Rho-Kinase as a Drug Target for the Treatment of Airway Hyperresponsiveness in Asthma

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Abstract: In asthma, inflammatory and structural cells contribute to increased bronchoconstriction acutely and more chronically to airway remodelling. Current asthma therapy doesn't inhibit these features satisfactorily. This review discusses Rho-kinase as a potential drug target, since increasing evidence suggests a central role for this pathway in acute and chronic airway hyperresponsiveness.

Keywords: Airway remodelling, airway smooth muscle, airway inflammation, Y-27632, fasudil.

INTRODUCTION

Asthma is an inflammatory airways disease characterised by exaggerated bronchoconstriction to neurotransmitters, inflammatory mediators and inhaled contractile stimuli. Airway hyperresponsiveness may be explained, in part, by increased shortening of airway smooth muscle, caused either by an intrinsic functional change in the muscle or by the presence of inflammatory mediators that acutely augment contraction to other agonists or induce the release of excitatory neurotransmitters, such as tachykinins and acetylcholine [1]. Inflammatory mediators can be released in the airways both by recruited inflammatory cells and by resident structural cells, including the airway smooth muscle. In addition to augmented contractile responses, chronic inflammation appears to drive irreversible remodelling of the airways and contribute significantly to the pathogenesis and severity of asthma. Airway remodelling includes several key features such as excessive deposition of extracellular matrix proteins (fibrosis) and a dramatic increase in the abundance of contractile airway smooth muscle encircling the bronchi.

Corticosteroids and β_2-adrenoceptor agonists currently constitute the first line drug therapy in asthma. Though both agents can elicit satisfactory responses acutely, they are only partially effective in inhibiting features of airway remodelling. For instance, corticosteroids prevent, but do not reverse airway wall remodelling [2]. In addition, the inhibitory effects of corticosteroids on airway smooth muscle proliferation *in vitro* are strongly impaired when cells are seeded on a collagen I matrix, the expression of which is increased in asthma [3]. Studies using animal models suggest only minimal effects of β_2-agonists on airway wall remodelling, despite of their effectiveness in inhibiting airway smooth muscle proliferation *in vitro* [4, 5]. The relative inability of these drugs to inhibit airway remodelling has prompted researchers to investigate alternative drug targets. For example, animal studies suggest a profound inhibitory effect of anticholinergics [6], and of leukotriene receptor antagonists [7-9]. Additional drug targets are also under investigation. This review will focus on the therapeutic potential of inhibitors of Rho-associated kinases, more commonly referred to as Rho-kinase. These drugs are already considered for the treatment of cardiovascular diseases and have clear beneficial effects on cardiovascular remodelling in animal models [10, 11]. In addition, accumulating evidence now suggests beneficial effects of these drugs on both acute and chronic airway hyperresponsiveness.

AIRWAY SMOOTH MUSCLE: AIRWAY HYPERRESPONSIVENESS AND REMODELLING

Chronically inflamed airways are subject to structural changes (airway remodelling) that are thought to play an important role in the development of persistent airway hyperresponsiveness and progressive decline of lung function. Deposition of matrix proteins associated with airway remodelling is driven by mesenchymal cells such as subepithelial and adventitial fibroblasts, myofibroblasts and airway smooth muscle cells [12-14]. Fibrosis may alter elastic forces of the tissue surrounding the airway and cause uncoupling of airway smooth muscle from parenchymal recoil, which may contribute to exaggerated bronchoconstriction [15, 16]. In contrast, stiffening of the subepithelial layer caused by extracellular matrix deposition may protect against excessive airway narrowing. The precise impact of excessive extracellular matrix deposition in the airway wall on lung function are not clear, however, as fibrosis leads to changes in airway diameter, this could be sufficient to limit airway capacity to the extent seen in asthmatics [17].

The remodelled airway wall of asthmatic subjects also contains increased airway smooth muscle mass, which may potentiate the response to bronchoconstricting agents and thus, contribute directly to airway hyperresponsiveness. Increased airway smooth muscle mass may be explained in part by increased cell number (hyperplasia) [18], which is in

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line with the observation that asthmatic airway smooth muscle cells proliferate faster in culture [19]. Both hyperplasia and increases in cell size (hypertrophy) have been noted in asthmatics, however [20]. The increase in airway smooth muscle mass caused by either hypertrophy or hyperplasia is in theory sufficient to comprise a major cause of exaggerated airway narrowing [21, 22]. Importantly, changes in airway wall structure due to smooth muscle accumulation and airway wall fibrosis increase with duration of disease, which may contribute to the progressive increase in severity of airway narrowing in long-term asthmatics [23].

Airway smooth muscle cells exhibit capacity for phenotypic and functional modulation that can be mediated by pro-inflammatory mediators associated with asthma [24]. Using *in vitro* systems the maturation of myocytes to a hypercontractile phenotype can be induced by growth arrest or insulin exposure [25-27]; these cells are characterised by increased contractility, and increased expression of contractile proteins such as smooth muscle-specific actin and myosin [28]. The abundance of muscarinic M_3 receptor and contraction regulatory proteins (e.g. myosin light chain kinase (MLCK), calponin) increase in hypercontractile myocytes as well [28, 29]. These events could be important to asthma pathogenesis and symptoms, as airway smooth muscle cells isolated from bronchial biopsies of asthmatic subjects have been reported to express increased MLCK and to contract more profoundly and more rapidly [30]. Passive sensitization of human bronchi with atopic serum increases maximal contractility and agonist-sensitivity *in vitro* [31], and this enhanced contractility is accompanied by increased MLCK expression [32]. Though it appears phenotype and functional changes in contractility and/or agonist-sensitivity of airway smooth muscle may contribute to airway hyperresponsiveness, a number of reports suggest there may be no major changes in contractility *in vitro* (cf. [33] for review). Therefore the extent to which hypercontractility may exist and it's (patho)physiological relevance still need to be fully elucidated.

