and novel (in BTSM: δ, ε and ζ) PKC isozymes, whereas Gö6976 inhibits conventional PKCs specifically [26;27]. Interestingly, pretreatment with either inhibitor abrogated the synergistic mitogenic response induced by bradykinin and EGF (Figure 8.3). Of note, GF109203X potentiated the EGF-induced response significantly (*P*<0.05), whereas basal and BK-induced responses were not affected.

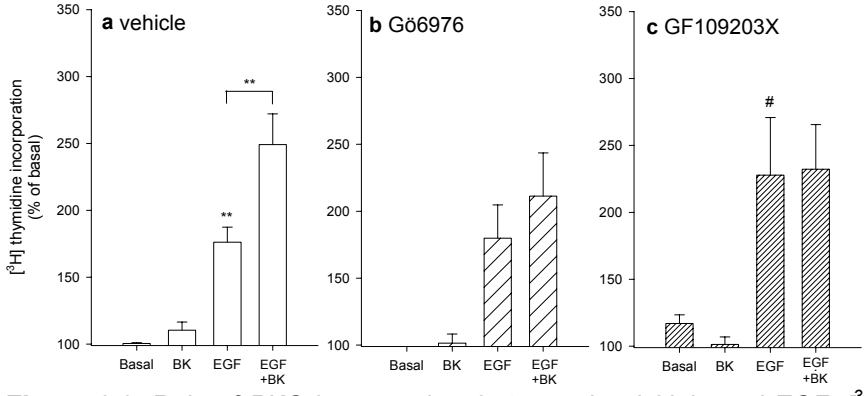

***Figure 8.3**: Role of PKC in synergism between bradykinin and EGF. [^{3}H]thymidine incorporation in cultured BTSM cells was measured to a fixed concentration of bradykinin (10 μM), EGF (10 ng/ml) and their combination in the presence of (A) vehicle, (B) Gö6976 (300 nM) or (C) GF109203X (10 μM). Data shown represent the means ± s.e.mean of 4-5 experiments each performed in triplicate. ** P< 0.01; # P< 0.05 compared to control EGF response.*

In support of a role for PKC in synergistic stimulation of DNA-synthesis, the PKC activator PMA potentiated the mitogenic responses to EGF in a synergistic fashion (*P*<0.05, Figure 8.4). In contrast to bradykinin, however, PMA induced a marked increase in [^{3}H]thymidine incorporation by itself, which was concentration-dependent (pEC_{50} = 7.5 ± 0.4 ; E_{max} = 2.7 ± 0.7-fold of basal, *P*<0.05). Interestingly, both the basal mitogenic effects of PMA and its synergistic interaction with EGF were completely insensitive to the conventional PKC inhibitor Gö6976, whereas GF109203X abrogated both responses (*P*<0.05, Figure 8.4).

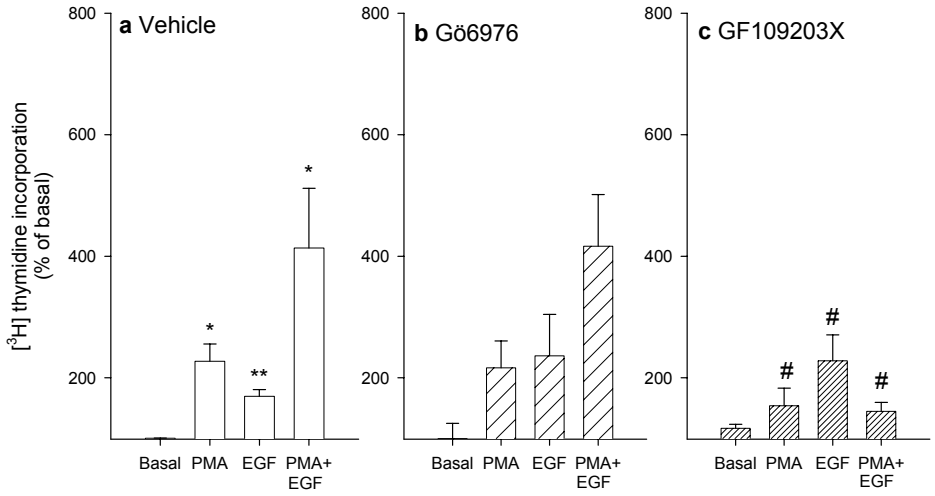

***Figure 8.4**: Role of PKC in synergism between PMA and EGF. [^{3}H]thymidine incorporation in cultured BTSM cells was measured to PMA (0.1 µM), EGF (10 ng/ml) and their combination in the presence of (A) vehicle, (B) Gö6976 (300 nM) or (C) GF109203X (10 µM). Data shown represent the means ± s.e.mean of 4-5 experiments each performed in triplicate. * P< 0.05; ** P< 0.01 compared to basal; # P< 0.05 compared to vehicle treated.*

To analyze the involvement of PKCs in p42/p44 MAP kinase activation, cells were stimulated with bradykinin, EGF or bradykinin in the absence and presence of GF109203X or Gö6976. p42/p44 MAP kinase activation by bradykinin was abolished by the conventional PKC-inhibitor Gö6976, whereas PMA-induced activation of p42/p44 MAP kinase was completely insensitive to this inhibitor. In contrast, GF109203X completely inhibited activation by either PMA or bradykinin. Of note, EGF-induced activation of the p42/p44 MAP kinase pathway was completely unaffected by the PKC inhibitors used (Figure 8.5).

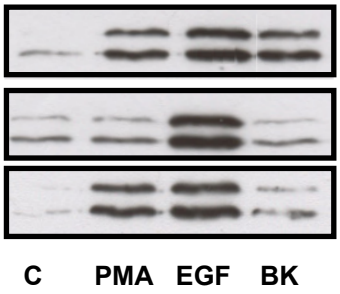

***Figure 8.5**: Role of PKC in p42/p44 MAP kinase activation by bradykinin, EGF and PMA. Cells were stimulated with bradykinin (10 μM), PMA (0.1 μM) or EGF (10 ng/ml) in the presence of (A) vehicle, (B) Gö6976 (300 nM) or (C) GF109203X (10 μM). Protein homogenates were subjected to electrophoresis and immunoblotted against the phosphorylated forms of p42/p44 MAP kinase. C: Control. Blot shown is representative for 4 experiments.*

Role of p42/p44 MAPK

Since MEK activates p42/p44 MAPK through direct phosphorylation, upstream inhibition of MEK can effectively prevent activation of the p42/p44 MAP kinase pathway [28;29]. The MEK inhibitors U0126 (3 μM) and PD98059 (30 μM), applied at selective concentrations, diminished both basal and EGF-induced [^{3}H]thymidine incorporation (P<0.001, Figure 8.6). In addition, the synergistic responses observed for the combination of EGF and bradykinin were abolished (P<0.001). Combined treatment with EGF and bradykinin did not activate the p42/p44 MAPK pathway synergistically in its early activation phase (t = 2-5 min), nor after 2 hours (Figure 8.7). In contrast to bradykinin, PMA activated the p42/p44 MAP kinase pathway in a sustained fashion. As observed for bradykinin, however, the combination of PMA and EGF did not activate the p42/p44 MAP kinase pathway synergistically, irrespective of the time-point studied (Figure 8.7).

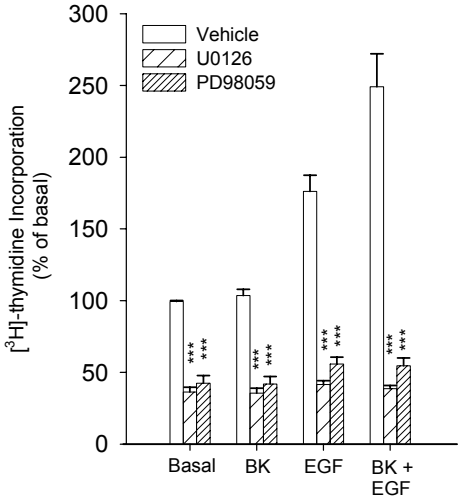

***Figure 8.6**: Role of p42/p44 MAPK in synergism between bradykinin and EGF. [^{3}H]thymidine incorporation in cultured BTSM cells was measured to bradykinin (10 μM), EGF (10 ng/ml) and their combination in the presence of vehicle (white bar), U0126 (3 μM, black bar) and PD98059 (30 μM, hatched bar). Data shown represent the means ± s.e.mean of 4 experiments each performed in triplicate. *** P<0.001 compared to absence of inhibitor.*

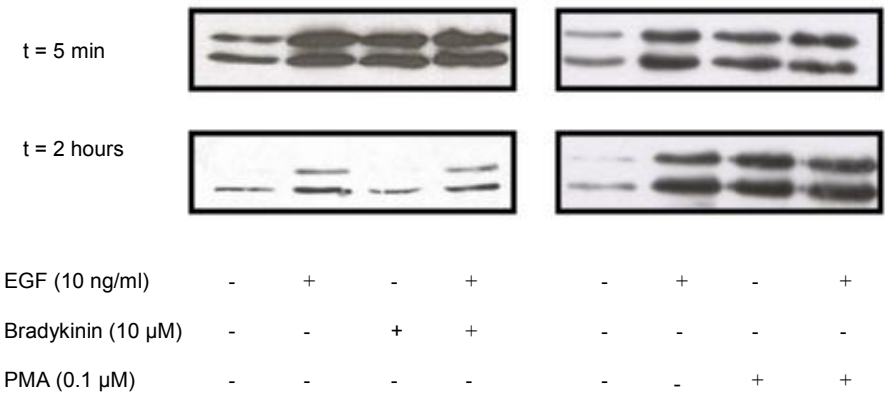

***Figure 8.7**: Time-dependent p42/p44 MAP kinase activation by bradykinin, EGF and PMA. Early phase (t = 2 min for bradykinin and 5 min for PMA) and late phase (t = 2 h for both) activation status was determined. Cells were stimulated with bradykinin (10 µM), PMA (0.1 µM) or EGF (10 ng/ml) or combinations hereof and protein homogenates were subjected to electrophoresis and immunoblotting against the phosphorylated forms of p42/p44 MAP kinase. Blot shown is representative for 4-6 experiments.*

Discussion

The results presented in this study demonstrate that bradykinin is mitogenic for BTSM when applied in combination with the peptide growth factor EGF. The high potency of bradykinin in augmenting EGF-induced responses (EC_{50} = 24 nM) is in agreement with other effects of bradykinin on airway smooth muscle, such as contraction of human bronchial preparations (EC_{50} = 20 nM) [14] and IL-6 release in human ASM cells (EC_{50} = 26 nM) [16]. However, bradykinin was not mitogenic by itself as also observed by others [17]; [18]. The bradykinin receptor involved in the synergistic response was of the B_2 subtype, shown using the B_2 receptor selective antagonist HOE140, which is not surprising in view of the absence of functional B_1 receptors in ASM [1]. The cultured cells were functionally responsive to bradykinin at least up to passage 3, in view of the strong calcium responses generated after receptor stimulation. This is in contrasts to muscarinic M_3 receptor expression which is lost rapidly in culture (c.f. Chapter 7).

Cyclo-oxygenase (COX) products have been implicated in various effects of bradykinin in human ASM cells: the COX-inhibitor indomethacin partially inhibits bradykinin-induced IL-6-production [16] and almost completely inhibits bradykinin-indued IL-8 production [24]. In addition, epithelium-dependent relaxation of guinea pig tracheal smooth muscle induced by bradykinin is, in part, caused by bradykinin-

induced production of PGE_2 [30]. However, PGE_2 is antiproliferative for guinea pig tracheal [31] and human airway smooth muscle cells [32]. Therefore, it seems highly unlikely that PGE_2 is involved in the observed increase in DNA-synthesis in combination with EGF in our study. Indeed, indomethacin did not affect the synergistic response induced by EGF and bradykinin.

The maximal response to the peptide growth factor EGF was augmented by bradykinin without large effects on its sensitivity, which corresponds to results, previously obtained using methacholine (Chapter 7). This indicates that maximal mitogenic signaling is a limiting factor, even for peptide growth factors that use receptor tyrosine kinases. Since cell division is an all-or-nothing response, bradykinin apparently triggers more cells into the cell cycle than EGF is capable of on its own. Bradykinin can either increase the statistical probability to enter the cell cycle, or trigger additional signaling in a selective number of cells only, since it has been found that a particular morphologically non-elongated subset of canine ASM cells in culture responds to bradykinin with increases in intracellular [Ca^{2+}] [33]. Such phenotypic selectivity may also exist for EGF, which is mitogenic for 'synthetic' (passage 2) BTSM cells as shown in this study, but nor for (hyper)contractile BTSM cells as shown previously in Chapters 2 and 3 [34;35].

Generally, GPCR agonists respond with only minor or even completely absent mitogenic responses unless used as a co-mitogen; for example, histamine [36], leukotriene D_4 [13], 5-hydroxytryptamine (unpublished observations), methacholine (Chapter 2) and endothelin-1 [37] have been found to be mitogenic for ASM cells in combination with growth factors only. This does not apply for all GPCR agonists however, since thrombin is extremely effective on its own [17]. Apparently, the ability to induce sustained activation of the p42/p44 MAP kinase pathway is needed to trigger cell division, since only a transient increase in p42/p44 MAP kinase activation is observed after non-mitogenic GPCR stimulation [38]; [39]. The transient p42/p44 MAP kinase activation induced by bradykinin and the sustained p42/p44 MAP kinase activation induced by PMA are therefore in line with their respective effects observed on [^{3}H]thymidine incorporation.

