Invited Review

The role of RhoA-mediated Ca\(^{2+}\) sensitization of bronchial smooth muscle contraction in airway hyperresponsiveness

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Abstract

Smooth muscle contraction is mediated by Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent pathways. The latter Ca\(^{2+}\)-independent pathway, termed Ca\(^{2+}\) sensitization, is mainly regulated by a monomeric GTP binding protein RhoA and its downstream target Rho-kinase. Recent studies suggest a possible involvement of augmented RhoA/Rho-kinase signaling in the elevated smooth muscle contraction in several human diseases. An increased bronchial smooth muscle contractility, which might be a major cause of the airway hyperresponsiveness that is a characteristic feature of asthmatics, has also been reported in bronchial asthma. Here, we will discuss the role of RhoA/Rho-kinase-mediated Ca\(^{2+}\) sensitization of bronchial smooth muscle contraction in the pathogenesis of airway hyperresponsiveness. Agonist-induced Ca\(^{2+}\) sensitization is also inherent in bronchial smooth muscle. Since the Ca\(^{2+}\) sensitization is sensitive to a RhoA inactivator, C3 exoenzyme, and a Rho-kinase inhibitor, Y-27632, the RhoA/Rho-kinase pathway is involved in the signaling. It is of interest that the RhoA/Rho-kinase-mediated Ca\(^{2+}\) sensitization of bronchial smooth muscle contraction is markedly augmented in experimental asthma. Moreover, Y-27632 relaxes the bronchospasm induced by contractile agonists and antigens \textit{in vivo}. Y-27632 also has an ability to inhibit airway hyperresponsiveness induced by antigen challenge. Thus, the RhoA/Rho-kinase pathway might be a potential target for the development of new treatments for asthma, especially in airway hyperresponsiveness.

Key words: asthma, airway hyperresponsiveness, bronchial smooth muscle, Ca\(^{2+}\) sensitization, RhoA, Rho-kinase

Introduction

Increased airway narrowing in response to nonspecific stimuli is a characteristic feature of human obstructive diseases, including bronchial asthma. This abnormality is an important symptom of the disease, although the pathophysiological variations leading to the hyperresponsiveness remain unclear. Several mechanisms have been suggested to explain airway hyperresponsiveness, such as alterations in the neural control of airway smooth muscle
(Boushey et al., 1980), increased mucosal secretions (Jeffery et al., 1993), and mechanical factors related to remodeling of the airways (Wiggs et al., 1990). In addition, it has also been suggested that one of the factors that contributes to the exaggerated airway narrowing in asthmatics is an abnormality in the nature of airway smooth muscle (Seow et al., 1998; Martin et al., 2000). The rapid relief from airway limitation in asthmatic patients by β-stimulant inhalation may also suggest an involvement of augmented airway smooth muscle contraction in the airway obstruction. Thus, it may be important for development of asthma therapy to understand changes in the contractile signaling of airway smooth muscle cells associated with the disease.

In the current brief review, we will discuss the role of RhoA-mediated Ca\textsuperscript{2+} sensitization of airway smooth muscle contraction in the pathogenesis of airway hyperresponsiveness.

**Ca\textsuperscript{2+}-dependent smooth muscle contraction**

Typically, smooth muscle contraction is mainly mediated by an increase in cytosolic Ca\textsuperscript{2+} via the activation of plasma membrane Ca\textsuperscript{2+} channels and/or Ca\textsuperscript{2+} release from sarcoplasmic reticulum (SR). Airway smooth muscle is predominantly innervated by vagal efferent nerves, which release acetylcholine (ACh) when stimulated and subsequently activate muscarinic cholinergic receptors. Five muscarinic receptor subtypes (m1–m5) have been cloned (Hulme et al., 1990), and three of them have been functionally characterized in airways; M1 receptors mediate bronchoconstriction through stimulation of parasympathetic ganglia (Beck et al., 1987), M2 autoreceptors on pulmonary parasympathetic nerve terminals inhibit vagally mediated ACh release (Fryer and Maclagan, 1984), and M3 receptors on airway smooth muscle cells mediate contraction through activation of phosphoinositide metabolism (Roffel et al., 1990). M3 receptors, that have been thought a main receptor subtype contributing to airway smooth muscle contraction (Barnes, 1990), are coupled to a heterotrimeric guanosine triphosphate (GTP) binding protein (G protein) termed G<sub>α</sub>. When ACh binds to M3 receptors, agonist-receptor-G protein coupling occurs and the exchange of guanosine diphosphate (GDP) for GTP in the α subunit is promoted. The binding of GTP to the α subunit induces a dissociation of the αβγ holomer to free, activated, GTP-bound α subunit. Subsequently, this GTP-bound α subunit activates phospholipase C (Linder and Gilman, 1992; Rodger and Pyne, 1992). This enzyme generates inositol 1,4,5-trisphosphate (IP<sub>3</sub>), which binds to its receptors of SR leading to Ca\textsuperscript{2+} release from SR (Rodger and Pyne, 1992). The increased cytosolic Ca\textsuperscript{2+} forms the 4Ca\textsuperscript{2+}-calmodulin-myosin light chain kinase (MLCK) complex and activates MLCK. The activated MLCK phosphorylates the 20 kDa myosin light chain (MLC), leading to smooth muscle contraction (Rodger and Pyne, 1992) (Fig. 1).