Increases in airway smooth muscle and (myo)fibroblast secretory function may also contribute to airway inflammation and airway remodelling. These cells are potent producers of cytokines, chemokines and extracellular matrix proteins [34-37]. Moreover, passively sensitized airway smooth muscle cells [38], and asthmatic fibroblasts [39] produce more extracellular matrix when compared to cells obtained from healthy controls and thus, exhibit a pro-fibrotic state. It is noteworthy that the profile of extracellular matrix proteins produced airway myocytes derived from

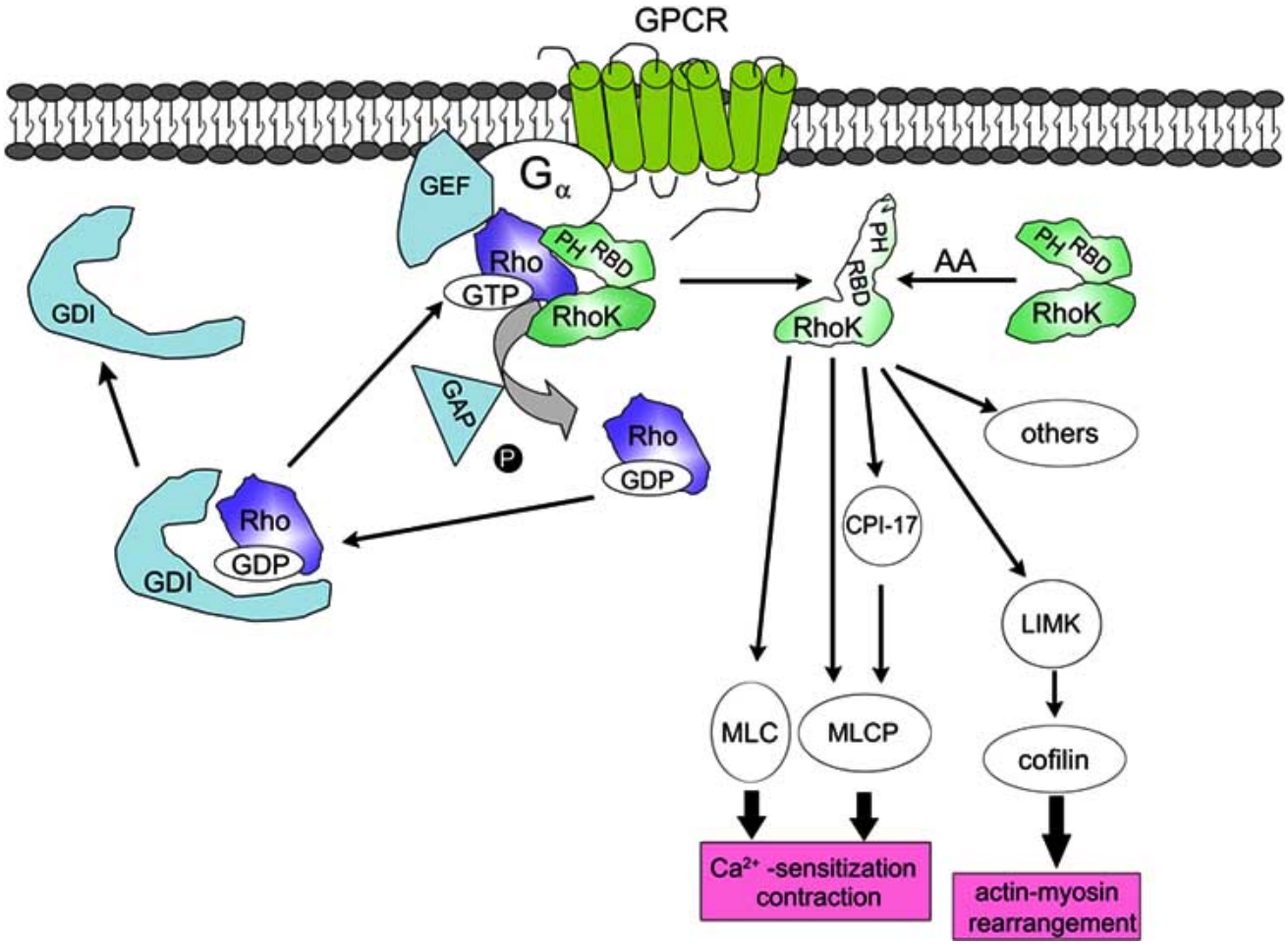

Fig. (1). Mechanisms by which agonists for G protein coupled receptors (GPCRs) activate the Rho/Rho-kinase pathway. Activated Rho-kinase can then phosphorylate various targets that contribute to airway smooth muscle contraction. One of the most important targets is thought to be myosin light chain phosphatase (MLCP), which upon phosphorylation by Rho-kinase is inactivated, causing calcium sensitization.

asthmatic subjects creates an environment that supports proliferation and thereby, may indirectly contribute to increased bronchial smooth muscle mass in asthma [40]. Collectively, these observations indicate that mesenchymal cells likely play a central role in the pathophysiology of asthma by modulating inflammation, extracellular matrix deposition and proliferation during periods of allergen exposure and through augmented contractile responses in the periods in between.