Activation of p42/p44 MAP kinase by bradykinin occurs *via* activation of PKC as demonstrated using GF109203X and Gö6976. Non-specific effects of the PKC inhibitors used can be excluded, as both basal and EGF-induced p42/p44 MAPK activity were unaffected. This non-mitogenic effect of bradykinin-induced PKC activation contrasts to the very effective proliferative response and sustained p42/p44 MAP kinase activation induced by PMA-induced PKC activation. Gö6976 specifically inhibits conventional PKC isozymes [26], showing that bradykinin-induced p42/p44 MAP kinase activation is fully dependent on these conventional PKC isozymes. In contrast, the responses to PMA were independent of conventional PKC isozymes in view of the lack of effect of Gö6976, but were fully inhibited by GF109203X. Therefore, it appears that PKC-isozyme-specificity exists with regard to the induction of sustained p42/p44 MAP kinase activity and concomitant induction of mitogenesis in ASM cells. The effectiveness of GF109203X (conventional and novel

PKCs, [26]) compared to Gö6976 suggests the involvement of novel PKC isozymes in PMA-induced mitogenic responses.

In addition, synergistic activation of mitogenesis by the combination of bradykinin and EGF was dependent on conventional PKC-isozymes, whereas PMA-induced synergy in combination with EGF was insensitive to Gö6976. This may suggest that the mitogenic signalling pathways activated by PMA are sufficient to overcome the inhibition of conventional PKCs. It is highly unlikely that PMA would not activate these conventional isozymes. Despite of this suggested functional relationship between PKC and p42/p44 MAP kinase however, and despite of the involvement of p42/p44 MAP kinase in both basal and mitogen-induced proliferative responses (determined using PD98059 and U0126), the MAP kinase enzymes were not activated synergistically, neither in its early phase, nor in its sustained phase by the combinations of bradykinin and EGF or PMA and EGF. Although synergistic activation of the p42/p44 MAP kinase pathway has been proposed as an explanation for synergism between GPCR agonists and growth factors in case of endothelin-1 and EGF in guinea pig ASM and for ATP and insulin in coronary arterial smooth muscle [40;41], others have not shown such synergistic activation in human ASM cells [36;42]. However, PKC signalling might be relevant for a synergistic interaction downstream of p42/p44 MAP since p70 S6K and the transcription factors AP-1, Elk-1 and Cyclin D_1 have all been reported to be induced synergistically by growth factors and GPCR agonists in human ASM cells [36;42].

EGF-induced mitogenesis and p42/p44 MAP kinase activation were not inhibited by GF109203X or Gö6976. Remarkably, GF109203X significantly potentiated the EGF-induced proliferative response, although no effects of GF109203X on EGF-induced p42/p44 MAP kinase activation status were measured. Yet, these results are entirely consistent with observations made by others that showed similar effects of PKC inhibition, using calphostin C and staurosporine, on insulin- and PDGF-induced mitogenesis in BTSM cells [43]. PKC isoform-specific effects may also explain this discrepancy: growth factors activate the inhibitory PKCδ in BTSM cells as a negative feedback mechanism [44].

In conclusion, this study has shown that, in BTSM cells, bradykinin is not mitogenic by itself but augments mitogenesis in combination with a peptide growth factor through B_2 receptor stimulation and subsequent activation of conventional PKC isozymes. The observation that bradykinin potentiates mitogenesis induced by growth factors in ASM may be relevant for the pathophysiology of asthma, as bradykinin is an important inflammatory mediator involved in this disease [1]. Persistent stimulation of ASM proliferation through such a mechanism during chronic inflammation may contribute to the increased ASM mass seen in asthmatics.

Acknowledgement

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Chapter 9

Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling *in vivo*

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Abstract

Recent findings have demonstrated that muscarinic M_3 receptor stimulation enhances airway smooth muscle (ASM) proliferation to peptide growth factors *in vitro*. Since both peptide growth factor expression and acetylcholine release are known to be augmented in allergic airway inflammation, it may be envisaged that anticholinergics protect against allergen-induced ASM remodeling *in vivo*. Therefore, we investigated the effects of treatment with the long-acting muscarinic receptor antagonist tiotropium on ASM changes in a guinea pig model of ongoing allergic asthma. Twelve weekly repeated allergen challenges induced an increase in ASM mass in the non-cartilaginous airways. This increase was not accompanied by alterations in ASM cell size, indicating that the allergen-induced changes were entirely due to increased ASM cell number. Morphometric analysis showed no allergen-induced changes in ASM area in cartilaginous airways. However, repeated OA challenge enhanced maximal contraction of open tracheal ring preparations *ex vivo*. This was associated with an increase in smooth muscle specific myosin expression in the lung, indicating that the ASM cells in the central airways acquired a more contractile phenotype. Treatment with inhaled tiotropium considerably inhibited the increase in ASM mass, myosin expression and contractility. These results indicate a prominent role for acetylcholine in allergen-induced ASM remodeling *in vivo*, a process that was thus far considered to be primarily caused by growth factors and other mediators of inflammation. Therefore, muscarinic receptor antagonists like the long-acting anticholinergic tiotropium bromide could be beneficial in preventing chronic airway hyperresponsiveness and decline in lung function in allergic asthma.

Introduction

The use of anticholinergics in obstructive airways diseases, like asthma and COPD, is primarily based on their acute bronchodilatory effects. Thus, muscarinic receptor antagonists provide acute relief from the increased levels of acetylcholine (ACh) released in the airways upon reflex vagal nerve stimulation during allergic airway inflammation [1]. Potential effects of anticholinergics on inflammation-induced structural changes in the airways, however, have not been considered thusfar. Nevertheless, it has recently been demonstrated that muscarinic receptor stimulation potentiates the mitogenic response of bovine tracheal smooth muscle (BTSM) cells to platelet-derived growth factor (PDGF), which was mediated by the G_q-coupled muscarinic M_3 receptor [2]. In addition, muscarinic receptor stimulation augmented the mitogenic responses of human airway smooth muscle (ASM) cells to epidermal growth factor (EGF) [3]. These two peptide growth factors can be released from structural cells in the airways, including ASM cells, as well as from infiltrated inflammatory cells [4]. This could indicate that, in addition to ACh, growth factor release may be augmented in allergic airway inflammation. Indeed, the expression levels of some peptide growth factors (eg. EGF and basic fibroblast growth factor, bFGF) have been found elevated in asthma [5;6]. Therefore, it may be envisaged that functional interaction of ACh with growth factors during chronic airway inflammation is involved in the development of ASM thickening, a pathological feature observed in asthmatics as well as in animal models of allergic asthma [7-9].

Airway smooth muscle thickening is considered to be involved in the development of chronic airway hyperresponsiveness in asthma [10].

Both in cell culture [11] and in organ culture [12] settings, growth factor-stimulated ASM growth has been tightly associated with ASM phenotypic plasticity. This phenotypic plasticity allows ASM to adapt to pro-mitogenic environments, resulting in diminished contractility and contractile protein expression but increased proliferative and synthetic properties [13]. Conversely, growth arrest can reconstitute a contractile or even a hypercontractile phenotype [14;15]. Therefore, ASM phenotypic plasticity may contribute to airway inflammation and airway remodeling during periods of allergen exposure and to increasesd contractility in the periods in between, thereby alternating changes in ASM mass and contractility. Importantly, a recent study showed the occurrence of allergen-induced ASM phenotype switching in Brown-Norway rats, indicating that phenotypic plasticity could indeed accompany ASM thickening *in vivo* [16]. The role of ACh in allergen-induced ASM phenotypic modulation, however, is presently unknown.

Therefore, in the present study we investigated the contribution of endogenous ACh to allergen-induced remodeling of ASM *in vivo*. For this purpose, the effects of treatment with the long-acting muscarinic receptor antagonist tiotropium bromide [17] was evaluated on ASM after repeated allergen challenge in a guinea pig model of allergic asthma, characterised by early and late asthmatic reactions, airway hyperresponsiveness after these reactions and airway inflammation [18]. As parameters for ASM remodeling, smooth muscle area, cell number, contractile protein expression and contractility were assessed *ex vivo* after twelve weekly repeated allergen exposures. It was demonstrated that allergen-induced augmentation of these parameters was considerably reduced by pre-treatment with tiotropium bromide.

Methods

Animals

Outbred, male, specified pathogen free Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom) weighing 250-300 g were sensitized to ovalbumin (OA) as described previously [18]. In short, 0.5 ml of an allergen solution containing 100 μg /ml ovalbumin and 100 mg /ml $Al(OH)_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck. The animals were used experimentally from 4 to 8 weeks after sensitization. The animals were group-housed in individual cages in climate controlled animal quarters and given water and food *ad libitum*, while a 12-h on/ 12-h off light cycle was maintained. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

Provocation procedures
Four weeks after sensitization, allergen-provocations were performed by inhalation of aerosolized solutions of ovalbumin or saline (control) as described previously [18]. Aerosols were produced by a DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA) with an airflow of 8 l/min resulting in an output of 0.33 ml/min. Provocations were carried out in a specially designed perspex cage (internal volume 9 l), in which the guinea pigs could move freely. Before the start of the experimental protocol, the animals were habituated to the provocation procedures on two sequential days. After an adaptation period of at least 30 min, three consecutive provocations with saline were performed, each provocation lasting 3 min, separated by 7 min intervals. Ovalbumin challenges were performed by inhalation of increasing concentrations of 0.05 %, 0.1 % and 0.3 % w/v ovalbumin in saline for 3 min each, with 7 min intervals. Allergen inhalations were discontinued when the first signs of respiratory distress were observed. No anti-histaminic was needed to prevent the development of anaphylactic shock.

Experimental protocol
Guinea pigs were divided into 4 groups: (1) OA-sensitized, saline-challenged controls, (2) OA-sensitized, OA-challenged animals, (3) OA-sensitized, saline-challenged, tiotropium-treated animals and (4) OA-sensitized, OA-challenged, tiotropium-treated animals. Guinea pigs were challenged with antigen once weekly as indicated, for 12 consecutive weeks. For tiotropium treatment animals received a nebulized dose of tiotropium bromide in saline (0.1 mM, 3min), 0.5 h prior to each challenge with saline or ovalbumin. The prolonged treatment with tiotropium bromide and/or ovalbumin did not affect growth of the animals. In week 12, animals weighed 1045 ± 95 g (12 x saline); 1017 ± 40 g (12 x OA); 1012 ± 26 g (12 x tiotropium) and 1029 ± 30 g (12 x OA + tiotropium).

Tissue acquisition
Twenty four h after the last challenge, guinea pigs were sacrificed by a sharp blow on the head, followed by rapid exsanguination. The lungs were immediately resected and kept on ice in a petri dish for further processing. In addition, the trachea was removed and transferred to a Krebs-Henseleit solution (37 ˚C), pregassed with 5 % CO_2 and 95 % O_2, pH 7.4. The lungs were divided into two equal parts. One part was frozen at –80 ˚C in isopentane and stored at –80 ˚C for histological purposes. The remaining part was snap frozen in liquid nitrogen and stored at –80 ˚C to be used for Western analysis.

Morphometric analysis of airway smooth muscle mass
Smooth muscle area was determined in 8 µm thick cryostat lung sections, with transverse cross-section of the main bronchus. To identify smooth muscle, the sections were stained for smooth muscle α-actin (sm-α-actin) or smooth muscle myosin heavy chain (sm-MHC), each in quadruplicate. Sections were dried for 30 min, fixed with acetone for 10 min and then washed three times in phosphate-buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4). Subsequently, sections were incubated for 1 h in PBS

supplemented with 1 % bovine serum albumin (BSA) and primary antibody (diluted 1:200 for sm-α-actin and 1:100 for sm-MHC) at room temperature. Sections were then washed three times with PBS after which endogenous peroxidase activity was blocked by treatment with PBS containing 0.075 % H_2O_2 for 30 min. Sections were washed for another three times with PBS, after which the horseradish peroxidase (HRP)-linked secondary antibody (Rabbit anti-mouse IgG, diluted 1:200) was applied for 30 min at room temperature. After another three washes, sections were incubated with diaminobenzidine (1 mg/ml) for 5 min in the dark, after which sections were washed and stained with hematoxylin. After rinsing with water the sections were embedded in Kaisers glycerol gelatin. Airways within each section were digitally photographed and classified as cartilaginous or non-cartilaginous. For both types of airways, sm-α-actin and sm-MHC positive areas were measured by a single observer in a blinded fashion. In addition, hematoxylin-stained nuclei within the ASM bundle were counted. Generally, 3 to 5 airways of each classification were analyzed per animal. Data were expressed as mm^2/mm basment membrane.