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In addition to the Ca\textsuperscript{2+}-dependent phosphorylation of MLC, the MLC phosphorylation is also regulated by MLC phosphatase, Ca\textsuperscript{2+}-independently, and thus further contraction occurs, which is termed Ca\textsuperscript{2+} sensitization (reviewed by Somlyo and Somlyo, 2003). The agonist-induced Ca\textsuperscript{2+} sensitization of smooth muscle contraction has been demonstrated by studies using the
Fig. 1. Regulation of smooth muscle contraction by myosin light chain kinase (MLCK) and MLC phosphatase. Various agonists, such as neurotransmitters and chemical mediators, bind to their specific G protein-coupled receptors (GPCR) to induce contraction in smooth muscle. Subsequently, phospholipase C (PLC) is activated via coupling with a heterotrimeric GTP-binding protein (G protein). Then the PLC produces two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG), from membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂). IP₃ causes Ca²⁺ release from sarcoplasmic reticulum (SR) via its specific receptors on SR. In addition, agonist stimulation also activates receptor-operated and voltage-dependent Ca²⁺ channels on the plasma membrane and induces Ca²⁺ influx into the cytosolic space. The cytosolic Ca²⁺ binds to calmodulin, leading to activation of myosin light chain kinase (MLCK) by forming 4Ca²⁺ · calmodulin · MLCK complex. The MLCK phosphorylates myosin light chain (MLC), leading to contraction of smooth muscle. In addition to the prototypical responses, Ca²⁺ sensitization, that is attributed to the inhibition of MLC phosphatase, is also initiated at the same time. The precise nature of the activation of RhoA by GPCR is not entirely clear but involves guanine nucleotide exchange factors (RhoGEFs, such as p115RhoGEF, PDZ-RhoGEF and LARG (reviewed by Somlyo and Somlyo, 2003). The RhoGEFs activate RhoA by exchanging GDP- to GTP-bound form of RhoA. The activated GTP-bound form of RhoA increases Rho-kinase activity, leading to an inhibition of MLC phosphatase. When the MLC phosphatase is inhibited, the phosphorylated MLC cannot be dephosphorylated, resulting in a promotion of contractile state, that is Ca²⁺ sensitization of smooth muscle contraction. In addition to the RhoA/Rho-kinase-mediated inhibition of MLC phosphatase, an involvement of CPI-17, which is activated by protein kinase C (PKC)-mediated phosphorylation, in the inhibition of MLC phosphatase has also been reported (e.g., Kitazawa et al., 2000).

Simultaneous measurements of force development and intracellular Ca²⁺ concentration (Sato et al., 1988), and chemically permeabilized preparations (Fujita et al., 1995) in various types of smooth muscle including airways (Ozaki et al., 1990; Chiba et al., 1999b). It has been demonstrated that agonist stimulation increases myofilament Ca²⁺ sensitivity in β-escin-permeabilized smooth muscle of the rat coronary artery (Satoh et al., 1994), guinea pig vas
deferens (Fujita et al., 1995), canine trachea (Bremerich et al., 1997), rat bronchus (Chiba et al., 1999b) and so on. A participation of a monomeric GTP binding protein, RhoA, and its downstream target, Rho-kinase, in the agonist-induced Ca\(^{2+}\) sensitization has been suggested by many investigators (e.g., Fujita et al., 1995; Otto et al., 1996; Gong et al., 1997; Chiba et al., 1999b).