PHARMACOLOGY OF Rho-KINASE INHIBITORS

Y-27632 and Analogues

The pyridine derivative Y-27632 ([(+)-(*R*)-trans-4-(1-aminoethyl)-*N*-4-pyridyl) cyclohexanecarboxamide dihydrochloride]) is one of the most commonly used Rho-kinase inhibitors in experimental settings. Y-27632 is cell permeable, and has been shown to induce bronchodilatory effects when delivered to guinea pigs by means of aerosols, resulting in only minimal side effects on systemic blood pressure. These effects of inhaled Y-27632 on lung function were fast in onset, and lasted for a period of approximately 4 hours [41]. Similar results were obtained in a rat model of hypoxia, in which effects of inhaled Y-27632 were compared to oral administration. It was found that inhaled, but not orally administered, Y-27632 produced selective inhibitory effects on pulmonary blood pressure that lasted for at least 6 hours, with little or no systemic effects [42].

Y-27632 is an ATP-competitive inhibitor, that inhibits both the Rho-associated protein kinases ROCK-I and ROCK-II *in vitro*, with similar inhibition constants (K_i = 0.22 μM for ROCK-I and 0.30 μM for ROCK-II). These concentrations correspond to the IC_{50} concentrations that are generally required to relax vascular and tracheal smooth muscles *in vitro* [43]. Other Rho effector kinases such as citron kinase and protein kinase N (PKN) are also inhibited, albeit at higher concentrations (K_i = 5.3 μM and 3.1 μM for citron kinase and PKN, respectively) [44]. Other ATP-dependent protein kinases, including PKCα, PKA and MLCK, are affected only by concentrations in the high micromolar range [44]. In addition, Y-27632 at a concentration of 10 μM does not affect protein kinases associated with regulation of cell cycle progression, including ERK2, S6K1, GSK3β, PDK1, PKBα and p38MAPK isoforms [45]. The compound can therefore be considered reasonably selective, and useful to study the role of Rho-kinase in events such as cell proliferation and differentiation, both *in vitro* and *in vivo*.

Several analogues of Y-27632 exist, with similarly high inhibitory constants for Rho-kinase and similar smooth muscle relaxant properties [43, 44]. Of those, Y-30141 and Y-30694 may be particularly worth mentioning, as their selectivity profiles with respect to PKC and MLCK are only slightly dissimilar from Y-27632. However, both Y-30141 and Y-30694 inhibit cAMP-dependent protein kinase at relatively low concentrations [43]. Y-30141 was also evaluated functionally and shown to inhibit lysophosphatidic acid-induced actin stress fiber formation in Swiss 3T3 cells [44]. For more detail and for the chemical structures of Y-27632 and its analogues, we refer to recent studies by Uehata and coworkers and Ishizaki and coworkers [43, 44].

Fasudil (HA-1077) and Analogues

Fasudil, or HA-1077 (1-(5-isoquinolinesulfonyl)-homopiperazine) has a similar affinity for Rho-kinase as Y-27632, as judged by the inhibition constant for Rho-kinase activity (0.33 μM) [46]. Its chemical structure is closely related to the protein kinase inhibitor H-7, which has a similar inhibition constant for Rho-kinase activity and selectivity profile for PKC, MLCK and cAMP dependent protein kinase [43]. However, HA-1077 is only 3-fold more selective for Rho kinase compared to PKA; inhibition constants for PKC and MLCK are 9.3 μM and 55 μM respectively [46]. Despite its lower selectivity compared to Y-27632, fasudil is widely used in animal models, and is currently the only Rho-kinase inhibitor available for clinical use; in Japan, fasudil is approved for the prevention of vasospasm in patients with subarachnoid hemorrhage [47]. Interestingly, a metabolite of fasudil, hydroxy fasudil (HA-1100), is also bio-active, and causes smooth muscle relaxation with EC_{50} similar to the parent compound [48].

H-1152P ((S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline) sulfonyl]-homopiperazine) is a derivative of HA-1077. It is, however, far more potent (K_i for Rho-kinase = 1.6 nM) and its selectivity profile (with respect to PKA, PKC and MLCK) is even be better than that of Y-27632 [46]. Despite of this, the drug has been less widely studied, which may relate to its novelty. For more detail on this class of Rho-kinase inhibitors and their chemical structures, we refer to a recent study by Sasaki and co-workers [46].

REGULATION OF Rho AND Rho-KINASE SIGNALLING

The main upstream activator of Rho-kinase is the monomeric G-protein RhoA, which is a member of the Rho (Ras-homologous)-subfamily of the Ras-superfamily [49]. Mechanisms of Rho activation are illustrated in Fig. (**1**). Activity of Rho is regulated by three groups of proteins: guanine dissociation inhibitors (GDIs), guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). In its inactive GDP-bound form Rho is localized to the cytoplasm, where it is complexed to a GDI that prevents nucleotide exchange and thereby activation [50]. Conversion of inactive GDP-bound Rho into active GTP-bound Rho is facilitated by the action of GEFs. Currently about 60 different GEFs for Rho family members have been identified [51]. Rho activation triggers it's translocation to specific plasma membrane sites, including caveolae in smooth muscle cells [52, 53], where it can interact with its effector proteins. In opposition to the role of GEFs, GAPs inactivate Rho by accelerating the intrinsic GTPase activity of the protein, resulting in the reconversion of GTP-bound Rho into GDP-bound Rho [49, 54]. Subsequently, inactive Rho relocates to the cytoplasm where it can re-associate with GDI proteins [50].