Western analysis of contractile protein expression

Lung homogenates were prepared by pulverizing tissue under liquid nitrogen and subsequent sonification in homogenization buffer (composition in mM: Tris.HCl 20.0, dithiothreitol (DTT) 0.1, phenyl methyl sulphonyl fluoride (PMSF) 0.2; pH 7.5, supplemented with 2 µg/ml leupeptin, 2 mg/ml aprotinin and 10 µg/ml soybean trypsin inhibitor). Homogenates were stored at –80 ˚C until further use. Protein content was determined according to Bradford [19]. Protein (50 µg per lane) was separated by SDS/PAGE on 6 % (sm-MHC) or 10 % (sm-α-actin) polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes, which were blocked overnight in blocking buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; dried milk powder 5 %; pH = 7.5). After two washes with washing buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; dried milk powder 2.5 %; pH = 7.5), membranes were incubated at room temperature with primary antibodies (anti-sm-MHC or anti-sm-α-actin, both diluted 1:200 in washing buffer). After three washes with washing buffer supplemented with 0.1 % Tween 20, membranes were incubated in HRP-labelled secondary antibodies (dilution 1:6000 in washing buffer) at room temperature for 1h, followed by another three washes. Antibodies were then visualised by enhanced chemiluminescence. Photographs of blots were analyzed by densitometry (Totallab ^{tm}; Nonlinear Dynamics, Newcastle, U.K.).

Isometric tension measurements

The trachea was prepared free of serosal connective tissue. Single open-ring, epithelium-denuded preparations were mounted for isometric recording in 20 ml water-jacked organ-baths, containing KH-buffer (pH 7.4), continuously gassed with 5 % CO_2 and 95 % O_2 at 37 ˚C. During a 90-min equilibration period, with washouts every 30 min, resting tension was graduallly adjusted to 0.5 g. Subsequently, muscle strips were precontracted with 20 mM and 40 mM KCl. Following two wash-outs, basal smooth muscle tone was determined by the addition of 0.1 µM isoprenaline and tension was re-adjusted to 0.5 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min, cumulative concentration

response curves (CRCs) were constructed to methacholine (1.10^{-9} M – 1.10^{-4} M using 0.5 log increments). When maximal tension was obtained, the strips were washed several times and resting tension was re-established using isoprenaline.

Data analysis
All data represent means ± s.e.mean from *n* separate experiments. The statistical significance of differences between data was determined by Bonferroni's Student's *t*-test or one-way ANOVA, as appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Materials
Methacholine hydrochloride was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Mouse monoclonal anti sm-MHC was from Neomarkers (Fremont, CA, USA). Mouse monoclonal anti sm-α-actin, rabbit anti-mouse IgG (peroxidase conjugate), soybean trypsin inhibitor, dithiothreitol, sodium-dodecyl sulphate, bovine serum albumin, diaminobenzidine, ovalbumin, aprotinin, leupeptin and (-)isoprenaline hydrochloride were from Sigma (St. Louis, MO, U.S.A.). Tiotropium bromide was from Boehringer Ingelheim (Ingelheim, Germany). Enhanced chemiluminescence reagents were from Pierce (Rockford, IL, USA). All other chemicals were of analytical grade.

Results

Effects of ovalbumin challenge and tiotropium treatment on ASM content.
Figure 9.1 shows representative lung sections stained for sm-MHC and sm-α-actin, containing the main bronchus as well as blood vessels. Although the ASM layer stained positively for contractile proteins, sm-α-actin and sm-MHC positive areas were slightly dissimilar, i.e. sm-MHC positive area was more discontinuous and appeared to be somewhat smaller. Indeed, morphometric analysis revealed that in saline challenged controls, sm-α-actin positive area was somewhat larger than sm-MHC positive area, both in cartilaginous (0.100 ± 0.006 vs. 0.073 ± 0.010 mm^2/mm basement membrane for sm-α-actin and sm-MHC, respectively; $P = 0.035$) and non-cartilagenous airways (0.064 ± 0.008 vs. 0.042 ± 0.006 mm^2/mm basement membrane for sm-α-actin and sm-MHC, respectively; $P = 0.010$; cf. Figures 9.2-9.3).

Repeated ovalbumin challenge did not change ASM content in the larger airways, irrespective of the contracile marker protein used. In non-cartilaginous airways however, OA challenge induced a significant increase in both sm-α-actin and sm-MHC positive area of 0.022 ± 0.006 mm^2/mm basement membrane (36 ± 3 % increase) and 0.024 ± 0.006 mm^2/mm basement membrane (57 ± 13 % increase), respectively, as compared to saline challenged, age-matched controls (Figures 9.2-9.3). This increase was largely prevented by treatment with tiotropium, for 75 ± 9 % for sm-α-actin positive area and for 76 ± 6 % for sm-MHC positive area. Tiotropium bromide treatment by itself did not induce significant changes in the morphometric parameters analysed, when compared to untreated saline challenged controls (Figures 9.2-9.3).

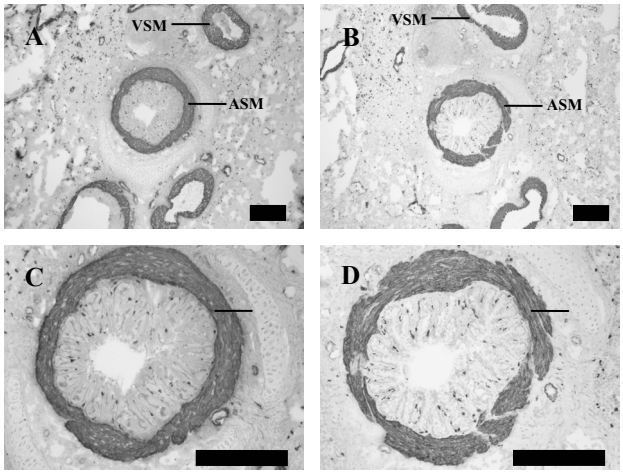

Figure 9.1 *Representative photomicrographs of serial lung sections containing transverse cross-sections of the main bronchus. Sections were stained for sm-α-actin (A,C) or sm-MHC (B,D). Only the area in the smooth muscle layers of airways and blood vessels stained positive for these proteins. Photographs were taken at 40 x (A,B) and 100 x (C,D) magnification. ASM, airway smooth muscle; VSM, vascular smooth muscle. Black bar represents 250 µm.*

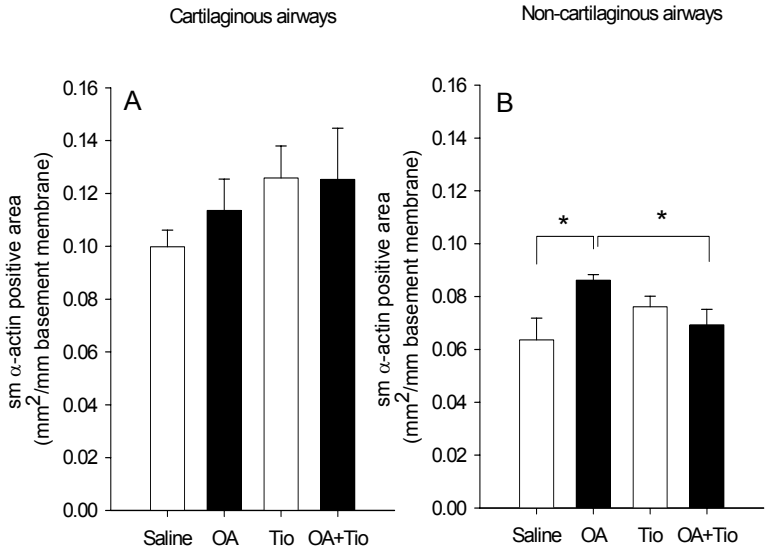

Figure 9.2 *Morphometric analysis of sm-α-actin expression in the guinea pig lung. Cartilaginous (A) and non-cartilaginous airways (B) were identified in sm-α-actin stained sections, after which sm-α-actin-positive area was measured and expressed as mm^2/mm basement membrane. Data shown represent means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). Three to five airways of each classification were analysed per animal. * P < 0.05.*

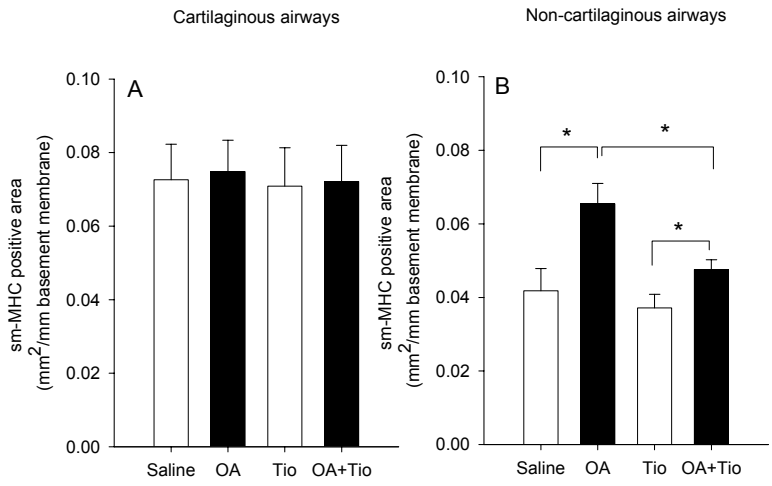

Figure 9.3 *Morphometric analysis of sm-MHC expression in the guinea pig lung. Cartilaginous (A) and non-cartilaginous airways (B) were identified in sm-MHC stained sections after which sm-MHC-positive area was measured and expressed as mm^2/mm basement membrane. Data shown represent means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). Three to five airways of each classification were analysed per animal. * P < 0.05.*

To determine the nature of the changes in ASM area within the non-cartilaginous airways, the number of nuclei that comprised the airway smooth muscle layer in these airways was counted. To account for differences in ASM content between airways as well as between treatment groups, data were expressed as the number of nuclei per mm^2 smooth muscle. With this data, the average apparent volume of the individual ASM cell was also calculated, assuming equal thickness of all sections (8 µm). For saline-challenged animals, 5107 ± 405 nuclei were counted per mm^2 smooth muscle, and an apparent volume of 1650 ± 121 μm^3 per ASM cell was calculated. No differences in either parameter were found between any of the treatment groups, indicating that the observed differences in ASM content were exclusively caused by changes in cell number, not in cell size (Figure 9.4).

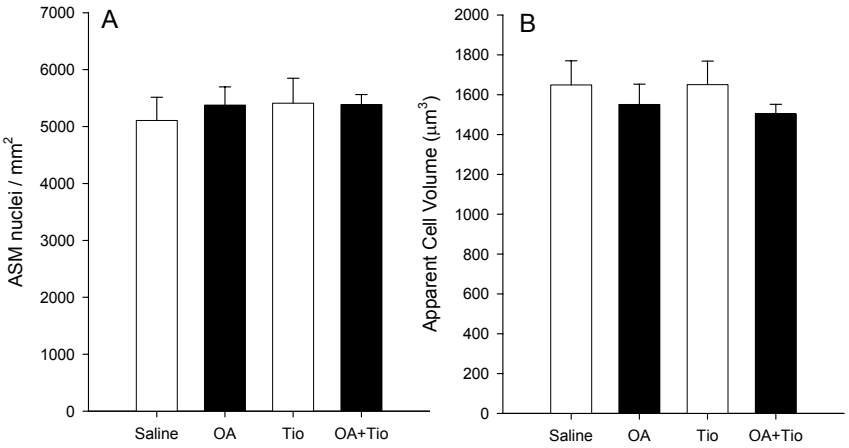

Figure 9.4 *An increase in ASM cell number accounts for the changes in ASM content. A: The number of hematoxyllin-stained ASM nuclei present in non-cartilaginous airways was counted and expressed relative to total ASM area. B: Based on these data, the apparent volume of the average individual ASM cell was calculated. Data shown represent means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). Two non-cartilaginous airways per animal were analysed.*

Effects of ovalbumin challenge and tiotropium treatment on contractile protein expression.

Changes in sm-α-actin and sm-MHC positive area could imply changes in contractile protein expression. Therefore, we used Western analysis to determine the relative contents of these contractile proteins in whole lung homogenates. For sm-α-actin expression, differences between treatment groups were small, indicating that the changes in sm-α-actin positive area in non-cartilaginous airways had only little impact on total sm-α-actin expression in the lung (Figure 9.5). Surprisingly therefore, large differences in sm-MHC expression were observed between the treatment groups. Repeated OA challenge strongly increased total sm-MHC expression in the lung to 422 ± 28 % of sm-MHC content in saline challenged controls. Pre-treatment with tiotropium attenuated this increase to 300 ± 22 % (P<0.01), corresponding to 38 ± 6 % inhibition of the OA-induced increase. Tiotropium by itself however, had no effect on sm-MHC expression (Figure 9.6).

