Respiratory Medicine (2008) **102**, 1173–1181

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/rmed

Molecular mechanisms underlying airway smooth muscle contraction and proliferation: Implications for asthma

Girolamo Pelaia [a], Teresa Renda [a,b], Luca Gallelli [a], Alessandro Vatrella [b], Maria Teresa Busceti [a], Sergio Agati [a], Mario Caputi [b], Mario Cazzola [c,*], Rosario Maselli [a], Serafino A. Marsico [b]

[a] *Department of Experimental and Clinical Medicine, University ''Magna Græcia'' of Catanzaro, Italy*
[b] *Department of Cardiothoracic and Respiratory Sciences, Second University of Naples, Italy*
[c] *Department of Internal Medicine, Unit of Respiratory Diseases, University of Rome ''Tor Vergata'', Via Montpellier 1, 00133 Rome, Italy*

Received 15 January 2008; accepted 26 February 2008
Available online 24 June 2008

KEYWORDS
Airway smooth muscle;
Contraction;
Proliferation;
Asthma

Summary
Airway smooth muscle (ASM) plays a key role in bronchomotor tone, as well as in structural remodeling of the bronchial wall. Therefore, ASM contraction and proliferation significantly participate in the development and progression of asthma. Many contractile agonists also behave as mitogenic stimuli, thus contributing to frame a hyperresponsive and hyperplastic ASM phenotype. In this review, the molecular mechanisms and signaling pathways involved in excitation–contraction coupling and ASM cell growth will be outlined. Indeed, the recent advances in understanding the basic aspects of ASM biology are disclosing important cellular targets, currently explored for the implementation of new, more effective anti-asthma therapies.

Introduction

Once believed to be just a contractile tissue, airway smooth muscle (ASM) is instead characterized, in addition to contraction, by many other biological and functional properties including regulation of bronchomotor tone, perpetuation and amplification of airway inflammation, as well as active participation in bronchial remodeling.[1] Therefore, ASM plays a central role in almost all the pathophysiologic and clinical aspects of asthma such as reversible airway obstruction, bronchial hyperresponsiveness (BHR) to direct and indirect contractile stimuli, chronic inflammation and airway structural changes. In this regard, during the last few years significant advances have been made in elucidating the molecular events underlying ASM functions related to calcium responses, synthetic phenotype and hyperplastic behaviour. Of course, a better knowledge of

* Corresponding author. Tel.: +39 348 6412311; fax: +39 06 72596621.
E-mail address: mcazzola@qubisoft.it (M. Cazzola).

0954-6111/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.rmed.2008.02.020

these phenomena may enable to improve our understanding of asthma and to develop new therapeutic strategies directly targeted to ASM. Within such a context, the aim of the present review is to briefly outline the basic mechanisms responsible for excitation—contraction coupling and ASM cell proliferation, with a particular focus on the relevance of these subjects to asthma.

Excitation—contraction coupling

ASM contraction plays an important physiologic role in that contributes to match ventilation with perfusion, confers mechanical stability to noncartilaginous airways and prevents toxic inhaled agents from reaching the alveolar air spaces. However, the exaggerated ASM contractile response characterizing BHR, a constant feature of asthma, is the main pathophysiologic factor involved in the development of reversible airway obstruction. BHR consists of a reduced threshold to a wide range of contractile stimuli acting either directly on ASM by activating specific cell membrane receptors, or indirectly through neural pathways and/or the release of bronchoconstrictive mediators from both inflammatory and structural cells.[2] Table 1 provides a list of the most important contractile agonists relevant to asthma (Fig. 1).

Similar to all contractile phenomena, ASM contraction also depends on calcium ions (Ca^{2+}). Nevertheless, ASM exhibits peculiar features that clearly differentiate it from skeletal and cardiac muscles, as well as from vascular smooth muscles. In such tissues, excitation—contraction coupling largely results from membrane depolarization leading to Ca^{2+} influx via voltage-dependent Ca^{2+} channels. In fact, Ca^{2+} channel blockers are very useful in modulating cardiac activity and treating hypertension. By contrast, these drugs exert only negligible effects on experimentally induced bronchoconstriction.[3,4] The modest contribution provided by extracellular Ca^{2+} sources to excitation—contraction coupling in ASM, may be at least in part explained by the electrical properties of ASM cells. Their membrane potential spontaneously oscillates thus giving rise to the so-called slow waves, whose amplitude and frequency are approximately 8—12 mV and 20—50 events min^{-1}, respectively.[5,6] This myogenic activity is likely due to a constitutive production of arachidonic acid metabolites in ASM.[7] However, slow waves usually cannot be converted into action potentials because of a strong outward rectification that counteracts any tendency to ASM depolarization. Such rectifying currents are mediated by the opening of large conductance Ca^{2+}-activated and, especially, delayed-rectifier K^+ channels, responsible for repolarizing or hyperpolarizing ion fluxes which confer a remarkable electrical stability to ASM.[8] Therefore, generation of spiking action potentials and the consequent activation of voltage-dependent Ca^{2+} channels can occur in ASM only under the experimental blockade of K^+ rectifying efflux, operated by depolarizing agents such as tetraethylammonium (TEA). This implies that calcium ions required for ASM contraction mostly come from intracellular stores.

Table 1 Main bronchoconstrictive mediators involved in asthma

Agonist	Receptor
Acetylcholine	M_3 muscarinic
Neurokinins	NK_1/NK_2
Cysteinyl leukotrienes ($LTC_4/D_4/E_4$)	$CysLT_1R$
Prostaglandin $F_{2\alpha}$	FP
Prostaglandin D_2	DP_2
Thromboxanes	TP
Histamine	H_1
Endothelin-1	ET_A/ET_B
Bradykinin	BK_1
Adenosine	A_1
Serotonin	$5\text{-}HT_{2a}/5\text{-}HT_{2c}$
Thrombin	PAR1/2/3