It is well established that RhoA, and in turn Rho-kinase, can be activated by a variety of G-protein coupled receptors, particularly by those coupled to $G_{12/13}$ proteins, through an

interaction with RhoGEFs [55, 56]. Interestingly, RhoGEFs may also inactivate these G proteins by increasing their GTPase activity [57, 58]. Recently, it was shown that agonist-stimulated $G_{q/11}$-coupled receptors are also capable of activating RhoA, and this is facilitated by a $G_{q/11}$-selective GEF, p63RhoGEF [59]. Stimulation of receptor tyrosine kinases can also lead to GEF activation and the modulation of Rho signalling. For example, upon insulin-like growth factor-1 binding, autophosphorylation of its receptor at tyrosine residues takes place, resulting in the formation of a complex with leukemia-associated Rho-guanine exchange factor (LARG), ultimately leading to the conversion of GDP-bound Rho into GTP-bound Rho and Rho-kinase activation [60]. Cytokine receptors and integrins have also been linked to RhoGEFs and Rho activation [61]. It appears therefore that activation of the Rho/Rho-kinase signalling pathway can occur through a variety of stimuli, including contractile agonists acting on GPCRs, growth factors acting on receptor tyrosine kinases (RTKs), cytokines acting on cytokine receptors and extracellular matrix proteins acting on integrins (Fig (***2***)). Furthermore, activation of the Rho/Rho-kinase pathway by KCl-induced membrane-depolarization has been described in airway smooth muscle [62-64].

Rho-kinase is one of the best-characterized effectors of Rho. It is a serine/threonine kinase that is activated by a direct interaction of a C-terminal Rho-binding domain (RBD) with GTP-bound RhoA [65, 66]. In addition to activation by RhoA, arachidonic acid (AA) can activate Rho-kinase in a RhoA-independent fashion [67]. It has been suggested that arachidonic acid binds to the C-terminal part of the coiled-coil domain of Rho-kinase, which acts as an autoinhibitor –domain [68], resulting in the release from its catalytic domain and subsequent activation [69]. A number of downstream targets for Rho-kinase have been identified, and they are associated with regulation of a broad range of cellular functions, including contraction, migration, gene transcription, cell adhesion, cytoskeletal remodelling, and proliferation [49].

Rho-KINASE AND AIRWAY HYPERRESPONSIVENESS IN ASTHMA

Airway Smooth Muscle Contraction

An agonist-dependent role of Rho-kinase in regulation of airway smooth muscle contraction appears to exist. Growth factor-induced contraction of human bronchial smooth muscle, for instance, appears to be completely dependent on Rho-kinase, whereas histamine-induced contractions, which are mediated through $G_{q/11}$-coupled H_1 receptors are not [70]. In addition, in guinea pig tracheal smooth muscle, potency and maximal contraction induced by $PGF_{2\alpha}$ are determined by Rho-kinase activity, whereas contractions elicited by histamine are not [71]. Although contractions induced by muscarinic receptor agonists and membrane depolarisation with KCl are both partially Rho-kinase-mediated, Rho-kinase inhibition reduced these responses to differential extents with surprisingly, the largest effects for KCl [63, 64, 72]. Clearly, complex interactions between receptors and downstream signal transduction cascades involving Rho and Rho kinase exist in airway smooth muscle cells.

Smooth muscle contraction is largely governed through phosphorylation of the 20kDa regulatory myosin light chain (MLC_{20}) [73]. MLC_{20} phosphorylation is induced after an increase in intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) and subsequent formation of Ca^{2+}-calmodulin leading to activation of MLCK. It has been shown that $[Ca^{2+}]_i$ does not always parallel the level of MLC_{20} phosphorylation and contraction. The extent of MLC_{20} phosphorylation is determined by the balance of activity of MLCK and myosin light chain phosphatase (MLCP) [74]. Activated Rho-kinase interferes with this equilibrium by phosphorylating the myosin binding subunit of MLCP. This results in an enhancement of MLC phosphorylation and hence an augmented level of contraction at a fixed $[Ca^{2+}]_i$; this state being called Ca^{2+}-sensitization [49, 54]. Rho-kinase may also directly phosphorylate MLC_{20} at Ser-19 *in vitro* [75]; this is the same site phosphorylated by MLCK, although this process may be of less importance in regulating MLC_{20} phosphorylation *in vivo* [74]. Rho-kinase can also target the PKC-potentiated inhibitor protein of 17 kDa (CPI-17) [76]. In parallel with PKC, Rho-kinase can phosphorylate CPI-17, which leads to inhibition of MLCP activity [76] (Fig (**1**)). It has been suggested that Rho-kinase-mediated phosphorylation of the actin filament-associated protein calponin, which in its unphosphorylated form binds to filamentous (F)-actin and inhibits the actin-activated myosin ATPase activity, could also contribute to smooth muscle contraction [77]. However, several studies, using calponin knockout mice, showed no significant role for calponin in the regulation of Ca^{2+}-sensitivity in smooth muscle [78, 79].