RhoA/Rho-kinase plays an important role in the regulation of MLC phosphatase activity. MLC phosphatase removes phosphate from phosphorylated MLC to induce smooth muscle relaxation. MLC phosphatase is a holoenzyme and consists of three subunits: a 37-kDa catalytic subunit, a 20-kDa variable subunit, and a 110- to 130-kDa myosin-binding subunit. The myosin-binding subunit, when phosphorylated, inhibits the enzymatic activity of MLC phosphatase, allowing the light chain of myosin to remain phosphorylated, thereby promoting contraction. Rho-kinase, a serine/threonine kinase, phosphorylates the myosin-binding subunit of MLC phosphatase, resulting in an inhibition of its activity and thus promoting the phosphorylated state of the MLC (Fig. 1). Pharmacological inhibitors of Rho-kinase, such as fasudil and Y-27632, block its activity by competing with the ATP-binding site on the enzyme and prevent RhoA-mediated MLC phosphatase inhibition, resulting in smooth muscle relaxation (e.g., Uehata et al., 1997; Nagumo et al., 2000; Chiba et al., 2001b).

**RhoA-mediated Ca\(^{2+}\) sensitization in diseased smooth muscle**

In experimental animal models of several human diseases, an augmented RhoA/Rho-kinase-mediated Ca\(^{2+}\) sensitization in smooth muscle contraction has been reported. Uehata et al. (1997) originally demonstrated an involvement of Rho-kinase signaling in the pathogenesis of hypertension. They showed that inhibition of Rho-kinase by Y-27632 reduced the elevated blood pressure in spontaneously hypertensive rats (SHR) and renal and deoxycorticosterone acetate-salt-induced hypertensive rats but not the normal blood pressure in normotensive control animals (Uehata et al., 1997). Mukai et al. (2001) reported an increase in Rho-kinase mRNA and activity in vascular smooth muscle of the SHR model. Furthermore, Seko et al. (2003) demonstrated an augmented activation of RhoA, *i.e.*, an increase in GTP-RhoA level, in vascular smooth muscle of various hypertension models including the SHR. In coronary arterial smooth muscle, Satoh et al. (1994) firstly demonstrated an augmented agonist-induced, G protein-mediated Ca\(^{2+}\) sensitization in coronary vasospasm of the SHR model, although the involvement of RhoA/Rho-kinase signaling had not yet been identified. Then Shimokawa et al. (1999) showed that hypercontraction and enhanced MLC phosphorylation induced by serotonin in a swine model of coronary artery spasm were inhibited by a Rho-kinase inhibitor, hydroxyfasudil, suggesting that the RhoA/Rho-kinase pathway plays a central role in the pathogenesis of coronary artery spasm. Recent studies demonstrated an upregulation of Rho-kinase by inflammatory stimuli, such as interleukin-1\(\beta\) and angiotensin II, in coronary artery smooth muscle (Kandabashi et al., 2000; Hiroki et al., 2004). RhoA/Rho-kinase signaling is also remarkable in cerebral vasospasm. Sato et al. (2000) reported that experimental cerebral vasospasm induced by subarachnoid hemorrhage is accompanied by elevated Rho-kinase activity and phosphorylation of myosin phosphatase at its myosin-binding subunit. In the SHR model, cerebral vasodilation induced by Y-27632 is significantly greater than that in the...
normotensive control (Chrissobolis and Sobey, 2001). It is thus possible that the RhoA/Rho-kinase-mediated signaling is the key for understanding the abnormal contraction of diseased vascular smooth muscles. Rho-kinase is now recognized as a therapeutic target and its inhibitors are clinically used for treating cerebral vasospasm (Sasaki et al. 2002).

Abnormalities of the RhoA/Rho-kinase signaling system have also been suggested in preterm labor and erectile dysfunction. During pregnancy, the uterus undergoes major functional and structural remodeling. The myometrium normally remains relatively quiescent but is able to generate powerful contractions at the time of parturition. Niino et al. (1997) reported an upregulation of RhoA/Rho-kinase associated with the augmented smooth muscle contractility in the rat myometrium during pregnancy. Similar results have also been obtained in pregnant rabbit myometrium (Cario-Toumaniantz et al., 2003). Penile erection is induced by raised corpus cavernosum pressure resulting from increased blood flow into the penis, which is mediated by relaxation of the smooth muscle cells in the cavernosal arterioles and sinuses. Chitaley et al. (2001) reported that Y-27632 increases corpus cavernosum pressure in an in vivo rat model, suggesting that RhoA/Rho-kinase-mediated Ca\(^{2+}\) sensitization of corpus cavernosum smooth muscle maintains the flaccid (contracted) state of the penis. This is further supported by recent investigations (Mills et al., 2001; Chitaley et al., 2002; Wang et al., 2002; Wingard et al., 2003). It is of interest that topical application of Y-27632 to the surface of the tunica albuginea or to the glans penis and surrounding skin causes penile erection in rats (Dai et al., 2004). Thus, the RhoA/Rho-kinase signaling pathway might also provide potential targets for the development of new treatments for preterm labor and erectile dysfunction.