Dynamics of Ca^{2+} responses

Studies performed with Ca^{2+}-sensitive fluorescent dyes have demonstrated that contractile agonists elicit in ASM, with regard to intracellular Ca^{2+}, a biphasic response.[9] Initially, cytosolic free Ca^{2+} concentration increases from baseline levels, ranging from 100 to 200 nM, up to 800—1000 nM. This sharp and rapid rise, responsible for tension development, is then followed by a sustained plateau characterized by Ca^{2+} levels slightly above the resting values, which is associated with tension maintenance. Moreover, contractile agents such as acetylcholine (ACh) induce regenerative and propagating Ca^{2+} oscillations,[10] whose functional role has not yet been fully understood. The initial Ca^{2+} spike is due to a release of Ca^{2+} from intracellular stores, especially the sarcoplasmic reticulum (SR), induced by inositol 1,4,5-trisphosphate (IP_3).[11] The latter originates from stimulation of contractile agonist receptors coupled to a Gq-type of G protein, responsible for activation of the β1 isoform of phospholipase C (PLC-β1), which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) thus producing the two second messengers IP_3 and 1,2-diacylglycerol (DAG).[12] IP_3 diffuses through the cytosol and binds to its specific receptor (IP_3R) located on the SR, thereby mobilizing the IP_3-sensitive pool of intracellularly stored Ca^{2+}, whereas DAG activates protein kinase C (PKC), which affects the sensitivity of the contractile apparatus to Ca^{2+}. Indeed, IP_3R is an agonist-operated Ca^{2+} channel, characterized by a homotetrameric structure consisting of four subunits, each having a molecular weight of about 260 kDa.[13] The fast and transient elevation of cytosolic Ca^{2+} concentration, induced by IP_3, is responsible for the sequential occupancy of the four Ca^{2+} binding sites of calmodulin (CaM). The Ca^{2+}—CaM complex in turn opens and activates the enzymatic domain of myosin light chain kinase (MLCK), which phosphorylates a specific amino acid residue (serine 19) of the regulatory, 20 kDa light chain (MLC_{20}) subunit of myosin. MLCK is overexpressed in ASM cells of asthmatic patients.[14] When MLCK is phosphorylated by 3′—5′ cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), for example as a consequence of β-adrenoceptor stimulation, its affinity for Ca^{2+}—CaM decreases, thus resulting in reduced MLCK activity and ASM contraction. MLC_{20} phosphorylation is indeed essential to trigger cross-bridge cycling, i.e. the movement of myosin heads along actin filaments. The contractile

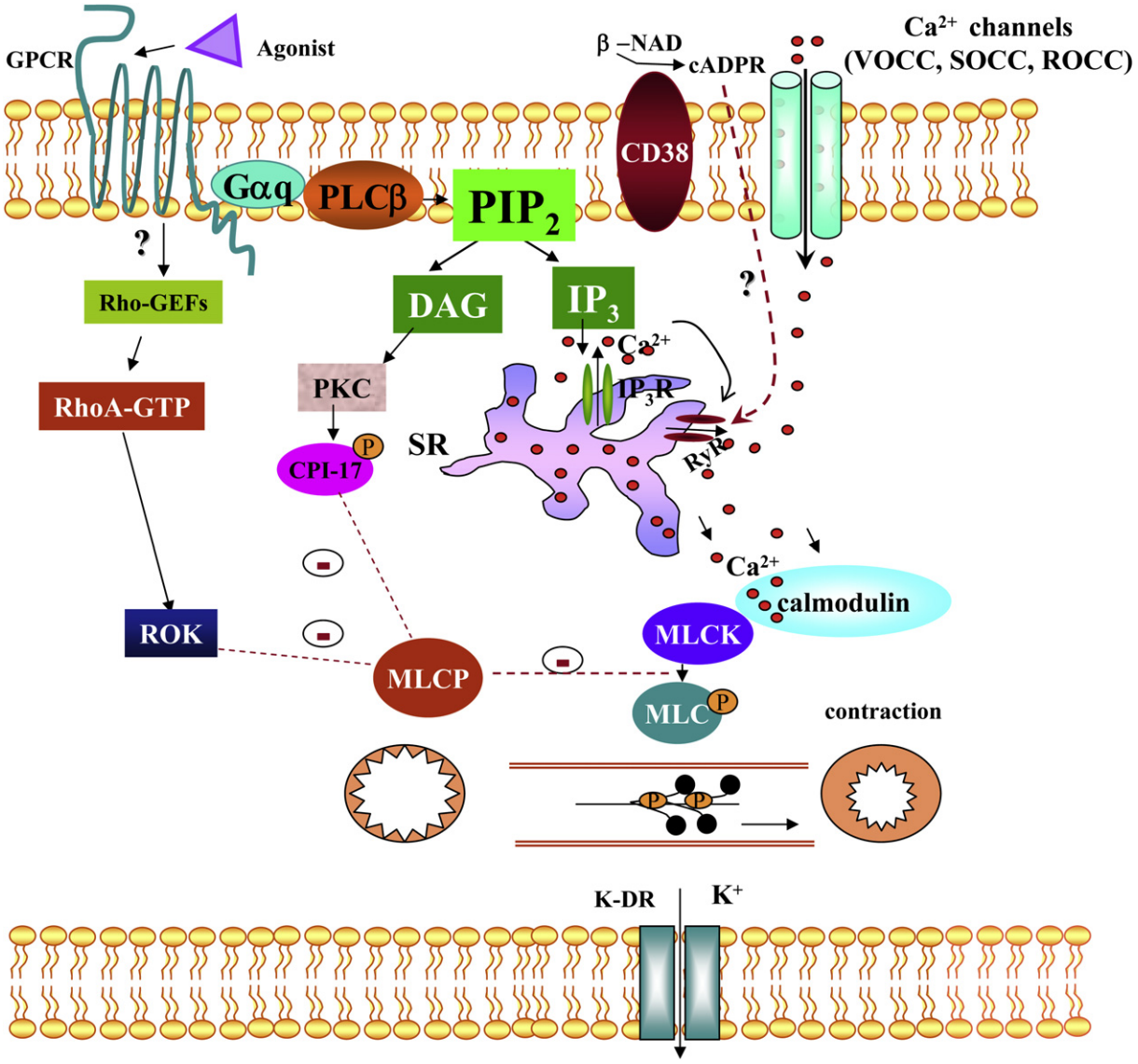

Figure 1 Signal transduction pathways involved in ASM contraction. Interaction of contractile agonists with their G protein-coupled receptors (GPCR) leads to activation of phospholipase C-β (PLCβ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), thus producing the two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor (IP_3R) on the sarcoplasmic reticulum (SR), thereby releasing Ca^{2+} that activates Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain (MLC), leading to ASM contraction, which is terminated by MLC phosphatase (MLCP). In addition to IP_3, Ca^{2+} release from SR is also stimulated by agonist-induced activation of CD38, producing cyclic ADP-ribose (cADPR) that presumptively (broken arrow with question mark) interacts with ryanodine receptors (RyR). Furthermore, Ca^{2+} can flow across plasma membrane through channels including store-operated Ca^{2+} channels (SOCC), receptor-operated Ca^{2+} channels (ROCC) and voltage-operated Ca^{2+} channels (VOCC). VOCC activation is, however, usually prevented by outward ion currents mainly due to opening of delayed-rectifier K^+ channels (K-DR). ASM contractile responses also depend on mechanisms of Ca^{2+} sensitization, mediated by MLCP inhibition induced by DAG/PKC/CPI-17 and Rho/Rho kinase (ROK) pathways; RhoA is activated by guanine nucleotide exchange factors (GEFs).