In addition to the effects of Rho and Rho-kinase on Ca^{2+} sensitization of the contractile apparatus, these effectors play a significant role in regulating actin cytoskeletal dynamics, that determine active force and shortening of airway smooth muscle [80, 81]. In human airway smooth muscle cells, it has been demonstrated that agonists for G_i and G_q protein coupled can induce actin polymerization (increasing the filamentous-to-globular (F/G) actin ratio) *via* a RhoA pathway [82, 83]. In addition, in cultured airway myocytes Rho-kinase can be induced by uniaxial cyclic mechanical strain or inhibited by biaxial cyclic strain, thus leading to an increase or decrease, respectively, in F:G actin [84, 85]. It is clear that mechanical plasticity and length adaptation of airway smooth muscle is, in part, modulated by pathways that regulate actin dynamics, thus it is likely that Rho plays a key role in determining contractile behaviour [86]. The precise downstream mechanisms of Rho/Rho-kinase-mediated effects on actin cytoskeletal organization in airway smooth muscle remain unresolved, though insight has been obtained from other cell systems, including endothelial cells [87] and vascular smooth muscle cells [88]. For example, Rho-kinase indirectly mediates phosphorylation and inactivation of the actin depolymerizing factor cofilin, presumably through the phosphorylation and activation of LIM-kinase (LIMK) [89] (Fig (***1***)). These observations strongly suggest that future studies aimed at dissecting the precise role of Rho and Rho kinase in regulating cytoskeletal dynamics during airway smooth muscle contraction are warranted.

Rho/Rho-kinase-mediated Ca^{2+}-sensitization appears to contribute to control of smooth muscle contraction under normal conditions [90, 91], and there is clear evidence that Ca^{2+}-sensitizing mechanisms may be enhanced by pathophysiological conditions. In vascular smooth muscle, for instance, increased activity of the Rho-kinase pathway has been implicated in the genesis of enhanced vasoconstriction in spontaneously hypertensive rats [92]. Also in humans it has been shown that Rho-kinase is likely involved in the pathogenesis of increased peripheral vascular resistance in systemic hypertension [93]. This pathophysiology-primed role for Rho-kinase likely also applies to airway diseases, since an augmented role of Rho-kinase in acetylcholine induced bronchial smooth muscle contraction after repeated allergen challenge is evident [94]. Moreover, it has recently been demonstrated that active allergic sensitization without subsequent allergen exposure can be sufficient to induce an enhanced role of Rho-kinase in guinea pig airway smooth muscle contraction *ex vivo* and airway responsiveness *in vivo* [71].

In passively sensitized guinea pigs, inhalation of the selective Rho-kinase inhibitor Y-27632 inhibits acetylcholine- and ovalbumin-induced elevations in airway resistance [41]. Also, Y-27632 suppressed airway hyperresponsiveness in mice repeatedly challenged with ovalbumin after active sensitization in the absence and presence of respiratory syncytial virus infection [95]. These findings indicate that Rho-kinase might be involved in the degree (and perhaps the development) of airway hyperresponsiveness. An increased functional role of Rho-kinase might involve increased levels of RhoA, the main upstream activator of Rho-kinase in smooth muscle. Indeed, the expression of RhoA is increased in bronchial smooth muscle from rats repeatedly challenged with allergen to induce airway hyperresponsiveness [94]. A similar observation has also been reported from studies using lung homogenates from actively sensitized guinea pigs that were not challenged with inhaled allergen [71]. Increased RhoA translocation to the cell membrane [96], and increased protein levels of $G\alpha_{12}$ and $G\alpha_{13}$ [97] have also been found in bronchial smooth muscle from rats that exhibit airway hyperresponsiveness. These mechanisms could underlie an augmented contribution for Rho/Rho-kinase signalling in airway smooth muscle after allergic sensitization and/or allergen challenge.

β_2-agonists are widely used in the treatment of asthma because of their potent bronchodilatory effects. However, it is well established that chronic β-agonist therapy can reduce the efficacy of these drugs, and may even cause adverse effects [98]. There is some evidence that Rho-kinase might be involved in the desensitization of the β_2-receptor. For instance, in guinea pig tracheal smooth muscle, continuous exposure to lysophosphatidylcholine augments homologous desensitization of the β-receptor presumably as a consequence of an increased Rho-kinase mediated Ca^{2+}-sensitization [99]. In line with this observation, Y-27632 can augment salbutamol- and terbutaline-induced relaxations of pre-contracted bovine tracheal smooth muscle [100]. These findings indicate that the combination of a Rho-kinase inhibitor with a β-agonist could be more effective than β-agonists alone.

Smooth Muscle Specific Gene Transcription

Airway remodelling in asthma includes a dramatically increased mass of contractile airway smooth muscle encircling the bronchi. Tissue hypertrophy evolves from myocyte hyperplasia and cellular hypertrophy, and may also result from phenotype maturation of myofibroblasts and "synthetic" airway smooth muscle cells [24]. Accumulation of contractile smooth muscle requires both the transcription of genes encoding proteins that mediate and control contraction, and subsequent translation of these transcripts. Only recently have studies using human airway myocytes revealed that protein translation of smooth muscle-specific contractile proteins is regulated by phosphatidylinositide-3-kinase signal transduction pathways involving p70 S6 kinase and PHAS-1 [101, 102]. Considerable understanding of pathways that regulate transcription of contractile smooth muscle specific genes such as smooth muscle myosin heavy chain (smMHC), smooth muscle α-actin, calponin, and SM22 has developed in the past decade and an essential role for RhoA and Rho-kinase has emerged [103-105].