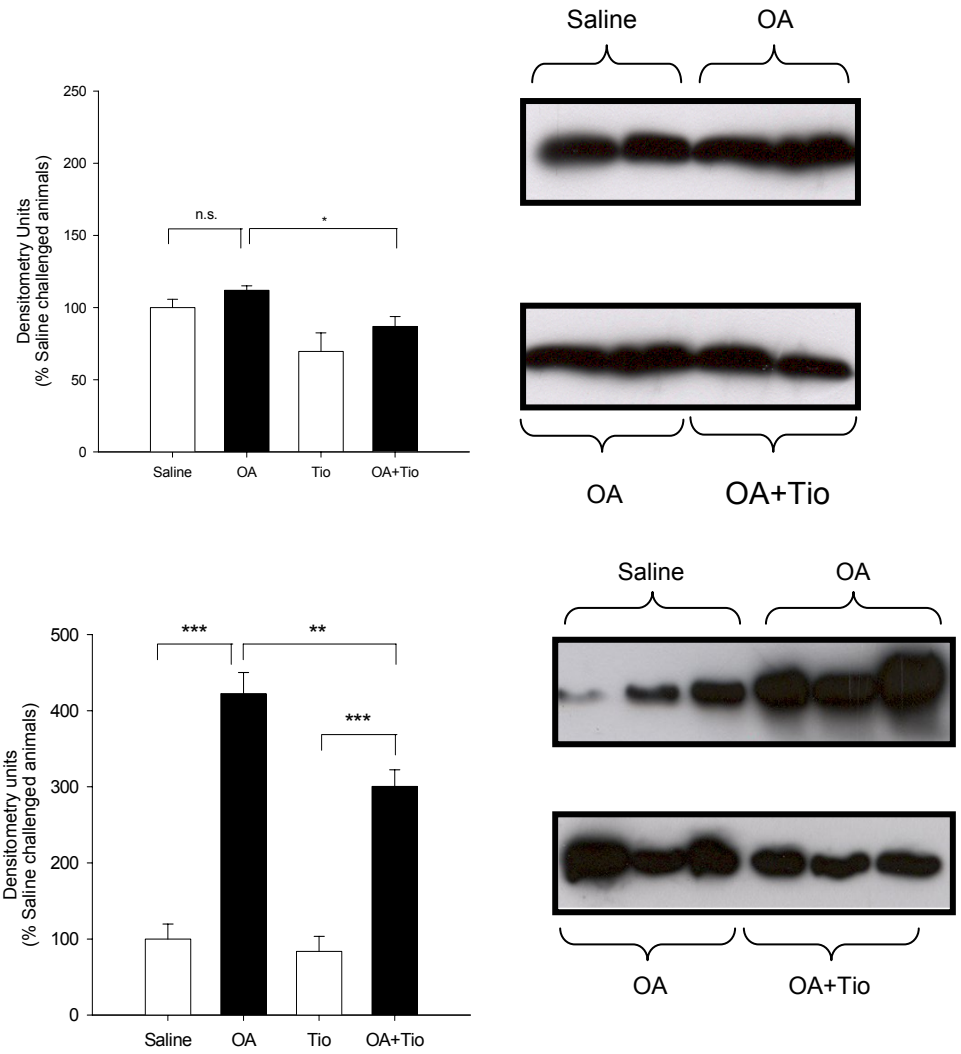

Figure 9.5 *Effects of repeated allergen challenge and tiotropium treatment on sm-α-actin (upper panel) and sm-MHC (lower panel) expression in the guinea pig lung. Protein matched lung homogenates were Western analysed for sm-α-actin. Data shown represent densitometric means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). * P < 0.05; ** P < 0.01; *** P < 0.001. n.s.: not significant. Blots shown are representative. Each lane represents a different animal.*

Effects of ovalbumin challenge and tiotropium treatment on tracheal smooth muscle contractility.

Even though OA-challenge-induced changes in ASM content were confined to non-cartilaginous airways, the observed changes in total sm-MHC expression could still allow for changes in contractility in the central airways. Indeed, repeated ovalbumin challenge enhanced methacholine-induced contraction of epithelium-denuded open-ring tracheal preparations from 2.1 ± 0.1 g in saline-challenged animals to 2.7 ± 0.2 g in ovalbumin-challenged guinea pigs (P<0.05; Figure 7). Sensitivity to methacholine, however, was not altered (pEC_{50} = 6.6 ± 0.1 and 6.5 ± 0.1 for saline- and ovalbumin-challenged animals, respectively). Basal smooth muscle tone tended to be somewhat higher in ovalbumin-challenged guinea pigs, but this was not statistically significant. Pre-treatment with tiotropium slightly decreased the sensitivity to methacholine in saline-challenged animals to a pEC_{50} value of 6.3 ± 0.1 (P<0.05), with no significant change in maximal contraction (1.8 ± 0.2 g). However, tiotropium pre-treatment completely prevented the increase in contractility induced by repeated OA challenge (1.6 ± 0.1 g; P<0.001; Figure 9.7).

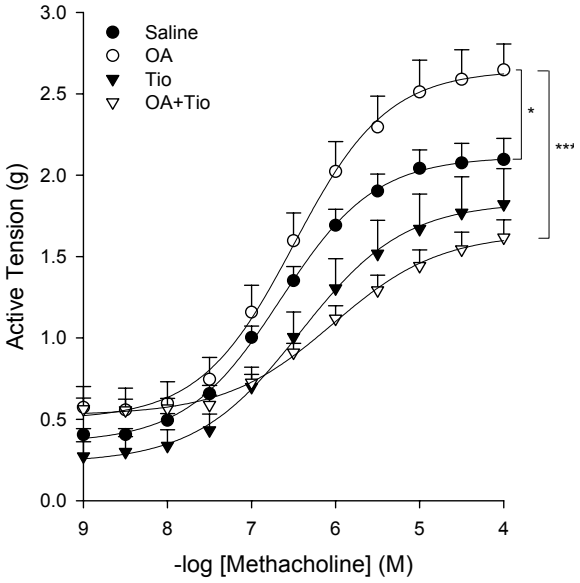

Figure 9.7 *Effects of repeated allergen challenge and tiotropium treatment on isometric contraction of epithelium-denuded, tracheal open-ring preparations. Data shown represent means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). * P < 0.05; *** P < 0.001.*

Discussion

The most important finding of this study is that changes in airway smooth muscle content, contractile protein expression and contractility induced by repeated allergen exposure can be partially or even fully prevented by tiotropium bromide, a long-acting muscarinic receptor antagonist, used for bronchodilation. In permanently instrumented, conscious and unrestrained guinea pigs, we have previously demonstrated that the tiotropium dose used in this study provides a sustained muscarinic receptor blockade, lasting over 96 h [20], which extends well beyond the

duration of allergen-induced early and late phase asthmatic reactions in this animal model [18]. Therefore, the effects of tiotropium described in this study are likely to represent the cumulative contribution of cholinergic activity to airway smooth muscle remodeling caused by the repeated allergen challenges. This indicates that ACh could have a major impact on the progression of airway remodeling in allergic asthma, a process that has thus far primarily been associated with mediators of inflammation and growth factors [21].

Nevertheless, inflammatory mediators and growth factors are likely to play a crucial part in the observed effects of ACh. Thus, tiotropium bromide was effective only in animals that were challenged with ovalbumin, indicating that ACh release affects structural changes in the airways in combination with allergic airway inflammation. This could, in part, be explained by augmented ACh release after allergen challenge. Thus, eosinophilic inflammation-derived polycations, such as major basic protein (MBP), are known to cause epithelial damage which can expose afferent sensory nerve endings to the airway lumen and increase vagal reflex activity in response to inhaled stimuli [22]. In addition, cholinergic afferents can be stimulated by a variety of mediators involved in allergen-induced airway inflammation (see Undem & Myers for extensive review [1]). Eosinophil-derived MBP can also increase vagally induced ACh release by inhibition of prejunctional auto-inhibitory M_2 receptors [23]. Importantly, both allergen-induced M_2 autoreceptor dysfunction and enhanced cholinergic reflex activity have been demonstrated in the guinea pig model of allergic asthma used in this study [24;25]. An additional mechanism that might contribute to increased levels of ACh after allergen exposure is its release from inflammatory and epithelial cells. This non-neuronal release of ACh may be elevated in conditions of allergic inflammation, as it was found to be increased in skin biopsies from patients with atopic dermatitis, a condition often associated with bronchial asthma [26;27].

Despite the enhanced release of ACh during allergic airway inflammation, it may be envisaged that ACh is ineffective in ASM remodeling by itself and that concerted action with mediators of inflammation and growth factors is required for the effect. This could explain the absence of tiotropium effect in the controls. Indeed, *in vitro*, muscarinic receptor stimulation does not or only modestly affect ASM proliferation by itself, but effectively augments growth factor-induced responses [2;3]. The latter mechanism may well be responsible for the allergen-induced increase in ASM mass observed in the non-cartilaginous airways, since an increase in cell number rather than cell size was the predominant cause of allergen-induced ASM thickening in these airways.

The mechanism of ACh-induced ASM thickening may also be relevant to airway remodeling in patients with asthma, since an increase in ASM mass in bronchial biopsies of patients with mild to moderate asthma was accompanied by a two-fold increase in cell number without a change in cell volume [28]. Other studies have indicated that hypertrophy may also contribute to increased ASM mass observed in asthmatics [29;30]. However, since no hypertrophy was observed in our model under the applied conditions, a possible role for ACh in this process remains unclear.

ASM content in the large pulmonary airways, including the main bronchi, did not change after repeated allergen challenge. Nevertheless, contractility of tracheal preparations was increased, suggesting a different nature of ASM remodeling in the central airways. This is also indicated by a previous study, demonstrating increased tracheal smooth muscle contraction after repeated allergen-challenge in guinea pigs, without concomitant changes in ASM mass [31]. These observations suggest that ASM cells in the central airways acquire a hypercontractile phenotype upon repeated allergen challenge. Moreover, the inhibitory effects of tiotropium bromide indicate that endogenous ACh contributes to the induction of this hypercontractile phenotype *in vivo*.

Regulation of contractile protein expression by ACh may be involved in the increased contractility, as our results demonstrate a selective increase in sm-MHC expression in the lung after allergen exposure, which was partially inhibited by tiotropium. Of note, changes in sm-MHC positive area in the non-cartilaginous airways may not be the sole cause of the increase in sm-MHC expression, since a 57 % increase in area in these airways as such cannot result in 322 % increase in whole lung myosin expression. Together with the unchanged sm-MHC positive area in the larger airways, increased sm-MHC expression per ASM cell seems to be more likely. However, the localization of this increased sm-MHC expression is as yet unknown.

Sm-α-actin expression did not increase significantly after allergen exposure. The discrepancy between the expression of these contractile proteins may be explained by relatively high sm-α-actin expression by cell types other than ASM cells (e.g. fibroblasts that express sm-α-actin but not sm-MHC [32]), which is supported by the observation that sm-α-actin positive area was larger than sm-MHC positive area even in the ASM layer. In addition, it may be envisaged that sm-α-actin and sm-MHC expression can be regulated independently and to different extents. Indeed, the induction of a hypercontractile canine ASM phenotype *in vitro* is accompanied by a much greater increase in sm-MHC exression (± 8-fold increase) as compared to sm-α-actin (± 2-fold increase) [15].

The effects of ACh on contractility may at least partially be explained by activation of the RhoA/Rho-kinase pathway, as this pathway has been described to regulate both ASM contractility [33] and smooth muscle specific gene transcription [34]. Moreover, muscarinic M_3 receptor-dependent activation of RhoA and Rho-kinase has been reported to induce smooth muscle specific gene transcription in ASM cells *in vitro* [35]. These RhoA-dependent effects may even be enhanced after repeated allergen exposure, which induces an increase in RhoA expression [36]. Nevertheless, prolonged (8 days) exposure of BTSM strips to high concentrations (≥ 10 μM) of methacholine results in a decline in contractility and contractile protein expression, caused by the prolonged elevation of $[Ca^{2+}]_i$ [37]. Therefore, Rho-dependent rather than Ca^{2+}-dependent mechanisms are likely to regulate ACh-induced alterations in contractility *in vivo*.

An important consideration is how the effects of tiotropium could relate to the effectiveness of anticholinergics in the long-term treatment of asthma. In asthma, β_2 adrenoceptor-agonists are usually more effective bronchodilators than anticholinergics [38]. Nonetheless, β_2-agonists appear to be at most modestly effective in inhibiting allergen-induced ASM proliferation *in vivo* [39], despite of their effectiveness in inhibiting ASM proliferation *in vitro* [40]. Moreover, chronic β_2-agonist exposure have even been reported to increase airway responsiveness to acetylcholine *in vivo* and *ex vivo* [31]. It appears therefore that anticholinergics could be more effective than β-agonists in preventing allergen-induced ASM remodeling. However, the effect of chronic treatment with anticholinergics on airway remodeling, responsiveness and changes in lung function in asthmatic patients is thus far unclear.

Corticosteroids have been reported to inhibit growth factor-induced ASM proliferation, cytokine production and extracellular matrix deposition *in vitro* [41-43], and to inhibit but not to reverse allergen-induced fibronectin deposition in rats *in vivo* [44]. Moreover, the inhibitory effects of corticosteroids on ASM proliferation *in vitro* are strongly inhibited when cells are cultured on collagen type I [45], which is increased in asthma [46]. Surprisingly, however, the effects of corticosteroids on ASM thickening has not yet been investigated in animal models. Future studies using animal models characterised by allergen-induced ASM proliferation could therefore be useful to compare different treatment strategies.