model of ASM is thereby consistent with a sliding-filament mechanism in which actomyosin cross-bridges, powered by ATP hydrolysis catalyzed by myosin ATPase, are responsible for tension development.[15] As a result of a homeostatic process, the increase in cytosolic free Ca^{2+} levels is then rapidly and almost completely reverted by a re-uptake into the SR mainly mediated by the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), that whereby refills the previously depleted intracellular Ca^{2+} stores; the latter are also repleted by transmembrane influxes of extracellular Ca^{2+} through voltage-activated Ca^{2+} channels, store-operated Ca^{2+} channels, and voltage-independent, non selective cation channels such as transient receptor potential channels.[4,7,16] On the other hand, SERCA is one of the cellular targets of β-adrenergic agonists, which relax ASM also by promoting Ca^{2+} re-sequestration into intracellular stores.[17] Moreover, β-sympathomimetics interfere with intracellular Ca^{2+} mobilization also by reducing

IP_3-induced Ca^{2+} release via cAMP/PKA-mediated phosphorylation-dependent inhibition of IP_3R.[18] Asthmatic subjects are much more dependent than normal individuals on endogenous modulation of bronchomotor tone exerted by catecholamines, as shown by the negative consequences of β-adrenergic blockade on both ASM contraction and BHR to methacholine.[19,20]

Hence, with regard to ASM contraction elicited by ACh and many other excitatory agents, IP_3 is crucial for initiation of intracellular Ca^{2+} oscillations. In particular, an important role in Ca^{2+} oscillations may be played by a periodical Ca^{2+} release through activated IP_3R, triggered by cyclical Ca^{2+} fluctuations within SR lumen.[21] Once initiated in ASM cells, however, these oscillations are not abolished by heparin, an IP_3R antagonist.[22] Also *in vivo*, inhaled heparin is able to attenuate, but not to completely suppress, bronchoconstriction induced in asthmatic patients by methacholine and other contractile stimuli.[23,24] ACh-induced intracellular Ca^{2+} oscillations can be inhibited by antagonists of the ryanodine receptor (RyR), such as ryanodine and ruthenium red.[25,26] These observations imply that Ca^{2+} release through RyR channels cooperates with IP_3-mediated Ca^{2+} mobilization to integrate the Ca^{2+} responses of ASM triggered by contractile agonists. In particular, the involvement of RyR channels, which are embedded in the SR membrane, is essential for propagation of intracellular Ca^{2+} oscillations in ASM cells. RyR is activated by the nucleotide metabolite cyclic ADP-ribose (cADPR) synthesized by CD38, a transmembrane glycoprotein which converts β-NAD (nicotinamide adenine dinucleotide) to cADPR.[22] However, the mechanism whereby stimulation of G protein-coupled receptors by contractile agonists leads to CD38 activation and cADPR production has not yet been elucidated. Moreover, it is not clear how extracellularly generated cADPR reaches its site of action, located on Ca^{2+} intracellular stores; the involvement of a specific carrier that shuttles cADPR across the plasma membrane, can thus be reasonably hypothesized.

cADPR contributes to Ca^{2+} release from SR via several different mechanisms. It could perhaps interact directly with RyR channels, but current evidence suggests that cADPR acts mainly indirectly, by binding to some accessory proteins.[22] In this regard, the 12.6-kDa tacrolimus (FK506)-binding protein (FKBP12.6) plays a very important role, in that it maintains RyR in an inactive state [27]; the interaction of cADPR with FKBP12.6 causes its dissociation from RyR, and the latter becomes thereby activated. Another indirect Ca^{2+} releasing process implies that cADPR can stimulate CaM, which in turn activates RyR either directly or indirectly, via RyR phosphorylation catalyzed by CaM-kinase II.[28] CaM-mediated mechanisms may explain the relevant contribution given by cADPR to the so-called phenomenon of Ca^{2+}-induced Ca^{2+} release,[29] which is very effective in propagating intracellular Ca^{2+} oscillations throughout the cytosol of ASM cells. cADPR-dependent mobilization of intracellular Ca^{2+} is not affected by heparin, thus ruling out any involvement of cADPR in IP_3R activation.

In light of all such considerations, it can be argued that IP_3 and cADPR act as Ca^{2+}-mobilizing second messengers for a multitude of contractile agonists, both being responsible in ASM for a synergistic coordination and integration of the intracellular Ca^{2+} responses underlying excitation—contraction coupling.

Effects of proinflammatory cytokines on generation of Ca^{2+}-mobilizing second messengers

Inflammatory cytokines can further enhance agonist-induced contractility, primarily by increasing the magnitude of Ca^{2+} responses mediated by intracellular second messengers. It is now clear that these mechanisms significantly contribute to the molecular pathogenesis of BHR.[30] In particular, it has been shown that tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are able to augment Ca^{2+} responses induced by carbachol, bradykinin and thrombin.[31] Moreover, Ca^{2+} responses to contractile agonists may also be increased by interleukin-13 (IL-13),[32] a T helper 2 (Th2)-derived cytokine that plays a key role in the development of allergic asthma. These cytokines exert such effects at least in part by inducing, in ASM cells, the expression of CD38 and the related production of cADPR.[16] In addition to cholinergic agents, also endothelin (ET) and bradykinin utilize the CD38/cADPR/Ca^{2+} signaling pathway,[31,33] which is hyperactivated in asthma as a result of the exposure of ASM cells to high levels of proinflammatory cytokines. The latter could also indirectly stimulate IP_3 generation through an up-regulation of the various components of the signaling machinery, such as Gq proteins and Gq-coupled receptors linked to phosphoinositide metabolism. For instance, IL-13 stimulates the expression of the CysLT1 receptor of leukotrienes,[34] thus possibly enhancing airway responsiveness to these mediators. Furthermore, in ASM cell cultures IL-1β is able to increase the density of B2 bradykinin receptors, as well as bradykinin-induced phosphoinositide turnover.[35] TNF-α stimulates ASM expression of PLC-activating Gq proteins, thereby promoting IP_3 production.[36] On the other hand, proasthmatic cytokines can also exert an indirect, negative interference on β-agonist functions, including β-adrenoceptor-mediated inhibition of PIP_2 hydrolysis and IP_3 synthesis. For example, IL-13 desensitizes ASM β_2-adrenergic receptors,[37] thus impairing the relevant role of β_2-sympathomimetics in modulation of intracellular Ca^{2+} fluxes.

Therefore, an inflammatory microenvironment like that characterizing asthmatic airways is likely to favour, via several different mechanisms involved in Ca^{2+} mobilization, the acquisition of a hypercontractile phenotype.