Transcription of contractile smooth muscle-specific genes is regulated by combinatorial control involving a number of key transcription factors [105]. Virtually all of these genes harbour a pair of essential CArG box elements $[CC(A/T)_6GG]$ in the 5' promoter region that binds dimers of the MADS transcription regulator family member, serum response factor (SRF) [106]. Binding of SRF is essential for promoter function, as mutation of these sites renders promoters for genes such as SM22 and smMHC inactive [103, 107, 108]. SRF activation is associated with its re-localization to the nucleus, where it can associate with co-factors including myocardin and MAL/MKL1 (megakaryocytic acute leukemia/megakaryoblastic leukemia) that direct its actions on smooth muscle gene promoters [103, 109-111]. Relocalization of SRF to the nucleus and the induction of smooth muscle specific genes are regulated by the RhoA / Rho-kinase pathway in airway smooth muscle [104]. Activation of the RhoA /Rho-kinase pathway also promotes actin polymerization that is required for SRF induction [108]. Actin polymerization leads to a loss of cytosolic globular actin, thus allowing nuclear translocation of MAL to further support SRF-driven transcription of contractile smooth muscle specific genes [109]. Collectively, these observations reveal that RhoA and Rho-kinase are required for contractile smooth muscle gene transcription and this effect involves co-ordinated control of actin cytoskeletal dynamics.

Intracellular control of RhoA/Rho kinase appears to be complex. An additional pathway that appears to modulate the effects of Rho-kinase on actin dynamics and smooth muscle gene transcription involves the protein kinase C (PKC) family. Activation of the $G\alpha_{q/11}$-coupled muscarinic M_3 receptor induces RhoA, likely *via* p63RhoGEF, and actin polymerization leading to transcription of smMHC and SM22 [82]. In contrast, PKC, which is also activated by the M_3 receptor, causes a loss of filamentous actin leading to diminished nuclear SRF and a reduction in transcriptional activity of SM22 and smMHC gene promoters [84]. Consistent with a role for PKC in balancing RhoA induced SRF-dependent smooth muscle gene transcription, we have observed a 6-fold increase in SM22 promoter activity in the

presence of pharmacological inhibitors of PKC in canine tracheal myocytes stimulated with acetylcholine (unpublished observation, AJH). Another consideration is that SRF is expressed ubiquitously, and does not bind exclusively to contractile smooth muscle specific genes; therefore elegant control is required to ensure effective cell responses. For example, the association of SRF with its CArG box co-factors, myocardin and MAL, can be greatly reduced by competitive binding of Ets transcription factor family members (eg. Elk-1) [111, 112]. In smooth muscle Elk-1 is phosphorylated by ERK1/2 in response to growth factor stimulation, which greatly increases its binding affinity to SRF. Elk-1/SRF complexes preferentially bind to serum response elements present in early response genes such as *c-fos* that promote cell proliferation. Thus, SRF binding to CArG boxes in contractile smooth muscle specific genes becomes reduced and transcription is attenuated. Mechanisms that modulate cross talk of Rho-kinase with ERK and PKC signal transduction pathways are not entirely clear, thus more insight in this area is clearly warranted.

Based on the requirement for Rho-kinase for transcription of contractile smooth muscle specific genes, Rho-kinase inhibitors appear to hold some potential for preventing hypertrophy of bronchial smooth muscle tissue that develops in long-term asthmatic patients. These effects coupled with the effects of Rho-kinase inhibition on reducing smooth muscle contractility [64] may also have potential for effectively preventing airways hyperresponsiveness due to acute constriction to agonists, and due to structural changes in the airway wall due to remodelling.

Airway Wall Thickening

In asthma airway remodelling includes increased numbers of fibroblasts and airway smooth muscle cells that contribute to airway wall thickening, and potentially to chronic airway hyperresponsiveness. In part, increased airway smooth muscle mass appears to be the result of myocyte proliferation driven by synergistic and additive effects of a number of growth factors, inflammatory mediators and neurotransmitters [113]. Peptide growth factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) are among the most effective inducers of mesenchymal cell proliferation, and may play a role in asthma [114]. Surprisingly, *in vitro* studies using cultured airway smooth muscle cells suggest no major role for Rho proteins or Rho-kinase in proliferation induced exclusively by PDGF [64] or EGF [115], despite of reports that suggest an essential role for RhoA and Rho-kinase in growth factor-induced proliferation of vascular smooth muscle cells [116]. On the other hand, the proliferative response of human airway smooth muscle cells to the G protein coupled receptor (GPCR) agonist lysophosphatidic acid (LPA) alone, and its strong synergistic effects with EGF can be abrogated by Rho inhibition [115]. This is explained by Rho-mediated control of the transcription factors nuclear factor (NF)-κB and activator protein-1 (AP-1), respectively. Intermediate signalling components were not identified, but could involve nuclear translocation of p42/p44 MAP kinase through activation of Rho-kinase as described for the GPCR agonist serotonin in pulmonary arterial smooth muscle cells [117]**.** Interestingly, though EGF alone induced little activation of Rho, LPA induced a 9-fold increase in Rho activity [115]; this suggests Rho and Rho-kinase indeed regulate myocyte proliferation, but that the level of activation and relative contribution of the pathway to proliferation is agonist-dependent. Since in addition to LPA, a number of other GPCRs have been associated with RhoA and Rho-kinase signal transduction, an important role for this pathway in synergistic effects of some GPCR agonists with peptide growth factors is implicated. Indeed the synergistic effects of M_3 muscarinic and $CysLT_1$ receptor activation on PDGF and EGF induced proliferation respectively have been reported [13, 118] though confirmation for a role of Rho signal transduction in addition to other key pathways such as those involving p70S6K, is still necessary [119].