In conclusion, we have demonstrated that tiotropium bromide inhibits allergen-induced airway remodeling in a guinea pig model of ongoing asthma. Therefore, endogenous ACh appears to play an important role in ASM remodeling, a process thusfar primarily associated with mediators of inflammation and growth factors. This could have important implications for the use of anticholinergics in the treatment of allergic asthma, by protecting against the development of chronic airway hyperresponsiveness and decline of lung function in addition to their acute bronchodilating effects.

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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 prejunctional 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, subtance P can possibly induce major basic protein release from eosinohils, causing M_2 dysfunction as described above [26]. In addition to reduced

M_2 autoreceptor function, inflammation-derived prostanoids including PGD_2, $PGF_{2\alpha}$ and TxA_2 can augment ACh release from cholinergic nerve endings by prejunctional faciliation [25]. Interestingly, ASM itself also represents a potential source of PGD_2, $PGF_{2\alpha}$ and TxA_2 [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 M_2 and G_q-coupled muscarinic M_3 receptors, the former being the predominant population, comprising ~80% of the total muscarinic receptor population [34;35]. G_q-coupled muscarinic M_1 receptors are not present, whereas the presence of G_i-coupled muscarinic M_4 receptors may be species specific. Thus, muscarinic M_4 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 M_2 and M_3 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 phosphatidyl inositol 3-kinase (PI 3-kinase) pathways, relevant for airway remodeling.

G_q-coupled muscarinic M_3 receptors in ASM activate phospholipase C, causing hydrolytic conversion of phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5-trisphosphate ($InsP_3$) and *sn*-1,2-diacylglycerol (DAG) [39]. $InsP_3$ is involved in the mobilization of Ca^{2+} from intracellular stores, which generates a rapid and transient increase in $[Ca^{2+}]_i$. DAG generated through muscarinic M_3 receptor

activation activates protein kinase C (PKC). Both Ca^{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^{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).

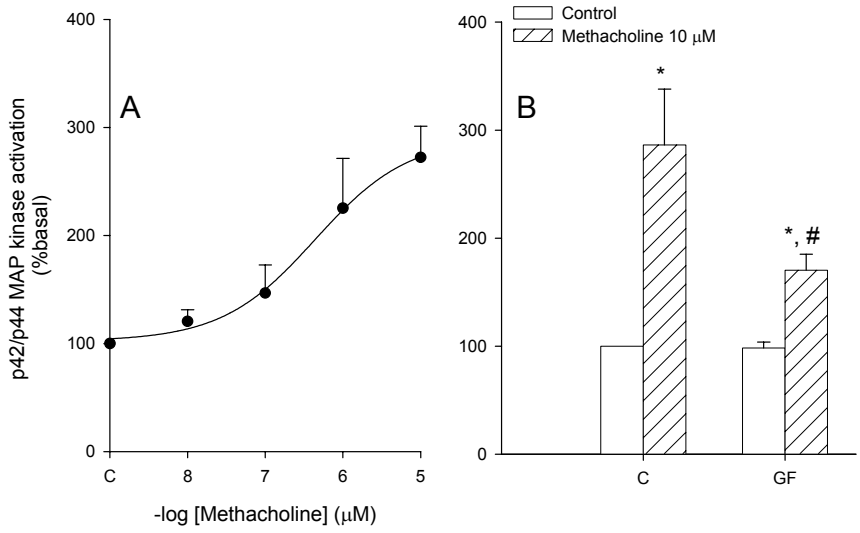

Figure 10.1 *Methacholine-induced p42/p44 MAPK activation in BTSM is concentration- and PKC dependent. A: Intact strips were stimulated with increasing concentrations of methacholine (5 min; 37 ºC), homogenised and immunoblotted against phosphorylated p42/p44 MAP kinase. Unstimulated strips were used 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_2 receptor activation has been reported in canine tracheal smooth muscle [44]. Presumably, this occurs via α_i 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_2 and M_3 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.

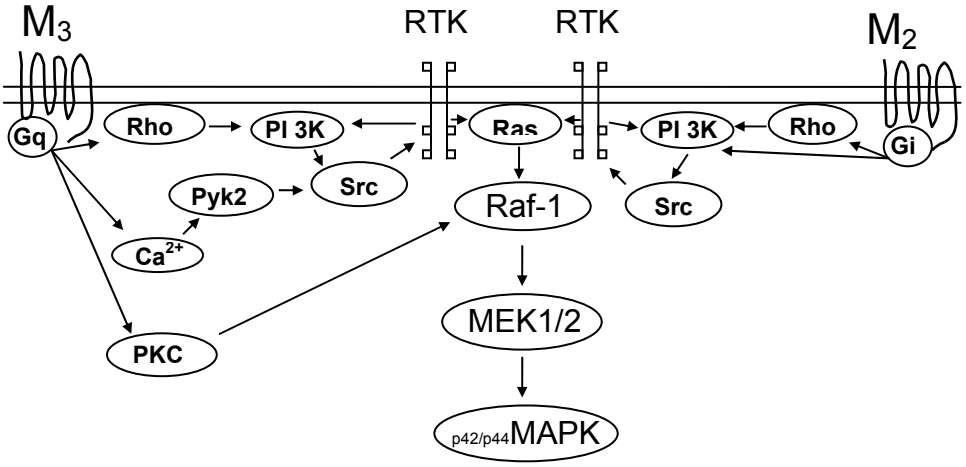

Figure 10.2 *Putative mechanisms of activation of p42/p44 MAP kinase, Rho and PI3-kinase by muscarinic M_2 and M_3 receptors in airway smooth muscle. These signalling pathways provide potential mechanisms for muscarinic receptors to cross-talk with growth factor-induced signal transduction, relevant 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 M_3 receptor expression is thought to increase under these conditions, since reconstitution of the contractile phenotype in culture also induces functional re-coupling of muscarinic M_3 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 M_3 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 M_3 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 M_3 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 M_2 and M_3 receptors, this potentiation was found to be mediated solely by muscarinic M_3 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 G_q coupled bradykinin B_2-receptors induces synergistic activation of mitogenesis when combined with EGF, which was dependent on conventional PKC isozymes (Chapter 8). In addition, the G protein-coupled receptor agonist lysophophatidic acid is synergistic with EGF by activating Rho [83]. Since muscarinic M_3 receptors activate both Rho and conventional PKC isozymes, 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

not been addressed, possibly because the G_q coupled muscarinic M_3 receptor loses its expression rapidly in culture [90]. Nevertheless, cholinergic regulation of ASM secretory function may be of great importance and warrants future investigation.

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, 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.

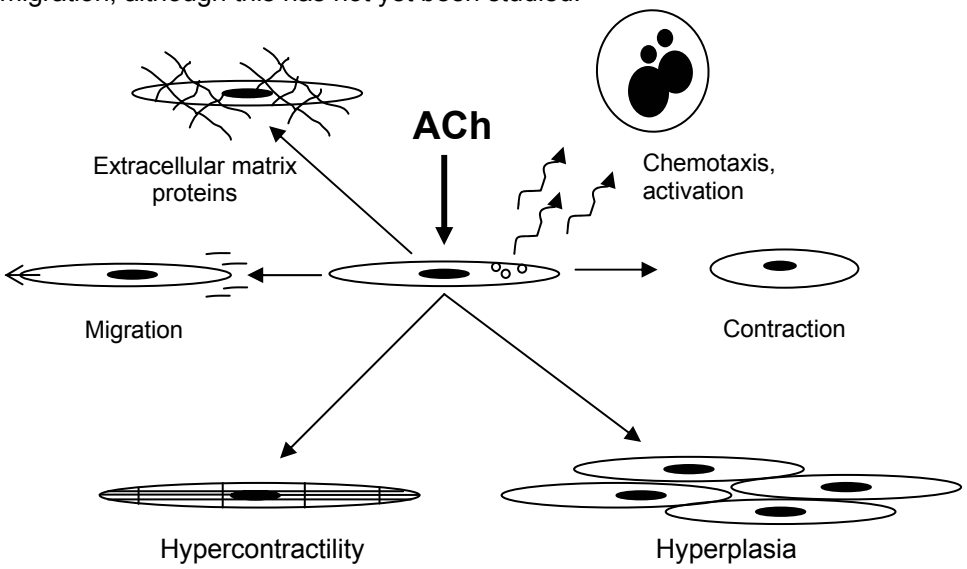

Figure 10.3 *Proposed mechanisms by which ACh could affect ASM remodelling. ACh has been shown to affect ASM contractility, contractile protein expression, pro-mitogenic signalling and proliferation. In addition, like several other G protein-coupled receptor agonists, ACh could also be involved in ASM cell migration, extracellular matrix protein production and secretion of cytokines and chemokines. Alltogether, these effects could contribute to airway remodelling in asthma and COPD.*

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 remodeling. 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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Chapter 11

General Discussion and

Summary

Growth factor-induced phenotypic modulation

Isolated smooth muscle cells in culture do not immediately start dividing, even in a medium containing all nutrients required. Before entering the cell cycle, the cells first accommodate their phenotype to their new environment. They lose their contractile properties and modulate to a proliferative and synthetic phenotype, characterised by decreased contractile protein and M_3 receptor expression, but enhanced expression of PKC and synthetic organelles [1;2]. This process of phenotypic modulation has been postulated to occur *in vivo* during periods of airway inflammation, in view of the increased airway smooth muscle (ASM) mass seen in asthmatics which is partly due to an increase in cell number [3]. A central role for phenotypic modulation in smooth muscle growth is also acknowledged in vascular remodeling. In atherosclerotic lesions, neointima formation is accompanied by modulation of smooth muscle cell phenotype to favour conditions of growth and extracellular matrix deposition [4].

ASM phenotypic modulation can be induced by exposing cells to high concentrations of fetal bovine serum (FBS). The main disadvantage of studying phenotypic plasticity in cell culture is, however, that cell to cell contacts and cell to matrix contacts are disrupted. Even at confluence, cell culture cannot mimic the three-dimensional context of intact muscle. The extracellular matrix in which the muscle cells are embedded has been found of major importance: vascular smooth muscle cells can be retained in their contractile phenotype when cultured in laminin-coated flasks or on matrigel, which contains solubilized basement membrane proteins [5;6]. In addition, human ASM cells cultured on laminin exhibit a contractile phenotype, whereas collagen I and fibronectin favour progression to a proliferative and less contractile phenotype [7].

To study the impact of the natural mix of extracellular matrix components on phenotypic modulation, Tao et al. cultured ASM cells on top of a ethanol-fixed layer of dead ASM cells, reasoning that the complex mix of extracellular matrix proteins synthesized by smooth muscle cells would create a more physiological environment [8]. They found that cells cultured under such conditions did not lose contractile responsiveness to methacholine, suggesting that phenotypic modulation is less prominent in this setting. Also, these cells did not spread to the extent that smooth muscle cells do when plated on glass and they did not acquire stress fibres. It is important to note, however, that the conditions used to create this matrix environment (smooth muscle cells only, stimulation with 10 % FBS) preclude a proper comparison to intact muscle.

Therefore, we used an organ culture approach in our studies, since this model has the advantage of having all endogenous extracellular matrix constituents and cell to cell contacts intact. Using this model, we showed that growth factor-induced phenotypic modulation can be induced in the intact muscle (Chapter 2). In summary, this study demonstrated that treatment of bovine tracheal smooth muscle (BTSM) strips with FBS caused a time-dependent ($t_{½}$ = 2.8 days) decrease in maximal contraction compared to serum-deprived control strips. Contractility was not

completely abrogated, however, even when exposure to FBS was as long as 8 days. In addition, muscarinic M_3 receptor expression was not affected as this would result in a decreased sensitivity of the muscle strip for methacholine. Importantly, growth factor (EGF, IGF-1, PDGF)-induced reductions in contractility were also observed. These were strongly correlated to their mitogenic responses in unpassaged primary cultures of BTSM cells, indicating that the growth factor-induced reductions in contractility may have been the consequence of growth-induced phenotypic modulation. This is supported by the observation that the reductions in contractility were evident for both receptor-dependent (methacholine) and -independent (KCl) contractions, suggesting that the effects occurred at the level of contractile protein expression or activity.

It is not clear whether the less prominent loss of contractility in organ culture as opposed to cell culture is caused by a less prominent phenotypic modulation of each individual smooth muscle cell. Presumably, only the fraction of cells that is committed to enter the cell cycle, undergoes phenotypic modulation. This concept of phenotypic heterogeneity is supported by the observation that regulation of contractility is inversely correlated to the mitogenic responses induced by the growth factors applied (Chapter 2). Cell division is an all-or-nothing response and the preceding switch in phenotype could be considered likewise.

Recently, a similar organ culture approach was used by Moir et al. for human bronchioli. Contractility of these rings was similarly sensitive to prolonged (3-6 days) FBS exposure, but no major changes in ASM content could be observed in these rings nor were there measurable changes in contractile protein expression [9]. The contraction regulatory protein calponin was decreased due to serum exposure in these rings however, which could be indicative of some degree of phenotypic modulation. Of note, calponin expression is more sensitive to phenotypic modulation when compared to contractile proteins in cultured canine ASM cells [1].