Mechanisms of Ca^{2+} sensitization

Upon ASM exposure to contractile agonists, the initial, transient peak in intracellular Ca^{2+} concentration is then rapidly followed by a decline to a steady-state pattern, characterized by a prolonged, but slight increase of cytosolic free Ca^{2+} above resting levels. Differently from the first phase of Ca^{2+} response, mostly due to Ca^{2+} release from SR, the steady-state plateau also depends on Ca^{2+} influx from the extracellular space.[38] In the absence of extracellular Ca^{2+}, in fact, intracellular Ca^{2+} oscillations can start, but cannot be maintained. Both Ca^{2+} re-uptake into the SR and Ca^{2+} influx from the extracellular environment, therefore, contribute to replete Ca^{2+} stores and to maintain intracellular Ca^{2+} oscillations. Anyway, similarly to other smooth muscles, contraction of ASM persists during

the steady-state phase of Ca^{2+} responses elicited by excitatory agents. This functional behaviour implies an enhanced sensitivity to Ca^{2+} of the contractile apparatus [15] that enables ASM to remain contracted even when intracellular Ca^{2+} levels are only slightly increased above baseline concentrations. It is currently thought that the main signaling mechanisms responsible for Ca^{2+} sensitization include the RhoA/Rho kinase pathway and the PKC/CPI-17 effector system.[39]

In addition to inducing the biosynthesis of IP_3 and cADPR, stimulation of Gq protein-coupled receptors by contractile agonists also leads, via not yet known molecular events, to activation of RhoA.[40] The latter is a small monomeric G protein which, under resting conditions, is associated with the guanine nucleotide GDP and complexed into the cytosol with the GDP dissociation inhibitor (GDI). Interaction between contractile agonists and their Gq-coupled receptors activates the so-called guanine nucleotide exchange factors (GEFs), which promote the substitution of GDP with GTP on RhoA.[41] Consequently, RhoA dissociates from GDI, and the RhoA—GTP complex can thus reach the inner side of plasma membrane, where it interacts with Rho kinase (ROK). This activation process is terminated by Rho GTPase activating proteins (RhoGAPs) that hydrolyze Rho-bound GTP to GDP, thereby enabling RhoA—GDP to reassociate with GDI and to be again located inside the cytosol. Binding of Rho-GTP to ROK produces a conformational change in the kinase domain, causing an activating autophosphorylation.[39] ROK sensitizes the contractile apparatus to Ca^{2+} through phosphorylation of the myosin binding subunit of MLC_{20} phosphatase (MLCP) that results in inhibition of its enzymatic activity.[42] As a consequence, MLC_{20} cannot be dephosphorylated thus remaining in its phosphorylated form, responsible for smooth muscle contraction. It is thus conceivable that RhoA/ROK-mediated Ca^{2+} sensitization plays a key role in the molecular pathogenesis of BHR. In fact, RhoA expression is enhanced in ASM of antigen-sensitized, hyperresponsive mice.[43] RhoA protein levels are also increased in case of TNF-α-mediated potentiation of ACh-induced rat ASM contraction.[44] Moreover, contractile agonists such as ACh and ET are able to promote Rho translocation from cytosol to plasma membrane in rat ASM cells.[45,46] Therefore, the RhoA/ROK signaling pathway can be considered as a suitable target for the development of new anti-asthma treatments, focused on modulation of BHR.

MLCP activity can also be modulated by the 17-kDa peptide CPI (C kinase-potentiated phosphatase inhibitor)-17 that upon PKC-dependent phosphorylation becomes a potent MLCP inhibitor.[47] Activation of the PKC/CPI-17 signaling route, induced by the second messenger DAG derived from phosphoinositide turnover, thereby represents another key mechanism involved in Ca^{2+} sensitization triggered by contractile agonists interacting with their Gq-coupled receptors expressed by ASM cells. For instance, CPI-17 expression and ACh-induced CPI-17 phosphorylation are significantly increased in ASM of hyperresponsive rats.[48] Phosphorylation of rat ASM CPI-17 can also be markedly enhanced by other contractile agonists such as ET-1.[49] On the other hand, it has been suggested that PKC activation, and of course the subsequent CPI-17 stimulation, can be blocked by 8-bromo-cAMP in rabbit tracheal smooth muscle contracted by carbachol, as well as in human bronchial smooth muscle exposed to leukotriene D_4 (LTD_4).[50] These observations imply that Ca^{2+} desensitization of the contractile apparatus, mediated by the cAMP/PKA pathway, may significantly contribute to the relaxing effects exerted on ASM by cAMP-elevating agents such as β-adrenergic agonists and phosphodiesterase (PDE) inhibitors.

Airway smooth muscle cell proliferation

In addition to chronic inflammation, reversible airflow limitation and BHR, another typical feature of asthma is airway remodeling that consists of structural changes spanning throughout the bronchial wall. In this regard it has been suggested that the natural, gradual disease progression can be at least in part explained by the development of airway remodeling.[51] Inflammation and remodeling are indeed the main characterizing elements of the asthmatic phenotype. However, although these two aspects are closely correlated and affect each other by reciprocal, positive feedbacks, structural modifications may occur independently of inflammation and often precede the onset of asthma symptoms.[52] Hallmarks of airway remodeling in asthma include epithelial shedding and goblet cell hyperplasia, subepithelial fibrosis with abnormal deposition of extracellular matrix, enhanced thickness of smooth muscle layer, and angiogenesis of bronchial vasculature.[53] Tissue remodeling could provide some potential benefits in asthma,[52] in that an increased stiffness might enable the airways to resist dynamic compression; furthermore, deposition of collagen fibres around ASM could produce a mechanical impedance to contraction. Nevertheless, these theoretical advantages are largely overwhelmed by the negative effects of airway remodeling, which results in narrowing of the bronchial lumen, fixed airflow obstruction with impaired response to bronchodilators, and progressive loss of lung function.[52] In particular, ASM bundle thickening, responsible for the reduced airway caliber and possibly associated with an exaggerated contractility, may represent a relevant contributing factor to BHR.[54] The increased mass of ASM layer is indeed correlated with airway responsiveness to methacholine and asthma severity.[55,56] On the other hand, hypertermic treatment of airways with radiofrequency waves (bronchial thermoplasty), performed through bronchoscopy and aimed to decrease ASM bulk, is able to improve BHR in patients with mild to moderate persistent asthma for at least 2 years after thermoplasty.[57] Within such a context, a crucial pathophysiologic role is thus played by the biological mechanisms leading to the oversized ASM mass, due in part to hypertrophy, but especially to hyperplasia (Fig. 2).