In addition to GPCR agonists, a recent report suggests that the extracellular matrix protein fibronectin can affect cell cycle progression in part by acting on Rho-kinase, which in coordinated activation with p42/p44 MAP kinase reduces the expression of the cell cycle inhibitory protein p21 [120]. Although these results were obtained using a non-small cell lung carcinoma cell line, this effect is of interest to airway remodelling in asthma, as wall fibrosis is a hallmark feature of airway wall remodelling. Fibrosis includes accumulation of a number of proteins including fibronectin, and myocytes derived from asthmatic subjects express an altered profile of matrix proteins secretion that appears to promote cell proliferation [40, 121]. Fibronectin enhances the proliferative response of airway smooth muscle cells to growth factors [122], *via* pathways mediated by β_1 integrins [123]. Parallel effects of fibronectin on immunomodulatory function of airway myocytes also exist, and the matrix protein significantly enhances IL1β-induced RANTES and GM-CSF, and thus could promote local inflammation [124]. However, the role of Rho-kinase in cytokine and/or chemokine secretion by these cells has not yet been studied.

Rho-kinase also has potential to regulate airway wall thickening by affecting the migratory response of airway smooth muscle cells and fibroblasts. Migration is thought to be an important component of tissue repair, and thus likely plays a role in airway remodelling. PDGF and leukotriene (LT) E_4-induced migration of airway smooth muscle cells can be inhibited by the Rho-kinase inhibitor Y-27632 [125]. Similar effects of Y-27632 have been found on the migratory response of airway smooth muscle cells to urokinase and PDGF [126]. The mechanism by which Rho-kinase inhibitors affect migration has not yet been elucidated in airway smooth muscle cells, but this likely involves modulation of myosin light chain phosphorylation, which is an important event in cytoskeletal dynamics that control the migratory responses of other cell types [127, 128].

Airway Inflammation

Rho-kinase inhibitors may have important inhibitory effects on chronic airway inflammation. Although airway remodelling persists after inflammation has ceased [129], the acute influx of inflammatory cells in response to allergic challenge may be an important initiation factor for this pathology. The number and type of inflammatory cells that