The study by Moir et al. also showed increases in calponin expression when human bronchioli were exposed to D-STIM, a medium formulated to maintain a contractile smooth muscle phenotype [9]. This medium is free of serum, which is interesting in view of the reported hypercontractility of cultured canine ASM cells upon prolonged serum deprivation [10;11]. However, both the mentioned D-STIM and the serum-free media used to induce hypercontractility in canine ASM cells contain high amounts of insulin (usually 1 μM). In Chapter 3, we demonstrated that organ cultured BTSM strips exposed to insulin respond with increased contractility to KCl and methacholine, as compared to fresh and serum-free treated BTSM strips. Furthermore, cultured BTSM cells pretreated with insulin were found less proliferative in response to peptide growth factors (EGF, PDGF, IGF-1), even though acute co-treatment of these growth factors with insulin synergistically activated proliferation. Thus, this study showed that the effects of insulin on ASM are time-dependent. Insulin acutely potentiates the mitogenic responses to other growth factors, but induces a hypercontractile and hypo-proliferative phenotype upon prolonged pretreatment. Therefore, the presence of insulin rather than the absence

of serum could have been responsible for the hypercontractile ASM phenotype as described by others. This observation is supported by studies demonstrating insulin-induced differentiation of vascular smooth muscle and skeletal muscle [12;13].

Regulation of contractility by Rho-kinase

Rho-kinase is involved in the regulation of smooth muscle specific gene transcription by controlling the subcellular localization of the transcription factor SRF [14;15]. To investigate whether these events also take place in intact ASM, we conducted the study described in Chapter 4, demonstrating that pretreatment with the Rho-kinase inhibitor Y27632 decreased overall contractility of organ cultured BTSM strips. This indicates that basal Rho-kinase activity is involved in controling ASM contractility. The contribution of Rho-kinase to ASM contraction was not dependent, however, on the phenotypic state of the muscle: *hyper*contractile BTSM strips, pretreated with insulin for 8 days and *hypo*contractile BTSM strips, pretreated with FBS for 8 days were equally susceptible to Rho-kinase inhibition for their contractile responses to methacholine and KCl as compared to serum-free pretreated controls.

Since Rho-kinase might also be involved in phenotypic modulation induced by growth factors, we studied the effects of Y27632 on PDGF-induced proliferation and PDGF-induced reductions in contractility as well. The latter processes were all completely insensitive to Y27632, in contrast to inhibitors of p38, p42 and p44 MAPK and PI 3-kinase. This supports the concept that the reciprocal relationship between contractility and growth (cf. Chapter 2) is prompted by growth-induced phenotypic modulation. In addition, the study described in Chapter 4 showed that Rho-kinase inhibition may result in an equally less contractile phenotype as observed for growth factors, without the concomitant detrimental effects on ASM remodeling,

In turn, this may suggest that growth factors do not activate RhoA and Rho-kinase, which contrasts to the results presented in Chapter 5, in which growth factor-induced contraction (IGF-1, angiotensin II) was shown to be extremely dependent on Rho-kinase in human bronchi. These apparently conflicting results are most likely explained by species differences as the growth factor applied in Chapter 4 (PDGF) does not induce BTSM contraction (unpublished observations). It can therefore not be excluded that Rho-kinase is able to counteract growth factor-induced reductions of human ASM contractility. The observed maintenance of contractility by Rho-kinase in Chapter 4 is nonetheless of potential importance in relation to chronic changes in ASM function in asthma. Rho-kinase dependent contraction has been reported to be increased in repeatedly allergen-challenged Brown-Norway rats [16]. This increase was accompanied by increases in RhoA protein expression, which suggests that repeated allergen challenge could also evoke increased contractility through such a mechanism.

Activation of Rho-kinase has been reported to occur upon stimulation of excitatory GPCRs [17]. In addition, although Rho-kinase is known to be activated by growth factors in human ASM cells, the response induced by GPCRs has been found more

pronounced [18]. Surprisingly therefore, histamine did not rely at all on Rho-kinase for its contraction whereas growth factor induced contraction was completely abolished (Chapter 5). This shows that kinase activation status does not necessarily have to parallel the impact on the physiological response. Most likely the transduction reserve of inositol-1,4,5-triphosphate generated by histamine is sufficient to overcome the absence of Rho-kinase mediated signalling. Weaker contractile agonists such as growth factors on the other hand, need all the transductional support they can get.

Regulation of contractility by GPCRs

Since GPCR agonists activate Rho-kinase, one might expect that prolonged treatment of BTSM strips with a GPCR agonist would increase contractility. As described in Chapter 6, however, prolonged treatment with methacholine dramatically decreased contractility and contractile protein expression (sm-α-actin, sm-MHC); these reductions in contractility and contractile protein expression were concentration dependent. However, the methacholine-induced reduction in contractility was not synergistic with the PDGF-induced reduction in contractility; nor was the decreased contractility accompanied by an increase in proliferative capacity, indicating that the methacholine-induced effects are distinct from the classical phenotype ´switch´ induced by growth factors. Likewise, these methacholine-induced effects did not rely on signalling pathways involving PI 3-kinase or MAPK. Rather, a prolonged elevation of $[Ca^{2+}]_i$ appeared to be responsible for the decrease in overall contractility, as selective elevation of cytosolic $[Ca^{2+}]$ by KCl could mimic the strong response induced by methacholine. It is not known whether these effects of methacholine represent physiologically relevant processes, since prolonged pretreatment with relatively high concentrations were required. In addition, phenotypic modulation of smooth muscle cells due to allergen-induced growth might alter the methacholine-induced signalling characteristics, including the regulation of $[Ca^{2+}]_i$ homeostasis. Nevertheless, the results presented in Chapter 6 imply that in phenotypically contractile ASM cells, direct stimulation with GPCR agonists is not likely to induce a hypercontractile phenotype.

These effects of GPCR agonists also provide a possible explanation for why the use of FBS in organ cultured smooth muscles has often failed to produce results that support a role for growth-induced phenotypic modulation in organ culture. FBS is a classical source for growth factors but also contains insulin and GPCR agonists like serotonin, which are responsible for the acute contractile effects of serum [19]. In the organ cultured rat renal artery, no effect of prolonged treatment with serum, decreasing contractility, could be demonstrated [19]. However, both in the human bronchiolus [9], rat tail artery [20], guinea pig ileum [21] and canine colon [22], treatment with FBS dramatically reduced contractility when compared to the absence of serum, although force responses still deteriorated over time in the absence of serum in some of these studies. In the rat tail artery and the guinea pig ileum, this serum-induced reduction of contractility appeared to be related to continuously elevated $[Ca^{2+}]_i$ levels rather than phenotypic modulation, since co-treatment with

verapamil could partially reverse the observed effects [21;23]. In addition, serum induced mitogenesis in these preparations appeared to be independent from its effects on contractility as verapamil did not inhibit the serum-induced incorporation of [^{3}H]thymidine. It should be noted though that even in the presence of verapamil some reduction in contractility persists [23], which is quantitatively comparable to the decrease in contractility observed by us due to growth factor exposure (Chapter 2). This shows that the treatment with serum in these preparations may have caused both GPCR / Ca^{2+} dependent reductions in contractility, comparable to our observations for methacholine (Chapter 6), as well as growth factor-induced phenotypic modulation.

Contractile agonists as growth factors

Even though the above mentioned results suggest that GPCR agonists are not likely to affect BTSM phenotype in a way similar to growth factors, contractile GPCR agonists may on the other hand affect ASM proliferation. Excitatory GPCR agonists were first recognized as potential contributors to the increase of ASM mass in asthma in 1990, when Panettieri et al. found that histamine could increase canine ASM cell number in a concentration-dependent fashion and to a similar extent as 10 % FBS [24]. Since then, the mitogenic responses to a vast number of GPCR agonists have been characterised (Table 11.1). Despite of the reported mitogenic effects of histamine, GPCR agonists in general are not effective or less effective compared to growth factors. However, they are generally considered effective in augmenting growth-factor induced proliferative responses.

This matches our results presented in Chapter 7. In this study, we demonstrated that the GPCR agonist methacholine (a derivative of acetylcholine) is not mitogenic for BTSM cells by itself, but concentration dependently augments PDGF-induced ASM proliferation. This mitogenic synergism was dependent on the cell culture stage used. It could be observed only in unpassaged BTSM cells, still functionally expressing G_q coupled M_3 receptors, whereas higher cell culture stages, not expressing M_3 receptors, did not have mitogenic responses to growth factors at all. The suggested relationship between G_q coupled M_3 receptors and mitogenic synergism was confirmed by the observation that the muscarinic receptor antagonists 4-DAMP and DAU5884, applied in M_3 selective concentrations, completely abrogated these responses. Notably, the G_i coupled muscarinic M_2 receptor was not at all involved, as the muscarinic M_2 receptor selective antagonist gallamine was without effect.

In line with these results, the study described in Chapter 8 demonstrates that the GPCR agonist bradykinin is not mitogenic by itself, but concentration-dependently potentiates the response to the peptide growth factor EGF in BTSM cells. This effect was mediated by the bradykinin B_2 receptor, which is a G_q coupled receptor. Further downstream, this effect appeared to be caused by conventional PKC iso-enzymes, as the synergistic responses were sensitive to both the pan-specific PKC inhibitor GF109203X and the conventional iso-enzyme specific inhibitor Gö6976. PKC-mediated activation of p42/p44 MAP kinase was not involved however, as these

latter enzymes were not activated synergistically by bradykinin and EGF. Therefore, the results from this study demonstrate that parallel rather than consecutive activation of conventional PKC iso-enzymes and p42/p44 MAP kinase may be responsible for the synergistic mitogenic response of a G_q coupled receptor agonist and a growth factor.

Table 11.1 *Effects of contractile GPCR agonists on ASM proliferation*

Stimulus	Mitogenic by itself		Mitogenic in combination with growth factor		Reference:
	Yes	No	Yes	No	
Histamine	Canine Human	Bovine Human	Human Bovine		[24;33;34]
Acetylcholine		Bovine Human	Bovine Human		[34] Chapter 7
Serotonin		Bovine			[35]
LTD_4		Human	Human		[36]
Endothelin-1	Bovine	Guinea Pig Human	Guinea Pig Human		[27;37;38]
Neurokinin A		Rabbit			[39]
Substance P	Rabbit Human				[29;39]
Thrombin	Bovine Human Rabbit		Bovine Human		[34;40]
TxA_2	Human Rabbit		Human		[41-43]
LPA	Human		Human		[28]
Bradykinin	Human	Bovine Human	Bovine		[30;44] Chapter 8

An important question that arises from the observations made in Chapters 7 & 8 is what underlies this apparent effectiveness of G_q coupled receptor as opposed to G_i, coupled receptors particularly because it has previously been postulated that G_i coupled receptor agonists are *more* mitogenic compared to G_q coupled receptor agonists. This postulate related to studies describing that G_i mediated activation of Ras is more effective than G_q mediated activation and to the observation that ASM mitogenesis is often pertussis toxin sensitive [25-28]. The selectivity of pertussis toxin for G_i may be considered questionable, however: pertussis toxin is known to inhibit mitogenesis to a variety of stimuli, irrespective of their selectivity to G_i, G_q (e.g. substance P, bradykinin) or even receptor tyrosine kinases (e.g. PDGF) [29-31]. In addition, pertussis toxin treatment has been described to downregulate the expression of the conventional PKCα iso-enzyme in pulmonary endothelial cells [32].

This has important implications, as synergistic induction of mitogenesis by the G_qPCR agonist bradykinin is dependent on conventional PKC iso-enzymes (Chapter 8). Furthermore, selective stimulation of receptors known to couple to G_i is necessarily not mitogenic, as observed by us for the M_2 receptor in Chapter 7.

Another important question is why some GPCR agonists are mitogenic and others not. For instance, muscarinic receptor agonists are generally found to be ineffective by themselves, which is desirable as the tonic presence of acetylcholine under normo-physiological conditions should not result in excessive airway narrowing over time. In contrast, thrombin is highly mitogenic (Table 11.1), but present only during periods of wound healing when proliferation of smooth muscle is required. Since thrombin is one of the most effective mitogenic GPCR agonists, it could be envisaged that its capability to activate multiple G proteins (G_i, G_q, $G_{12/13}$) induces 'auto-synergy'. This is illustrated by the fact that two GPCR agonists can respond in a synergistic mitogenic fashion, which is more profound when G_i and G_q coupled receptor agonists are combined [28]. However, methacholine activates G_i and G_q as well, yet is not mitogenic by itself and dependent on G_q coupled M_3 receptors only for it synergistic mitogenic response (Chapter 7). In addition, recent evidence suggests that thrombin is not even dependent on PAR receptors for its mitogenic effect [45]. A selective focus on thrombin as a model for GPCR agonists in general is therefore not completely appropriate.