An increased proliferation of ASM cells has been shown in both asthmatic patients and animal models of allergic airway inflammation.[58,59] ASM mitogens include growth factors, contractile agonists, and perhaps proinflammatory cytokines. Among the first ones, a relevant proliferative action is exerted by epidermal growth factor (EGF), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF)-2. Interestingly, some of the agents that induce ASM contraction, such as histamine, ET-1, substance P, serotonin, α-thrombin, thromboxane A_2 and LTD_4, are also

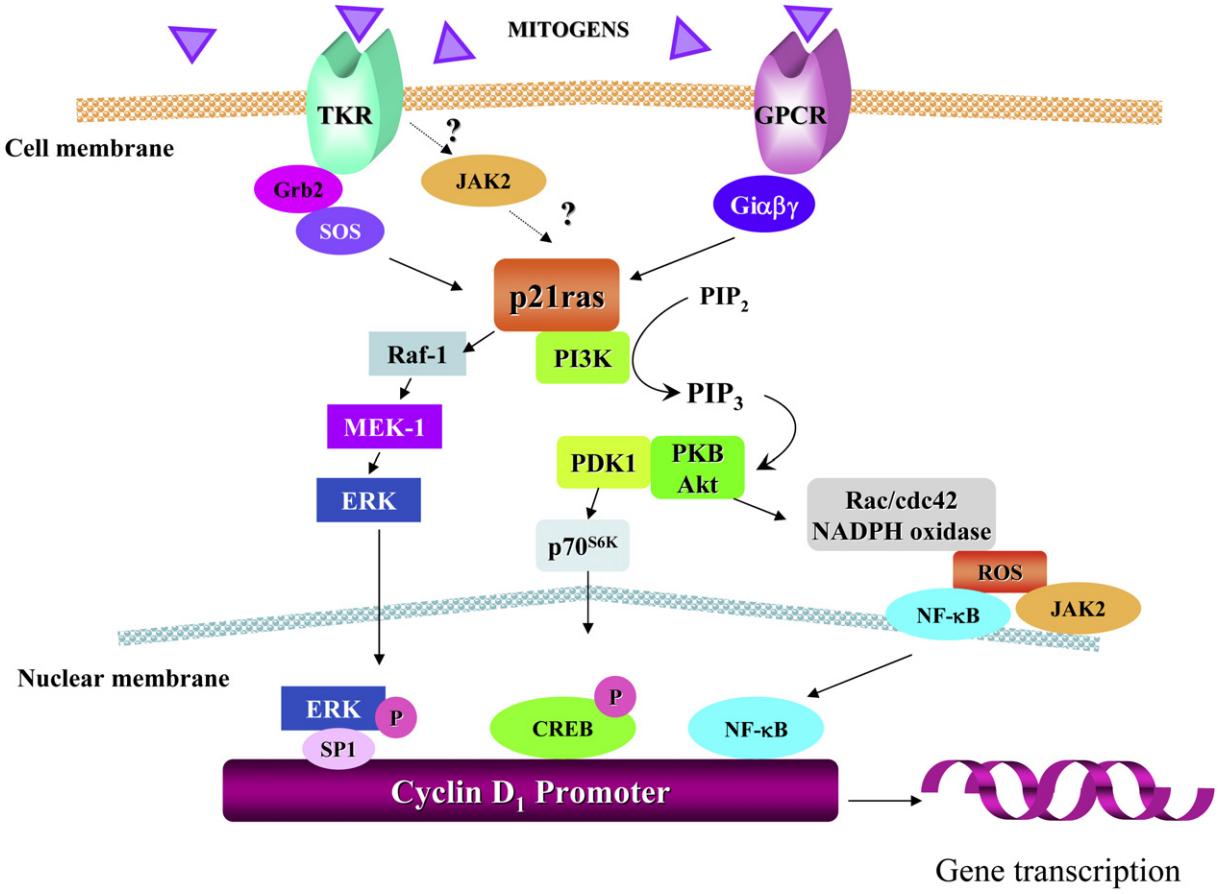

Figure 2 Signal transduction pathways involved in ASM proliferation. ASM proliferation is induced by mitogens acting mainly via tyrosine kinase receptors (TKR) and G protein-coupled receptors (GPCR). Stimulation of these receptors leads to activation of p21ras proteins, which interact with downstream pathways including extracellular signal-regulated kinase (ERK) cascades and phosphoinositide 3-kinase (PI3K). ERK and PI3K-dependent signaling molecules (PDK1, PKB/Akt, $p70^{S6K}$, Rac1, etc.) cooperate in the activation of transcription factors (SP1, CREB, NF-κB) required for cyclin D_1 gene expression and cell growth.

able to behave as mitogenic stimuli. Furthermore, in ASM derived from asthmatic patients, when compared to normal subjects, low levels have been found of the bronchodilating prostaglandin E_2 (PGE_2),[60] which is also an inhibitor of ASM cell growth.[61] More controversial is the potential proliferative role of inflammatory cytokines like IL-1β, IL-6, and TNF-α, whose mitogenic action has been shown in ASM of some animal species.[1] Moreover, IL-13 is able *in vitro* to potentiate the proliferative effects induced by LTD_4 on human ASM.[34]

The transduction pathways that originate from plasma membrane and through the cytoplasm reach the nucleus, activated by the interactions of several different mitogens with their respective ASM surface receptors, converge on a relatively limited number of intracellular signaling modules, mainly including mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K).[62] In particular, growth factors bind to receptors with intrinsic tyrosine kinase activity, whereas contractile agonists stimulate G protein-coupled receptors. Downstream from receptor activation, the molecular events triggered by both growth factors and contractile agonists lead to activation of the 21-kDa small GTPase, p21ras protein.[63] Activated p21ras binds PI3K and the 74-kDa cytoplasmic, serine–threonine kinase Raf-1.[59] The latter is responsible for activation of MAPK cascades via phosphorylation of MEK1 (MAPK/ERK kinase 1), which in turn phosphorylates and activates extracellular signal-regulated kinases (ERK) 1 and 2. Activated ERK1/2 is able to induce cyclin D1 expression in airway myocytes,[64] thus promoting a progression of the cell cycle from G1 to S phase and ASM proliferation. In fact, activation of the nuclear complexes constituted by cyclins and cyclin-dependent kinases (cdk) is required for hyperphosphorylation of the retinoblastoma restriction protein (Rb), resulting in its reduced affinity for the elongation factor E2F [59]; dissociation from Rb activates E2F, which is responsible for stimulation of DNA polymerase and transcriptional induction of specific S phase-dependent genes. In human ASM, a prolonged ERK activation is indeed correlated with DNA synthesis and cell proliferation, which can be markedly attenuated by the MEK1 inhibitors PD98059 and U0126, respectively.[65,66] Furthermore, ASM mitogenesis is significantly inhibited by antisense oligodeoxynucleotides targeted to ERK1/2 mRNAs.[67] In addition to ERK, also other members of the MAPK superfamily such as p38 and c-Jun N-terminal kinase (JNK) may be involved in induction of ASM growth. In human cultured ASM myocytes, Rb phosphorylation, DNA synthesis, and cell proliferation induced by basic FGF are mediated by p38 MAPK activation, thus suggesting that the antimitogenic effects of the p38 inhibitor SB203580 are due to an arrest of

cell cycle at late G1 phase.[68] In ASM of sensitized mice, p38 MAPK plays indeed a key role in mediating the proliferative response elicited by allergen challenge.[69] Moreover, JNK has also been shown to induce ASM mitogenesis in allergen-sensitized rats and mice [70,71]; therefore, in asthma this MAPK pathway could synergize with ERK and p38 in promoting the acquisition of a hyperplastic ASM phenotype.