**RhoA-mediated Ca\(^{2+}\) sensitization in airway smooth muscle of asthmatic animal models**

Asthmatic patients have an increased contractility of airway smooth muscle (Roberts et al., 1984), which might be a major cause of airway hyperresponsiveness. Asthmatic animal models also have hyperresponsiveness of airway smooth muscle (Gavett and Wills-Karp, 1993; Lee et al., 1994). Similarly, an increased responsiveness of bronchial smooth muscle has been demonstrated in a rat model of airway hyperresponsiveness induced by repeated antigen inhalation (Misawa and Chiba, 1993; Chiba and Misawa, 1995a, b). In this animal model of airway hyperresponsiveness, the bronchial smooth muscle contraction induced by receptor agonists such as ACh, but not by high K\(^+\) depolarization, is markedly augmented (Misawa and Chiba, 1993; Chiba and Misawa, 1995a, b). Moreover, it has also been demonstrated that muscarinic receptor density and antagonist affinity of airway smooth muscle are at normal levels (Chiba and Misawa, 1995a). Thus, it is possible that the mechanisms responsible for the airway hyperresponsiveness exist, at least in part, in the downstream pathway of muscarinic receptor signaling, including agonist-mediated Ca\(^{2+}\) sensitization.

Ca\(^{2+}\) sensitization of airway smooth muscle has been reported in canine (Bremerich et al., 1997), porcine (Croxton et al., 1998) and rabbit trachea (Yoshii et al., 1999) and human bronchus (Yoshii et al., 1999; Yamagata et al., 2000). Likewise, Ca\(^{2+}\) sensitization is also inherent in rat bronchial smooth muscle (Fig. 2), as determined by permeabilized muscle strips. Since the Ca\(^{2+}\) sensitization induced by ACh is sensitive to C3 exoenzyme (Chiba et al., 1999b)
Fig. 2. Upper panel: A typical trace showing acetylcholine-induced Ca²⁺ sensitization of β-escin-permeabilized rat intrapulmonary bronchial smooth muscle. After the 10⁻⁶ M Ca²⁺ (pCa-6)-induced contraction reached plateau, acetylcholine (10⁻⁵–10⁻³ M) induced a further concentration-dependent contraction in the presence of 100 μM GTP, that is Ca²⁺ sensitization. Lower panel: Effect of Y-27632 (10⁻⁵ and 10⁻⁴ M) on the acetylcholine-induced Ca²⁺ sensitization of β-escin-permeabilized rat intrapulmonary bronchial smooth muscle. After permeabilization with β-escin (for 30 min, at room temperature) in the presence of 10 μM A23187, the muscle strips were equilibrated. Twenty min after Y-27632 or its vehicle treatment (control), Ca²⁺ (pCa-6)-induced contraction was measured. When the Ca²⁺-induced contraction reached plateau, acetylcholine-induced contractions (in the presence of GTP) were measured cumulatively, in the presence of Y-27632 or vehicle. pCa; -log[Ca²⁺]. Data represent the mean ± S.E.M. from 6 experiments. **P<0.01 vs. control by Dunnett’s multiple analysis. From Chiba et al. (2001b).

and Y-27632 (Fig. 2), the RhoA/Rho-kinase pathway is involved in the signaling. RhoA and Rho-kinases are also expressed in rat bronchial smooth muscle (Chiba et al., 1999b; 2001b; 2003). Activation of RhoA by ACh stimulation has also been demonstrated in the bronchial smooth muscle of the rat (Figs. 3 and 4) (Chiba et al., 2001a; 2004).

An increase in responsiveness of airway smooth muscle to muscarinic agonists has been demonstrated in both animal models of airway hyperresponsiveness (Gavett and Wills-Karp, 1993; Lee et al., 1994; Chiba and Misawa, 1995; Chiba et al., 2000) and asthmatic patients (Roberts et al., 1984), although no change in the levels of plasma membrane receptors was observed (Gavett and Wills-Karp, 1993; Lee et al., 1994; Chiba and Misawa, 1995). Moreover, the agonist-induced increase in cytosolic Ca²⁺ level has been reported to be normal level even in
Fig. 3. Time course of acetylcholine (ACh)-induced translocation of RhoA in bronchial smooth muscle of nonsensitized normal rats. The isolated main and intrapulmonary bronchi were stimulated by ACh (1 mM), and homogenized to prepare cytosolic and membrane fractions after stopping the reaction by liquid nitrogen at the time indicated. Western blotting was performed by using these fractions both on RhoA and β-actin in the identical transferred membrane. a) Representative western blots of membrane RhoA (21 kD) and β-actin. b) Relevance of the time courses of ACh (1 mM)-induced increase in membrane RhoA and decrease in cytosolic RhoA. c) Relevance of the time courses of ACh (1 mM)-induced contraction and translocation of RhoA. Values are means ± S.E.M. from 5 experiments in duplicate. *P<0.05 and **P<0.01 vs. respective Time 0 (no stimulation). From Chiba et al. (2001a).