Others have considered the possibility that differences in signaling kinetics may underlie the discrepancy between mitogenic and non-mitogenic GPCR agonists. This approach appears to be more successful: several studies have indicated a requirement for prolonged p42/p44 MAPK activation for mitogenesis in both human and bovine ASM [29;35;46], irrespective of the type of stimulus applied (GPCR agonist, RTK agonist or kinase activator). Also in Chapter 8 we showed that the transient p42/p44 MAP kinase activation by bradykinin was not mitogenic, whereas the sustained PMA-induced p42/p44 MAP kinase activation resulted in the induction of mitogenesis. The key to this stimulus-specificity was the differential activation of PKC isozymes: selective inhibition of conventional PKC isozymes by Gö6976 could fully inhibit bradykinin-induced p42/p44 MAPK activation, but had not even the slightest effect on PMA-induced responses. GF109203X however, which inhibits both conventional and novel PKCs, could fully inhibit the PMA effects, which suggests the involvement of novel PKC isozymes [47]. It would be interesting to investigate this agonist-specificity for mitogenic and non-mitogenic GPCR agonists as this may provide insight in the role that these PKC isozymes have in the kinetics of p42/p44 MAP kinase activation.

Nevertheless, conventional PKC isozymes appear to be sufficient for a synergistic interaction with growth factors in view of the bradykinin-induced effects in combination with EGF. Moreover, the synergistic activation of proliferation is not accompanied by a synergistic activation of p42/p44 MAP kinase, as demonstrated by us (Chapter 8) and others [34;48]. This suggests that GPCR-induced proliferation

and GPCR-induced potentiation of growth-factor induced proliferation are mechanistically completely different effects.

Importantly, both ASM phenotypic plasticity and the synergistic mitogenic interaction of GPCR agonists with growth factors reported in the previous Chapters may also occur *in vivo*, as described in Chapter 9. In a guinea pig model of ongoing asthma, repeated allergen challenges were shown to induce ASM hyperplasia in the non-cartlaginous airways and to result in phenotypically hypercontractile ASM cells in the cartilaginous airways. Moreover, the long-acting muscarinic receptor antagonist tiotropium bromide could inhibit allergen-induced ASM hyperplasia, demonstrating that endogenous acetylcholine contributes to ASM growth *in vivo* presumably through mechanisms similar to those observed *in vitro*. Moreover, the other features of allergen-induced airway smooth muscle remodeling, being the increases in contractility (tracheal ring preparations) and the increases in contractile protein expression (lung homogenates) were also reduced by treatment with tiotropium bromide. These results for the first time indicate that endogenous ACh may be involved in allergen-induced airway remodelling *in vivo*. Since prolonged neuronal and non-neuronal release of ACh may be induced by various inflammatory mediators as observed in asthma and COPD, a role for ACh in airway remodelling should be envisaged, which is elaborated on in Chapter 10.
Taken together, the studies described in this thesis have revealed that:

- Phenotypic plasticity of airway smooth muscle is no epi-phenomenon of cell culture settings, but occurs in organ-cultured intact airway smooth muscle preparations in response to growth factors, as well as *in vivo* in response to repeated allergen challenges (Chapters 2 & 9).
- Insulin is able to induce a hypercontractile airway smooth muscle phenotype (Chapter 3).
- Phenotypic modulation of airway smooth muscle induced by growth factors is dependent on pathways involving p38, p42 and p44 MAP kinase, as well as PI 3-kinase, but not Rho-kinase (Chapters 2 & 4)
- Basal Rho-kinase activity is involved in the maintenance of airway smooth muscle contractility (Chapter 4).
- Rho-kinase is a major contributor to growth factor-induced airway smooth muscle contraction (Chapter 5).
- Long-term treatment of intact airway smooth muscle with GPCR agonists such as methacholine decreases contractility and contractile protein expression through mechanisms presumably involving prolonged elevations of cytosolic [Ca^{2+}]. Of importance, this modulation of smooth muscle contractility by methacholine does not involve the phenotype ´switch´ as it is not accompanied by increases in proliferative capacity (Chapter 6).
- GPCR agonists, such as methacholine and bradykinin, are generally not mitogenic by themselves but are effective in potentiating growth factor-induced proliferation, both *in vitro* and *in vivo* (Chapters 7 - 9). M_3 (methacholine) and B_2 (bradykinin) receptors are responsible for this synergistic effect, with no involvement at all for M_2 (methacholine) receptors,

which implicates a more prominent role for G_q as compared to G_i coupled receptors (Chapters 7 & 8).

- Activation of novel but not of conventional PKC iso-enzymes leads to sustained p42/p44 MAP kinase activation and proliferation of airway smooth muscle (Chapter 8).
- Parallel rather than consecutive activation of PKC and p42/p44 MAPK appear to be crucially important in the regulation of proliferation synergy by a GPCR agonist and a growth factor (Chapter 8).
- The long-acting muscarinic receptor antagonist tiotropium bromide attenuates airway smooth muscle remodeling (increased contractility, contractile protein expression and airway smooth muscle content) induced by repeated allergen challenges in a guinea pig model of ongoing asthma, suggesting a significant role for endogenous acetylcholine in the development and/or progression of chronic asthma (Chapter 9).

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Nederlandse Samenvatting

Allergisch astma is een chronische luchtwegaandoening, die onder meer wordt gekarakteriseerd door overgevoeligheid van de luchtwegen voor obstructieve prikkels (luchtweg hyperreactiviteit). Dit houdt in, dat astmapatiënten sterk reageren op prikkels die in gezonde personen niet of nauwelijks luchtwegvernauwing veroorzaken. Deze overgevoeligheid is voor een belangrijk deel toe te schrijven aan processen die, direct of indirect, de contractiele functie van de luchtweg gladde spieren bevorderen. Deze spierlaag regelt de interne diameter van de luchtweg en daarmee de hoeveelheid ingeademde lucht.

Ontstekingsprocessen spelen een belangrijke rol bij luchtweghyperreactiviteit. Ontstekingscellen die bij allergisch astma betrokken zijn, zoals mestcellen en eosinofielen, zijn in staat mediatoren uit te scheiden die zelf contractie induceren en bovendien de contractiele effecten van andere verbindingen (bijvoorbeeld neurotransmitters) versterken. Bovendien leidt het ontstekingsproces tot schade aan het luchtwegepitheel, dat normaliter zowel passief (barrièrefunctie) als actief (bijvoorbeeld door productie van het relaxerende stikstofmonoxide) bescherming biedt tegen luchtwegvernauwing. Bovendien legt epitheelschade sensibele zenuwuiteinden bloot, die bij prikkeling door bijvoorbeeld koude lucht, mist, (roet)deeltjes of allergische mediatoren, via een reflexreactie de afgifte van contractiele neurotransmitters kunnen bevorderen.

Op de langere termijn gaan ook andere processen een rol spelen. Chronische luchtwegontsteking bij allergisch astma leidt tot structurele veranderingen van de luchtwegen, waarvan wordt aangenomen dat ze bijdragen aan luchtweghyperreactiviteit. Zo is de luchtwegwand bij astma sterk verdikt, onder meer door toename van de gladde spiermassa en door afzetting van matrixeiwitten onder de epitheellaag. Ook is het basaalmembraan, waaraan epitheelcellen gehecht zijn, verdikt, is er een overmatige slijmproductie en is de doorbloeding van de luchtweg toegenomen. De gebruikelijke term die al deze structurele veranderingen samen weergeeft, is ‘luchtwegremodelling’.

De studies die in dit proefschrift zijn beschreven, richten zich op de processen die betrokken zijn bij remodelling van de gladde spierlaag. Centraal staat de gedachte dat de individuele gladde spiercel actief kan bijdragen aan luchtwegremodelling door zijn fenotype aan te passen. Door zijn fenotype te veranderen kan de gladde spiercel zijn functie overschakelen van contractiel (nadruk op luchtwegvernauwing) naar overwegend proliferatief (nadruk op toename in massa door celdeling) en synthetisch (nadruk op uitscheiden van mediatoren). Deze verandering gaat gepaard met veranderingen in eiwitexpressie die de functieverschuiving ondersteunen. Zo brengt het contractiele fenotype meer contractiele eiwitten zoals actine en myosine tot expressie, terwijl het proliferatieve en synthetische fenotype meer protein kinase C (PKC; o.a. betrokken bij proliferatie) en Golgi eiwitten (o.a. betrokken bij eiwitsynthese) tot expressie brengt.

Enkele jaren geleden is de hypothese geformuleerd dat groeifactoren het fenotype van de gladde spiercel dusdanig kunnen veranderen dat de proliferatieve en synthetische functies bevorderd worden. Omgekeerd zou de afwezigheid van groeifactoren een contractiel of zelfs hypercontractiel fenotype kunnen induceren, waarbij de proliferatieve en synthetische functies juist afnemen. Aangezien wisselende episodes van allergeenblootstelling bij astma de concentratie groeifactoren in de luchtwegen afwisselend doen toe- en afnemen, hebben wij gepostuleerd dat bij astma de gladde spiercel herhaaldelijk van fenotype verandert. Dit herhaaldelijk wisselen van contractiel naar proliferatief / synthetisch en vice versa zou zo kunnen bijdragen aan een pulsatief toenemende massa en contractiliteit. De gevolgen voor luchtwegremodelling gaan zelfs verder. Door het fenotype te veranderen naar synthetisch kunnen gladde spiercellen mogelijk bijdragen aan afzetting van bindweefsel onder de epitheellaag door actief matrixeiwitten te gaan uitscheiden. Bovendien dragen ze mogelijk bij aan de vorming van nieuwe bloedvaten door de productie van angiogene groeifactoren, en aan chronische luchtwegontsteking door het uitscheiden van moleculen die ontstekingscellen aantrekken.

Proliferatie van luchtweg gladde spiercellen wordt in belangrijke mate gereguleerd door intracellulaire signaaltransductieroutes waarbij MAP kinases en PI 3-kinase betrokken zijn. Deze kinases worden normaliter geactiveerd door groeifactorreceptoren en zijn betrokken bij een reeks effecten, die door groeifactoren worden gereguleerd, waaronder celdeling en differentiatie. Dezelfde signaaltransductie-routes worden ook aangestuurd door agonisten, die hun effect vooral uitoefenen via G-eiwit gekoppelde receptoren. Voorbeelden van dergelijke agonisten zijn de neurotransmitters acetylcholine, neurokinine A en substance P, alsmede ontstekingsmediatoren zoals histamine en bradykinine. Van deze agonisten is beschreven dat ze zelf ook proliferatie van de luchtweg gladde spiercel kunnen induceren en/of het proliferatieve effect van groeifactoren kunnen versterken.

Gebaseerd op het bovenstaande hebben wij tevens gepostuleerd dat fenotypeveranderingen en proliferatie van de luchtweg gladde spier gereguleerd kunnen worden door groeifactoren en G-eiwit gekoppelde receptoragonisten en dat interacties tussen deze twee betrokken zijn bij luchtwegremodelling. Om dit te bestuderen is gebruik gemaakt van celkweken, orgaankweken en een diermodel. Deze methodologisch brede aanpak maakt het mogelijk om het belang van bevindingen op cellulair en moleculair niveau te vertalen naar intacte biologische systemen. Dit laatste is belangrijk, omdat in het intacte orgaan cel-celcontacten en cel-matrixcontacten behouden blijven. Cel-matrixcontacten in het bijzonder hebben een duidelijke regulerende invloed op gladde spierfenotype en -proliferatie.

Desalniettemin zijn er tot dusverre geen studies uitgevoerd op intacte orgaansystemen, die fenotypeveranderingen beschrijven. Dit onderstreept het belang van de studie beschreven in Hoofdstuk 2. Hierin hebben we gebruik gemaakt van gladde spierpreparaten van de rundertrachea, die tot 8 dagen in kweek zijn

gehouden in af- en aanwezigheid van groeifactoren. Lange termijn behandeling met foetaal runderserum (FBS), dat veel groeifactoren bevat, was in staat de contractiliteit van deze gladde spierstrippen te verminderen, terwijl de afwezigheid van groeifactoren de contractiliteit juist deed toenemen. Dit proces was tijdsafhankelijk
($t_{½}$ = 2.8 dagen) en kon ook worden geïnduceerd door de groeifactoren PDGF en IGF-1, terwijl EGF geen effect had. Bovendien was de mate waarin deze groeifactoren de contractiliteit onderdrukten sterk gecorreleerd (r = 0.97) met hun proliferatieve respons. Dit geeft aan dat de vermindering in contractiliteit veroorzaakt is doordat de individuele cellen in het gladde spierpreparaat een proliferatief fenotype hebben aangenomen.

Tegenover het proliferatieve fenotype staat het (hyper)contractiele fenotype. Verhoging van contractiliteit en inductie van contractiele eiwitexpressie kan worden bewerkstelligd door luchtweg gladde spiercellen langdurig te depriveren van groeifactoren. Media die normaal gebruikt worden voor dergelijke experimenten bevatten doorgaans insuline. Hoofdstuk 3 laat zien, dat insuline acuut proliferatie van luchtweg gladde spiercellen induceert en zelfs de effecten van andere groeifactoren synergistisch kan versterken. Op lange termijn worden de proliferatieve eigenschappen echter onderdrukt en worden contractiele eigenschappen bevorderd. Dit toont aan dat insuline een hypercontractiel fenotype induceert. In vivo zou in feite zelfs dit mechanisme en niet deprivatie van groeifactoren verantwoordelijk kunnen zijn voor eerder genoemde hypercontractiliteit. Bovendien zouden deze bevindingen belangrijke gevolgen kunnen hebben voor de toepassing van geïnhaleerd insuline. Deze toedieningsroute wordt door de farmaceutische industrie overwogen bij de behandeling van type I en II diabetes mellitus.