Together with MAPK, PI3K is the other main signaling pathway activated by mitogenic stimuli in ASM. Upon p21ras-dependent activation, PI3K phosphorylates phosphatidylinositol membrane lipids at the D-3 position of the inositol ring, thus producing the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3).[72] PIP_3 binds and recruits to the plasma membrane a group of signaling molecules such as serine—threonine kinases (Akt/PKB, PDK1) and exchange factors for GTP binding proteins (Grp1, Rac), which act as important regulators of cell cycle and cell survival/proliferation. In particular, protein kinase B (PKB) phosphorylates Bad thus inhibiting its proapoptotic functions, and also represses transcription of the cdk inhibitor $p27^{kip1}$, thereby allowing cell cycle transition.[73] A relevant downstream target of phosphoinositide-dependent kinase 1 (PDK1) is the 70 kDa ribosomal S6 kinase (p70S6K), whose rapamycin-mediated inhibition leads to a reduction in cyclin D1 mRNA and protein levels,[74] as well as to a marked attenuation of growth factor-induced DNA synthesis in both bovine and human ASM.[75,76] Furthermore, the PIP_3-regulated Rac1 factor is also required for cyclin D1 expression in bovine tracheal myocytes.[77] Therefore, the PI3K effector system plays a crucial role in inducing ASM mitogenesis, as it has been shown with regard to the proliferative actions of PDGF and thrombin.[78] However, maximal proliferation of ASM is probably dependent on parallel activation of both PI3K and MAPK pathways.[59]

ASM growth is not only stimulated by mitogenic mechanisms, but also finely modulated by antiproliferative signals, among which a relevant function is exerted by the transcription factor CCAAT/enhancer binding protein-α (C/EBPα). C/EBPα controls the rate of ASM proliferation by inducing the synthesis of the cell cycle inhibitor $p21^{waf1/cip1}$. In this regard, it has been reported that the simultaneous addition to ASM cell growth medium of a glucocorticoid (budesonide) and a β_2-agonist (formoterol) results in a synergistic activation of the $p21^{waf1/cip1}$ gene promoter, paralleled by a marked antiproliferative effect.[79] However, the antimitogenic action of glucocorticoids has not been confirmed in primary cultures of bronchial smooth muscle cells obtained from asthmatic patients, which do not express C/EBPα.[80] Therefore, it can be argued that a deficit of C/EBPα in ASM of subjects with asthma favours the acquisition of a hyperplastic phenotype and impairs the potential antiproliferative effect of glucocorticoids.[81] This latter action can indeed be restored by experimentally introducing, through an expression vector, the C/EBPα protein inside asthmatic ASM cells.[80]

Conclusions

Current evidence clearly indicates that ASM plays a key role in the pathogenesis of asthma. In particular, both contractile and proliferative responses significantly contribute, together with the synthetic properties of ASM cells, to the pathophysiological changes underlying BHR and airway remodeling. Therefore, elucidation of the complex signaling pathways involved in excitation—contraction coupling and cell growth is essential to understand how ASM is actively implicated in shaping the biological asthmatic microenvironment. Within such a context, the recent insights into the molecular mechanisms responsible for ASM contraction and proliferation may unveil new potential targets (e.g. cADPR, ROK, MAPK, PI3K, C/EBPα) for the development of future, more effective bronchodilating, anti-hyperresponsive and anti-remodeling therapeutic strategies, eventually capable of affecting the natural history of asthma.

Conflict of interest statement

None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Lazaar AL, Panettieri RA. Airway smooth muscle as a regulator of immune responses and bronchomotor tone. *Clin Chest Med* 2006;**27**:53—69.
2. Marsico SA, Pelaia G. Airways smooth muscle response to biological stimuli. *Monaldi Arch Chest Dis* 1998;**53**:97—9.
3. Marsico SA, Pelaia G, Grembiale RD, Pugliese C, Cellamare P. Calcium ions and airway smooth muscle contraction. In: Olivieri D, Bianco S, editors. *Airway obstruction and inflammation. Progress in respiration research*, vol. 24. Basel: Karger; 1990. p. 26—34.
4. Sanderson MJ, Delmotte P, Bai Y, Perez-Zogbhi JF. Regulation of airway smooth muscle cell contractility by Ca^{2+} signaling and sensitivity. *Proc Am Thorac Soc* 2008;**5**:23—31.
5. Tomita T. Electrical properties of airway smooth muscle. In: Coburn RF, editor. *Airway smooth muscle in health and disease*. New York: Plenum Press; 1989. p. 151—67.
6. Thirstrup S. Control of airway smooth muscle tone. I — electrophysiology and contractile mediators. *Respir Med* 2000;**94**:328—36.
7. Hirota S, Helli P, Janssen LJ. Ionic mechanisms and Ca^{2+} handling in airway smooth muscle. *Eur Respir J* 2007;**30**:114—33.
8. Pelaia G, Gallelli L, Vatrella A, Grembiale RD, Maselli R, De Sarro GB, et al. Potential role of potassium channel openers in the treatment of asthma and chronic obstructive pulmonary disease. *Life Sci* 2002;**70**:977—90.
9. Murray RK, Kotlikoff MI. Receptor-activated calcium influx in human airway smooth muscle cells. *J Physiol* 1991;**435**:123—44.
10. Nuttle LC, Farley JM. Frequency modulation of acetylcholine-induced oscillations in Ca^{2+} and Ca^{2+}-activated Cl^- current by cAMP in tracheal smooth muscle. *J Pharmacol Exp Ther* 1996;**277**:753—60.
11. Berridge MJ. Inositol trisphosphate and calcium signaling. *Nature* 1993;**361**:315—25.
12. Hall IP. Second messengers, ion channels and pharmacology of airway smooth muscle. *Eur Respir J* 2000;**15**:1120—7.
13. Patel S, Joseph SK, Thomas AP. Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium* 1999;**25**:247—64.
14. Ma X, Cheng Z, Kong H, Ma X, Cheng Z, Kong H, et al. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol* 2002;**283**:L1181—9.

15. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994;**372**:231–6.
16. Jude JA, Wylam ME, Walseth TF, Kannan MS. Calcium signaling in airway smooth muscle. *Proc Am Thorac Soc* 2008;**5**:15–22.
17. Janssen LJ, Tazzeo T, Zuo J. Enhanced myosin phosphatase and Ca^{2+}-uptake mediate adrenergic relaxation of airway smooth muscle. *Am J Respir Cell Mol Biol* 2004;**30**:548–54.
18. Johnson EN, Druey KM. Heterotrimeric G protein signaling: role in asthma and allergic inflammation. *J Allergy Clin Immunol* 2002;**109**:592–602.
19. Grieco MH, Pierson RN. Mechanism of bronchoconstriction due to beta-adrenergic blockade. *J Allergy Clin Immunol* 1971;**48**: 143–52.
20. Vatrella A, Parrella R, Pelaia G, Biscione GL, Tranfa CME, De Sarro GB, et al. Effects of non-bronchoconstrictive doses of inhaled propranolol on airway responsiveness to methacholine. *Eur J Clin Pharmacol* 2001;**57**:99–104.
21. Berridge MJ. Inositol trisphosphate and calcium oscillations. *Biochem Soc Symp* 2007;**74**:1–7.
22. Deshpande DA, White TA, Dogan S, Walseth TF, Panettieri RA, Kannan MS. CD38/cyclic ADP-rybose signalling: role in the regulation of calcium homeostasis in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2005;**288**:L773–88.
23. Ceyhan B, Celikel T. Effect of inhaled heparin on methacholine-induced bronchial hyperreactivity. *Chest* 1995;**107**: 1009–12.
24. Tranfa CME, Vatrella A, Parrella R, Pelaia G, Bariffi F, Marsico SA. Effect of inhaled heparin on water-induced bronchoconstriction in allergic asthmatics. *Eur J Clin Pharmacol* 2001;**57**:5–9.
25. McPherson PS, Campbell KP. The ryanodine receptor/Ca^{2+} release channel. *J Biol Chem* 1993;**268**:13765–8.
26. Prakash YS, Kannan MS, Walseth TF, Sieck GC. Role of cyclic ADP-ribose in the regulation of $[Ca^{2+}]_i$ in porcine tracheal smooth muscle. *Am J Physiol Cell Physiol* 1998;**274**:C1653–60.
27. Berridge MJ, Bootman MD, Roderick HL. Calcium signaling: dynamics, homeostasis and remodeling. *Nat Rev Mol Cell Biol* 2003;**4**:517–29.
28. Takasawa S, Ishida A, Nata K, Nagakawa K, Noguchi N, Tohgo A, et al. Requirement of calmodulin-dependent protein kinase II in cyclic ADP-ribose mediated intracellular Ca^{2+} mobilization. *J Biol Chem* 1995;**270**:30257–9.
29. Lee HC. Potentiation of calcium- and caffeine-induced calcium release by cyclic ADP-ribose. *J Biol Chem* 1993;**268**: 293–9.
30. Amrani Y, Tliba O, Deshpande DA, Walseth TF, Kannan MS, Panettieri Jr RA. Bronchial hyperresponsiveness: insights into new signaling molecules. *Curr Opin Pharmacol* 2004;**4**:230–4.
31. Deshpande DA, Walseth TF, Panettieri RA, Kannan MS. CD38-cyclic ADP-rybose-mediated Ca^{2+} signaling contributes to airway smooth muscle hyperresponsiveness. *FASEB J* 2003; **2003**(17):452–4.
32. Tliba O, Deshpande D, Van Besien C, Chen H, Kannan M, Panettieri RA, et al. IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. *Br J Pharmacol* 2003;**140**:1159–62.
33. White TA, Kannan MS, Walseth TF. Intracellular calcium signaling through the cADPR pathway is agonist specific in porcine airway smooth muscle. *FASEB J* 2003;**17**:482–4.
34. Espinosa K, Bossé Y, Stankova J, Rola-Pleszczynski M. Cys-LT1 receptor upregulation by TGF-β and IL-13 is associated with bronchial smooth muscle cell proliferation in response to LTD4. *J Allergy Clin Immunol* 2003;**111**:1032–40.
35. Schmidlin F, Scherrer D, Daeffler L, Bertrand C, Landry Y, Gies JP. Interleukin-1β induces bradykinin B2 receptor gene expression through a prostanoid cyclic AMP-dependent pathway in human bronchial smooth muscle cells. *Mol Pharmacol* 1998;**53**:1009–15.
36. Hotta K, Emala CW, Hirshman CA. TNF-α upregulates Giα and Gqα protein expression and function in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 1999;**276**: L405–11.
37. Shore SA, Moore PE. Regulation of β-adrenergic responses in airway smooth muscle. *Respir Physiol Neurobiol* 2003;**137**: 179–95.
38. Murray RK, Fleischmann BK, Kotlikoff MI. Receptor-activated Ca influx in human airway smooth muscle: use of Ca imaging and perforated patch-clamp techniques. *Am J Physiol Cell Physiol* 1993;**264**:C485–90.
39. Somlyo AP, Somlyo AV. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;**83**:1325–58.
40. Chiba Y, Misawa M. The role of RhoA-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in airway hyperresponsiveness. *J Smooth Muscle Res* 2004;**40**:155–67.
41. Sylvester JT. The tone of pulmonary smooth muscle: ROK and Rho music? *Am J Physiol Lung Cell Mol Physiol* 2004;**287**: L624–30.
42. Ito S, Kume H, Honjo H, Katoh H, Kodama I, Yamaki K, et al. Possible involvement of Rho kinase in Ca^{2+} sensitization and mobilization by MCh in tracheal smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2001;**280**:L1218–24.
43. Chiba Y, Ueno A, Shinozaki K, Takeyama H, Nakazawa S, Sakai H, et al. Involvement of RhoA-mediated Ca^{2+} sensitization in antigen-induced bronchial smooth muscle hyperresponsiveness in mice. *Respir Res* 2005;**6**:4.
44. Sakai H, Otogoto S, Chiba Y, Abe K, Misawa M. Involvement of p42/44 MAPK and RhoA protein in augmentation of ACh-induced bronchial smooth muscle contraction by TNF-α in rats. *J Appl Physiol* 2004;**97**:2154–9.
45. Sakai H, Shirai T, Yamamoto M, Chiba Y, Misawa M. Involvement of M_3 muscarinic receptors in ACh-induced increase in membrane-associated RhoA of rat bronchial smooth muscle. *Biol Pharm Bull* 2005;**28**:625–8.
46. Chiba Y, Sakai H, Misawa M. Endothelin-1-induced translocation of RhoA is mediated by endothelin ET_A receptors in rat bronchial smooth muscle. *Eur J Pharmacol* 2005;**517**: 182–5.
47. Eto M, Ohmori T, Suzuki M, Furuya K, Morita F. A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. *J Biochem* 1995;**118**:1104–7.
48. Sakai H, Chiba Y, Hirano T, Misawa M. Possible involvement of CPI-17 in augmented bronchial smooth muscle contraction in antigen-induced airway hyper-responsive rats. *Mol Pharmacol* 2005;**68**:145–51.
49. Sakai H, Chiba Y, Misawa M. Augmentation of endothelin-1-induced phosphorylation of CPI-17 and myosin light chain in bronchial smooth muscle from airway hyperresponsive rats. *Biol Pharm Bull* 2006;**29**:1897–9.
50. Endou K, Iizuka K, Yoshii A, Tsukagoshi H, Ishizuka T, Dobashi K, et al. 8-Bromo-cAMP decreases the Ca^{2+} sensitivity of airway smooth muscle contraction through a mechanism distinct from inhibition of Rho kinase. *Am J Physiol Lung Cell Mol Physiol* 2004;**287**:L641–8.
51. Fixman ED, Stewart A, Martin JG. Basic mechanisms of development of airway structural changes in asthma. *Eur Respir J* 2007;**29**:379–89.
52. Tang MLK, Wilson JW, Stewart AG, Royce SG. Airway remodelling in asthma: current understanding and implications for future therapies. *Pharmacol Ther* 2006;**112**:474–88.
53. Pascual RM, Peters SP. Airway remodeling contributes to the progressive loss of lung function in asthma: an overview. *J Allergy Clin Immunol* 2005;**116**:477–86.
54. Cockcroft DW, Davis BE. Mechanisms of airway hyperresponsiveness. *J Allergy Clin Immunol* 2006;**118**:551–9.