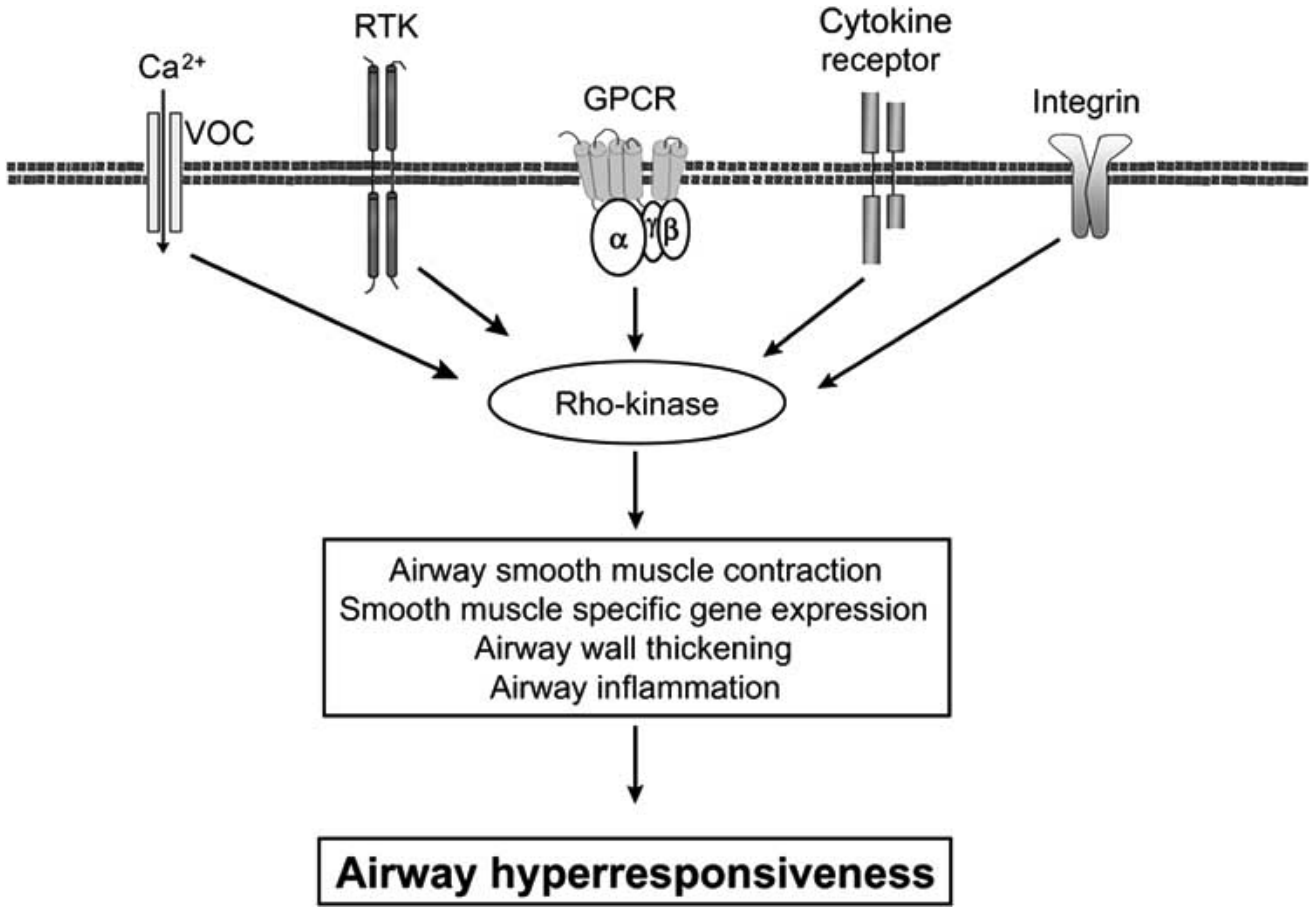

Fig. (2). A central role for Rho-kinase in airway hyperresponsiveness. Rho-kinase can be activated by a variety of stimuli and is implicated in the biology of virtually all cell types present in the airways. Because of this central role, Rho-kinase could play a role in many processes that underlie acute and chronic airway hyperresponsiveness in asthma.

are recruited to the airways is variable; however, the accumulation of $CD4^+$ Th2 lymphoctes and eosinophils is generally seen in allergic asthma [130]. Although the role of the eosinophil in allergic asthma is challenged by some studies, other studies have provided compelling evidence for a role in allergic airways disease [131]. As a source of basic granule proteins, growth factors, lipid mediators, pro-inflammatory cytokines and chemokines, eosinophils have for instance been associated with the induction of epithelial damage, M_2 autoreceptor dysfunction on airway nerves, airway remodelling, and airway hyperresponsiveness [132-135].

The importance of the eosinophil to airway hyperresponsiveness in allergic asthma is potentially relevant for the putative beneficial effects of Rho-kinase inhibitors on allergic airway inflammation and related airway hyperresponsiveness, since in a mouse model of allergic inflammation, Y-27632 effectively inhibits the pulmonary influx of eosinophils after ovalbumin challenge [136]. Y-27632 also inhibits airway hyperresponsiveness to methacholine and serotonin in allergic mice [95]. The effects of Y-27632 are consistent with findings that demonstrate a major role for RhoA and Rho-kinase in eotaxin-induced chemotaxis of eosinophils [137], and in the migration of eosinophils through endothelial barriers *in vitro* [138]. A large number of studies have demonstrated the importance of RhoA and Rho-kinase in the migration and/or function of other inflammatory cells such as neutrophils, lymphocytes, dendritic cells, mast cells and monocytes [139-142]. Some of these effects are related to effects on endothelial barrier integrity. Actomyosin mediated endothelial cell contraction is key for inflammatory cell-induced reduction in endothelium integrity, which is necessary for tissue infiltration by inflammatory cells. Reduction of myosin phosphorylation in HUVEC endothelial cells by Rho-kinase or myosin light chain kinase inhibition protects endothelial barrier integrity and prevents inflammatory cell transit [140]. Though studied *in vitro*, these effects could be important *in vivo* as well, since neutrophil infiltration of the lungs in a murine endotoxin-induced acute lung injury model is inhibited by Y-27632 [143]. Since neutrophil influx is observed in asthma [130], and since neutrophils represent an important source of proteases, lipid mediators and pro-inflammatory cytokines and chemokines, these effects could contribute to an anti-remodelling effect of Rho-kinase inhibitors as well.

CONCLUDING REMARKS

By potentiating airway smooth muscle contraction, differentiation of myofibroblasts and maturation of airway smooth muscle cells, proliferation and migration of airway

wall mesenchymal cells, and migration and function of inflammatory cells, Rho-kinase appears to play a central role in many of the key processes observed in chronic inflammatory airways diseases such as asthma (Fig. (**2**)). Thus, the pharmacotherapeutical potential of inhibitors for Rho-kinase in the treatment of asthma holds promise. In addition, Rho-kinase inhibitors may be useful for the treatment of neurological disorders, including stroke, inflammatory and demyelinating diseases, Alzheimer's disease and neuropathic pain [144], vascular disorders, including arteriosclerosis, pulmonary arterial hypertension, and coronary and cerebral vasospasm [47, 145, 146], renal interstitial fibrosis [147], erectile dysfunction [148-150] and elevated intraocular pressure [151, 152].

The benefits of Rho-kinase inhibitors in preventing and reversing airway hyperresponsiveness and airway remodelling will however need to be weighed against any side-effects, as virtually all cells express Rho-kinase and rely on its function in some way. However, the Rho-kinase inhibitor fasudil appears to be well tolerated without serious adverse side effects in clinical trials [153]. In addition, there is evidence that RhoA and Rho-kinase expression and function is enhanced by inflammation, implying that Rho-kinase inhibitors could be reasonably selective for inflamed portions of the airways without major effects on healthy airways or other target organs. Such selectivity for pathophysiological conditions is consistent with salient studies that demonstrate selective inhibitory effects of Rho-kinase inhibitors on systemic blood pressure in hypertensive patients compared to normotensive patients [93]. Furthermore, in vasospastic angina, Rho-kinase inhibitors are effective inhibitors of contraction in spastic segments of affected blood vessels but do not appear to affect healthy segments of blood vessels within the same patient [154]. Further studies that focus more specifically on airways disease are needed with focus on their effects on all aspects of acute and chronic asthma, both *in vitro* and *in vivo*. Currently, Rho-kinase represents a potentially important novel pharmacotherapeutical target for the treatment of acute and chronic airways diseases.

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