Rho-kinase is betrokken bij de regulatie van contractiele eiwitexpressie in luchtweg gladde spiercellen en zou als zodanig dus betrokken kunnen zijn bij de regulatie van het fenotype. Aangezien Rho-kinase activiteit ook bijdraagt aan contractie, is in Hoofdstuk 4 de rol van Rho-kinase bij fenotypeveranderingen bestudeerd in intacte luchtweg gladde spier preparaten. Inhibitie van Rho-kinase had vergelijkbare effecten op contractie van controle weefsel als van het (in Hoofdstuk 2 beschreven) *hypo*contractiele fenotype en van het (in Hoofdstuk 3 beschreven) *hyper*contractiele fenotype. Ook bleek Rho-kinase niet betrokken te zijn bij het induceren van fenotype-veranderingen door groeifactoren. In overeenstemming hiermee had remming van Rho-kinase ook geen effect op groeifactor-geïnduceerde proliferatie van luchtweg gladde spiercellen. Kinases waarvan bekend is dat ze hier wel bij betrokken zijn, zoals p38 MAP kinase, p42/p44 MAP kinase en PI 3-kinase, bleken allen ook een rol te hebben in het reduceren van contractiliteit, wat in overeenstemming is met de theorie dat proliferatie en fenotypeverschuiving aan elkaar gekoppeld zijn.
Niettemin is er wel een rol voor basale Rho-kinase activiteit in het onderhouden van contractiliteit. Lange termijn behandeling (8 dagen) met een Rho-kinase remmer

bleek namelijk de maximale methacholine-geïnduceerde contractie van spierpreparaten te verminderen tot een niveau dat vergelijkbaar is met dat na behandeling met groeifactoren. Rho-kinase is daarmee mogelijk een interessant target voor de behandeling van obstructieve luchtwegziekten, aangezien wel de gunstige hypocontractiele toestand wordt bereikt zonder dat proliferatieve eigenschappen worden geïnduceerd.

Hoofdstuk 5 laat zien dat de acute groeifactor-geïnduceerde contractie van *humaan* bronchiaal glad spierweefsel afhankelijk is van Rho-kinase wat suggereert dat groeifactoren Rho-kinase wel degelijk kunnen activeren. Mogelijk is activering van Rho-kinase door groeifactoren dus maar van korte duur.

Contractiele agonisten die aangrijpen op bepaalde G-eiwit gekoppelde receptoren worden geacht in staat te zijn om ook Rho-kinase te activeren. Desondanks was de door histamine geïnduceerde contractie van humaan bronchiaal glad spierweefsel niet afhankelijk van Rho-kinase (Hoofdstuk 5). Bovendien induceerde lange termijn behandeling met methacholine, dat zowel G_i-gekoppelde M_2 receptoren als G_q-gekoppelde M_3 receptoren activeert, geen toename in contractiliteit. Contractiliteit en contractiele eiwitexpressie waren na behandeling met methacholine zelfs sterk afgenomen, zoals beschreven staat in Hoofdstuk 6. Deze vermindering van contractiliteit ging echter niet gepaard met een toename in proliferatie; er was ook geen interactie met de groeifactor-geïnduceerde afname in contractiliteit. Dit geeft aan dat de afname niet het gevolg is geweest van een fenotypeverschuiving, zoals die optreedt na behandeling met een groeifactor. Bovendien was de afname in contractiliteit niet of slechts gedeeltelijk afhankelijk van de mechanismen die wel verantwoordelijk zijn voor de effecten van groeifactoren; PKC bleek in het geheel niet betrokken; p42/p44 MAP kinase en PI 3-kinase slechts in beperkte mate. Selectieve blokkade van M_3 receptoren remde het effect echter volledig; bovendien kon ook langdurige behandeling met KCl, dat verhoogde intracellulaire Ca^{2+} spiegels veroorzaakt, de effecten van methacholine nabootsen. Hoofdstuk 6 laat daarom zien, dat langdurige stimulatie van contractie een negatieve feedback aanstuurt op het niveau van contractiele eiwitexpressie en dat contractiele (G_q en G_i gekoppelde) receptoragonisten waarschijnlijk geen hypercontractiliteit induceren.

Het bovenstaande betekent echter niet dat contractiele agonisten geen effect zouden kunnen hebben op proliferatie van luchtweg glad spierweefsel. Het is meerdere keren beschreven dat contractiele G-eiwit gekoppelde receptoragonisten zelf proliferatie kunnen veroorzaken en/of het effect van groeifactoren kunnen versterken. Ook onze resultaten, zoals beschreven in de hoofdstukken 7 en 8, ondersteunen deze stelling. Hoofdstuk 7 laat zien, dat stimulatie van muscarine receptoren door methacholine ervoor zorgt dat de PDGF-geïnduceerde proliferatie van runder tracheale gladde spiercellen wordt bevorderd, zonder dat stimulatie van muscarine-receptoren zelf effect heeft. Dit synergistisch effect verloopt in het geheel via de G_q gekoppelde M_3-receptor, daar de M_3-selectieve antagonisten 4-DAMP en DAU5884 het synergistische effect volledig kunnen remmen, terwijl de M_2-selectieve antagonist gallamine geen enkel effect heeft. Bovendien heeft methacholine alleen

een synergistisch effect in cellen, die ook de M_3-receptor functioneel tot expressie brengen. Naast de prominente rol voor G_q-gekoppelde receptoren die uit deze resultaten spreekt, kan geconcludeerd worden dat G_i-gekoppelde receptoren minder of zelfs niet relevant zijn.

In overeenstemming met deze resultaten wordt in Hoofdstuk 8 getoond dat de G_q gekoppelde receptoragonist bradykinine in staat is de EGF-geïnduceerde proliferatie van runder tracheale gladde spiercellen te bevorderen. Dit effect bleek volledig afhankelijk te zijn van door B_2-receptoren geactiveerde conventionele PKC iso-enzymen (i.e. PKCα en/of β) . p42/p44 MAP kinase bleek hierbij ook betrokken, ook al werd dit kinase niet synergistisch geactiveerd door EGF en bradykinine. Dit toont aan, dat parallelle en niet sequentiële activering van p42/p44 MAP kinase en PKC betrokken is bij de synergistische proliferatierespons.

Activering van conventionele PKC's alleen bleek echter niet voldoende voor een eigen proliferatieve respons. Dit leek opvallend, aangezien de PKC-activator PMA een potente mitogene verbinding is. Het kan echter verklaard worden door functionele verschillen tussen de verschillende klassen PKC iso-enzymen. In tegenstelling tot activering van conventionele PKC's, leidt activering van zgn. novel PKC's (i.e. PKC δ,ε en /of θ) wel tot een langdurige p42/p44 MAP kinase activering, wat een voorwaarde is voor een proliferatieve respons. De resultaten in dit hoofdstuk bieden daarom ook een mogelijke verklaring voor de verschillen in de mitogene capaciteit van verschillende contractiele G-eiwit gekoppelde receptoragonisten.

Hoe verhouden deze *in vitro* bestudeerde effecten van groeifactoren en G-eiwit gekoppelde receptoragonisten zich nu tot allergisch astma? Om antwoord te geven op deze centrale vraag is in Hoofdstuk 9 het effect van herhaalde allergeenprovocaties op remodelling van de luchtweg gladde spier (contractiliteit, contractiele eiwitexpressie, massa) bestudeerd in een caviamodel van allergisch astma. Tevens is het effect bestudeerd van behandeling met tiotropiumbromide. Dit is een langwerkend anticholinergicum dat gebruikt wordt bij de behandeling van obstructieve luchtwegaandoeningen zoals astma en COPD. Tiotropiumbromide blokkeert de muscarine-receptoren en heeft mogelijk een selectiviteit voor de M_3-receptoren. Aangezien de endogene muscarine-receptoragonist, acetylcholine, verhoogd wordt afgegeven bij allergisch astma, werd het op basis van de resultaten in de hoofdstukken 6 en 7 mogelijk geacht dat tiotropiumbromide de allergeen-geïnduceerde luchtwegremodelling zou kunnen remmen.

Gevonden werd dat herhaalde allergeen provocaties (eenmaal per week gedurende 12 weken) een toegenomen spiermassa, veroorzaakt door hyperplasie (toename in aantal gladde spiercellen; met name in de kleine luchtwegen) en hypercontractiliteit (met name in de grote luchtwegen) induceerde. Opvallend was de waarneming dat behandeling met tiotropiumbromide de hyperplasie met ca. 75 % kon remmen. Bovendien waren de andere kenmerken van luchtwegremodelling, namelijk de toename in contractiliteit (trachea gladde spier) en de toename in myosine-expressie

(longweefsel), sterk verminderd na behandeling met tiotropium. Deze resultaten laten voor het eerst zien dat endogeen acetylcholine betrokken is bij allergeen-geïnduceerde luchtwegremodelling. Aangezien ontstekingsmediatoren zowel de neuronale als de niet-neuronale afgifte van acetylcholine kunnen potentiëren bij astma en COPD, zou het blokkeren van muscarine receptoren met langwerkende anticholinergica de progressie van deze chronische luchtwegaandoeningen mogelijk kunnen verminderen. Deze stelling wordt ook besproken in Hoofdstuk 10.

De belangrijkste conclusies van dit proefschrift zijn kort samengevat:

- Onder invloed van groeifactoren (en deprivatie van groeifactoren) treden fenotypeveranderingen op in de intacte luchtweg gladde spier, in vitro en in vivo.
- Insuline induceert een hypercontractiel luchtweg gladde spierfenotype.
- Groeifactor-geïnduceerde fenotypeveranderingen zijn afhankelijk van p38 MAP kinase, p42/p44 MAP kinase en PI 3-kinase, maar niet van Rho-kinase.
- Rho-kinase is betrokken bij de groeifactor-geïnduceerde contractie van de humane bronchus.
- De G-eiwit gekoppelde receptoragonisten methacholine en bradykinine kunnen de proliferatieve effecten van groeifactoren zowel *in vitro* als *in vivo* potentiëren. Activering van G_q eiwitten is hierbij belangrijker dan van G_i eiwitten. Voor deze potentiëring is parallelle en niet sequentiële activering van PKC en p42/p44 MAP kinase verantwoordelijk.
- Activering van ‘novel’ maar niet van conventionele PKC iso-enzymen in luchtweg glad spierweefsel leidt tot langdurige p42/p44 MAP kinase-activering en proliferatie.
- Het langwerkende anticholinergicum tiotropiumbromide remt allergeen-geïnduceerde remodelling van de luchtweg gladde spier (toegenomen contractiliteit, contractiele eiwitexpressie en spiermassa) in een diermodel voor chronisch astma. Dit suggereert een belangrijke rol voor endogeen acetylcholine in de ontwikkeling en/of progressie van chronisch astma.

List of abbreviations

ACh	acetylcholine
AngII	angiotensin II
ASM	airway smooth muscle
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
BTSM	bovine tracheal smooth muscle
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine mono phosphate
ChAT	choline acetyltransferase
COPD	chronic obsructive pulmonary disease
COX	cyclo-oxygenase
CRC	concentration response curve
CTGF	connective tissue growth factor
DAG	diacylglycerol
DMEM	Dulbecco's modification of Eagle's medium
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular signal regulated kinase
FBS	fetal bovine serum
GPCR	G protein coupled receptor
HRP	horseradish peroxidase
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IGFBP	insulin-like growth factor binding protein
IL	interleukin
IRS	insulin receptor substrate
KH	Krebs-Henseleit
LIF	leukemia inhibitory factor
LT	leukotriene
MAPK	mitogen activated protein kinase
MBP	major basic protein
MEK	MAPK ERK kinase
MHC	myosin heavy chain
MLCK	myosin light chain kinase
MMP-1	matrix metalloproteinase-1
OA	ovalbumin
PAR	protease activated receptors
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PDK	PIP_3 dependent protein kinase
PG	prostaglandin
PI 3-kinase	phosphatidyl inositol 3-kinase

PIP_3	phosphatidyl inositol 3,4,5 tris-phosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PTX	pertussis toxin
Pyk2	protein tyrosine kinase 2
RTK	receptor tyrosine kinase
SRF	serum response factor
TNFα	tumor necrosis factor α
TGF-β	transforming growth factor-β
Tx	thromboxane
VEGF	vascular endothelial growth factor
VOC	voltage operated calcium channel

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Curriculum Vitae

The author of this thesis was born in Roosendaal, the Netherlands, on the 10th of September 1977. He moved to Groningen in 1995 to study pharmacy at the University of Groningen. During his studies, he was a student assistant (1999-2000) in a practical pharmacology course for year 3 Pharmacy Students at the University of Groningen. He performed his graduation project on agonist-induced constitutive β_2-adrenergic receptor activity at the Department of Molecular Pharmacology and graduated in march 2000. Later that year (September 2000) he started his PhD study at the same department, where he worked on a research project funded by the Netherlands Asthma Foundation, entitled: 'airway remodelling in asthma: functional interactions of neurotransmitters and growth factors', the results of which are presented in this thesis. In the forthcoming two years, he will work as a post-doc at the Department of Physiology at the University of Manitoba (Winnipeg, Canada).

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