55. Sapienza S, Du T, Eidelman DH, Wang NS, Martin JG. Structural changes in the airways of sensitised brown Norway rats after antigen challenge. *Am Rev Respir Dis* 1991;**144**:423—7.
56. Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P, et al. Differences in airway remodeling between subjects with severe and moderate asthma. *J Allergy Clin Immunol* 2005; **116**:544—9.
57. Cox G, Miller JD, McWilliams A, Fitzgerald JM, Lam S. Bronchial thermoplasty for asthma. *Am J Respir Crit Care Med* 2006;**173**: 965—9.
58. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, et al. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001;**164**:474—7.
59. Hirst SJ, Martin JG, Bonacci JV, Chan V, Fixman ED, Hamid QA, et al. Proliferative aspects of airway smooth muscle. *J Allergy Clin Immunol* 2004;**114**:S2—17.
60. Chambers LS, Black JL, Ge Q, Carlin SM, Au WW, Poniris M, et al. PAR-2 activation, PGE2, and COX-2 in human asthmatic and nonasthmatic airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2003;**285**:L619—27.
61. Belvisi MG, Saunders M, Yacoub M, Mitchell JA. Expression of cyclo-oxygenase-2 in human airway smooth muscle is associated with profound reductions in cell growth. *Br J Pharmacol* 1998;**125**:1102—8.
62. Zhou L, Hershenson MB. Mitogenic signaling pathways in airway smooth muscle. *Respir Physiol Neurobiol* 2003;**137**: 295—308.
63. Ammit AJ, Kane SA, Panettieri RA. Activation of K-p21ras and N-p21ras, but not H-p21ras, is necessary for mitogen-induced human airway smooth muscle proliferation. *Am J Respir Cell Mol Biol* 1999;**21**:719—27.
64. Ramakrishnan M, Musa NL, Li J, Liu PT, Pestell RG, Hershenson MB. Catalytic activation of extracellular signal-regulated kinases induces cyclin D_1 expression in primary tracheal myocytes. *Am J Respir Cell Mol Biol* 1998; **18**:736—40.
65. Fernandes D, Guida E, Koutsoubos V, Harris T, Vadiveloo P, Wilson JW, et al. Glucocorticoids inhibit proliferation, cyclin D1 expression, and retinoblastoma protein phosphorylation, but not activity of the extracellular-regulated kinases in human cultured airway smooth muscle. *Am J Respir Cell Mol Biol* 1999;**21**:77—88.
66. Orsini MJ, Krymskaya VP, Eszterhas AJ, Benovic JL, Panettieri Jr RA, Penn RB. MAPK superfamily activation in human airway smooth muscle: mitogenesis requires prolonged p42/p44 activation. *Am J Physiol Lung Cell Mol Physiol* 1999; **277**:L479—88.
67. Lee JH, Johnson PR, Roth M, Hunt NH, Black JL. ERK activation and mitogenesis in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2001;**280**:L1019—29.
68. Fernandes DJ, Ravenhall CE, Harris T, Tran T, Vlahos R. Contribution of the $p38^{MAPK}$ signalling pathway to proliferation in human cultured airway smooth muscle cells is mitogen-specific. *Br J Pharmacol* 2004;**142**:1182—90.
69. Nath P, Leung SY, Williams A, Noble A, Chakravarty SDS, Luedtke GR, et al. Importance of p38 mitogen-activated protein kinase pathway in allergic airway remodelling and bronchial hyperresponsiveness. *Eur J Pharmacol* 2006;**544**:160—7.
70. Eynott PR, Nath P, Leung SY, Adcock IM, Bennett BL, Chung KF. Allergen-induced inflammation and airway epithelial and smooth muscle cell proliferation: role of Jun N-terminal kinase. *Br J Pharmacol* 2003;**140**:1373—80.
71. Nath P, Eynott P, Leung SY, Adcock IM, Bennett BL, Chung KF. Potential role of c-Jun NH2-terminal kinase in allergic airway inflammation and remodelling: effects of SP600125. *Eur J Pharmacol* 2005;**506**:273—83.
72. Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002;**296**:1655—7.
73. Wymann MP, Zvelebil M, Laffargue M. Phosphoinositide 3-kinase signalling: which way to target? *Trends Pharmacol Sci* 2003;**24**:366—76.
74. Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, Ferrari S. Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J Biol Chem* 1998;**273**:14424—9.
75. Scott PH, Belham CM, al-Hafidh J, Chilvers ER, Peacock AJ, Gould GW, et al. A regulatory role for cAMP in phosphatidylinositol 3-kinase/p70 ribosomal S6 kinase-mediated DNA synthesis in platelet-derived-growth-factor-stimulated bovine airway smooth muscle cells. *Biochem J* 1996;**318**:965—71.
76. Krymskaya VP, Penn RB, Orsini MJ, Scott PH, Plevin RJ, Walker TR, et al. Phosphatidylinositol 3-kinase mediates mitogen-induced human airway smooth muscle cell proliferation. *Am J Physiol Lung Cell Mol Physiol* 1999;**277**:L65—78.
77. Page K, Li J, Hodge J, Liu P, Vanden Hoek T, Becker L, et al. Characterization of a Rac1 signaling pathway to cyclin D1 expression in airway smooth muscle cells. *J Biol Chem* 1999; **274**:22065—71.
78. Walker TR, Moore SM, Lanson MF, Panettieri Jr RA, Chilvers ER. Platelet-derived growth factor-BB and thrombin activate phosphoinositide 3-kinase and protein kinase B: role in mediating airway smooth muscle proliferation. *Mol Pharmacol* 1998;**54**: 1007—15.
79. Roth M, Johnson PRA, Rudiger JJ, King GG, Ge Q, Burgess JK, et al. Interaction between glucocorticoids and β_2-agonists on bronchial airway smooth muscle cells through synchronised cellular signaling. *Lancet* 2002;**360**:1293—9.
80. Roth M, Johnson PRA, Borger P, Bihl MP, Rudiger JJ, King GG, et al. Dysfunctional interaction of C/EBPα and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004;**351**:560—74.
81. Borger P, Tamm M, Black JL, Roth M. Asthma: is it due to an abnormal airway smooth muscle cell? *Am J Respir Crit Care Med* 2006;**174**:367—72.