hyperresponsive bronchial smooth muscle (Jiang et al., 1995; Chiba et al., 1999a), reminding us that the Ca^{2+} sensitization induced by agonist stimulation might be elevated in airway hyperresponsiveness. Indeed, an augmented ACh-induced, RhoA-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in a rat airway hyperresponsiveness model has been suggested by the following findings (Chiba et al., 1999b): 1) the ACh-induced Ca^{2+} sensitizing effect, measured in permeabilized muscle strips under a constant Ca^{2+} concentration, was augmented in the airway hyperresponsive rats (Fig. 5), 2) this Ca^{2+} sensitizing effect was
Fig. 4. Typical confocal immunofluorescent images of RhoA and α-actin in freshly isolated rat bronchial smooth muscle cells at rest (left panels) and stimulated by 10^{-3} M acetylcholine (ACh; for 10 min; right panels). A and E; transmitted light images, B and F; RhoA immunostainings indicated by green fluorescence of Alexa Fluor 488, C and G; overlays of A and E with B and F, respectively, and D and H; α-actin immunostainings indicated by red fluorescence of Alexa Fluor 546. Original magnification: × 600. From Chiba et al. (2004).

blocked by pretreatment with C3 exoenzyme, and 3) the RhoA protein expression in the bronchial smooth muscle was markedly increased in the airway hyperresponsive rats (Fig. 6). The same findings have also been observed in a murine model of antigen-induced airway hyperresponsiveness (Chiba et al., submitted data). It is thus possible that RhoA/Rho-kinase-mediated signaling is the key to the understanding of the augmented bronchial smooth muscle contraction in asthma. If RhoA proteins are activated by receptors other than muscarinic receptors, this might account for the 'non-specific' airway hyperresponsiveness, which is a common feature of allergic asthmatics. Selective inhibition of this pathway may be an effective asthma treatment as Y-27632 can relax the bronchospasm induced by contractile agonists and antigens in vivo (Iizuka et al., 2000; Tokuyama et al., 2002). Moreover, Y-27632 inhibits the
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Fig. 5. Comparison of ACh-induced Ca\(^{2+}\) sensitization in β-escin-permeabilized intrapulmonary bronchial smooth muscle from normal rats (Control; n=6) and the antigen-induced airway hyperresponsive rats (AHR; n=5). The contractile responses induced by 10\(^{-6}\) M Ca\(^{2+}\) in the presence (closed column) and absence (open column) of 100 μM ACh and 100 μM GTP are expressed as % of maximal contraction induced by 10\(^{-5}\) M Ca\(^{2+}\). Data represent the mean ± S.E.M. *P<0.05 and **P<0.01 vs. respective Ca\(^{2+}\)-induced contraction in the absence of ACh and GTP. ***P<0.01 vs. ACh-induced Ca\(^{2+}\) sensitization in the control group. From Chiba et al. (1999b).

Fig. 6. The levels of RhoA protein in intrapulmonary bronchi from normal rats (Control) and the antigen-induced airway hyperresponsive rats (AHR). Left panel: typical immunoblot. Lane 1: Control, Lane 2: AHR, Markers; protein molecular weight markers, and GAPDH; glyceraldehyde-3-phosphate dehydrogenase as a tissue marker. The bands were analyzed by a densitometer and normalized by loading protein, and the data are summarized as shown in the right panel. The data represent the mean ± S.E.M. from 4 individual experiments, respectively. **P<0.01 vs. Control. From Chiba et al. (1999b).

murine airway hyperresponsiveness induced by antigen challenge (Hashimoto et al., 2002).

Conclusion

As described in this brief review, there is increasing evidence that upregulation of RhoA/ Rho-kinase signaling is widely involved in the enhanced contraction of diseased smooth muscle
including bronchial smooth muscle in asthma. Thus, the RhoA/Rho-kinase pathway might be a potential target for the development of new treatments for asthma, especially for airway hyperresponsiveness.

References
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