Published in final edited form as:

Can J Physiol Pharmacol. 2015 February ; 93(2): 129–136. doi:10.1139/cjpp-2014-0388.

A novel role for RhoA GTPase in the regulation of airway smooth muscle contraction

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Abstract

Recent studies have demonstrated a novel molecular mechanism for the regulation of airway smooth muscle (ASM) contraction by RhoA GTPase. In ASM tissues both MLC phosphorylation and actin polymerization are required for active tension generation. RhoA inactivation dramatically suppresses agonist induced tension development and completely inhibits agonist induced actin polymerization, but only slightly reduces MLC phosphorylation. The inhibition of MLC phosphatase does not reverse the effects of RhoA inactivation on contraction or actin polymerization. Thus, RhoA regulates ASM contraction through its effects on actin polymerization rather than MLC phosphorylation. Contractile stimulation of ASM induces the recruitment and assembly of paxillin, vinculin and FAK into membrane adhesion complexes (adhesomes) that regulate actin polymerization by catalyzing the activation of cdc42 GTPase by the GIT-Pak PIX complex. Cdc42 is a necessary and specific activator of the actin filament nucleation activator, N-WASp. The recruitment and activation of paxillin, vinculin and FAK is prevented by RhoA inactivation, thus preventing cdc42 and N-WASp activation. We conclude that RhoA regulates ASM contraction by catalyzing the assembly and activation of membrane adhesome signaling modules that regulate actin polymerization, and that RhoA mediated assembly of adhesome complexes is a fundamental step in the signal transduction process in response to a contractile agonist.

Keywords

Actin polymerization; Myosin light chain phosphorylation; Adhesion junction; vinculin; paxillin; cdc42

The small GTPase RhoA is widely recognized as an important regulator of contractility and shortening in smooth muscle tissues in response to stimulation with contractile agonists (Somlyo and Somlyo 2003;Puetz *et al.* 2009). RhoA can regulate phosphorylation of the 20KD regulatory light chain of myosin II by inhibiting of the catalytic activity of MLC phosphatase, which leads to an increase in MLC phosphorylation. RhoA activates Rho kinase (ROCK), which inhibits MLC phosphatase activity by phosphorylating its regulatory subunit MYPT1 or by phosphorylating the inhibitory peptide of MLC phosphatase CPI-17

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(Somlyo and Somlyo 2003;Puetz, Lubomirov, and Pfitzer 2009;Ito *et al.* 2004). In vascular smooth muscle, the stimulation of MLC phosphorylation resulting from the RhoA-mediated inhibition of MLC phosphatase is widely regarded as an important mechanism for the regulation of contraction in response to agonist stimulation (Somlyo and Somlyo 2003;Puetz, Lubomirov, and Pfitzer 2009;Ito, Nakano, Erdodi, and Hartshorne 2004).

RhoA is also recognized as a key regulator of actin cytoskeletal dynamics and organization in most cell types. In airway smooth muscle and a number of other smooth muscle tissue types, actin polymerization plays a critical role in the regulation of active tension development (Walsh and Cole 2013;Yamin and Morgan 2012;Zhang and Gunst 2008a;Gunst and Zhang 2008). Contractile stimulation triggers the polymerization of a small pool of actin and catalyzes the dynamic reorganization of the actin cytoskeletal system. Agonist induced force development is markedly reduced when actin polymerization is inhibited using either pharmacologic or molecular interventions. Studies of airway smooth muscle using a variety of molecular interventions to inhibit upstream cytoskeletal processes required for actin polymerization uniformly find that the polymerization of a small pool of actin is necessary for agonist-induced tension generation (Zhang *et al.* 2012;Zhang and Gunst 2008b;Zhang *et al.* 2010;Gunst and Zhang 2008;Tang*et al.* 2003 ;Opazo Saez A. *et al.* 2004;Tang and Gunst 2001).

Mechanisms for the regulation of actin polymerization and its role in regulating activation of the contractile apparatus and tension development have been analyzed extensively in airway smooth muscle tissues (Herrera *et al.* 2004;Zhang, Du, and Gunst 2010;Gunst and Zhang 2008;Zhang *et al.* 2005;Dowell *et al.* 2005;Halayko and Stelmack 2005;An *et al.* 2002;Gunst *et al.* 2003;Zhang and Gunst 2008a). The inhibition of either actin polymerization or of MLC phosphorylation in airway smooth muscle tissues depresses tension development in response to stimulation with ACh (Zhang, Du, and Gunst 2010) (Fig. 1A). However, the inhibition of actin polymerization using either molecular or pharmacologic approaches has little or no effect on the increase in MLC phosphorylation in response to agonist stimulation (Fig. 1B) (Gunst and Zhang 2008;Zhang, Du, and Gunst 2010;Zhang, Wu, Du, Tang, and Gunst 2005;Mehta and Gunst 1999). Conversely, agonist induced actin polymerization is not suppressed when MLC phosphorylation is inhibited (Fig. 1C)(Zhang, Du, and Gunst 2010). Thus, in airway smooth muscle, actin polymerization and smooth muscle MLC phosphorylation appear to be distinct cytoskeletal processes that are independently regulated during contractile stimulation.

Studies of airway smooth muscle also demonstrate that the molecular processes that catalyze actin polymerization occur at the cell cortex, suggesting that the actin filaments polymerized in response to stimulation with contractile agonists are localized to a cortical network of filaments (Zhang, Huang, and Gunst 2012;Zhang and Gunst 2008a;Gunst and Zhang 2008;Opazo Saez A., Zhang, Wu, Turner, Tang, and Gunst 2004). While the function of this pool of actin in smooth muscle has not been established, it may stabilize the attachment of actin filaments to cell membrane junctions; thus strengthening these sites for the transmission of force between the contractile apparatus and the extracellular matrix. Cortical actin filaments may also provide a scaffold for the support and activation of signaling

modules critical for the transduction of signals from extracellular stimuli to the cytoskeleton (Gunst and Zhang 2008;Zhang, Huang, and Gunst 2012).

Multiple studies have documented a role for RhoA GTPase in regulating actin polymerization in airway smooth muscle (Hirshman and Emala 1999;Iizuka *et al.* 1999;Lesh *et al.* 2001;Togashi *et al.* 1998;Lesh, Emala, Lee, Zhu, Panettieri, and Hirshman 2001); thus RhoA activation might potentially affect airway smooth muscle tension development by modulating either actin polymerization or MLC phosphorylation. We evaluated the relative contributions of RhoA in the regulation of myosin light chain phosphorylation and actin polymerization during airway smooth muscle contraction and tension development. RhoA was inhibited in tracheal smooth muscle tissues by expressing inactive RhoA mutant protein (RhoA T19N) and by treating the tissues with C3 exoenzyme (Zhang, Du, and Gunst 2010). Both of these treatments were confirmed to inhibit the activity of endogenous RhoA in the intact tracheal smooth muscle tissues. When RhoA activity was inhibited, contractile tension development in response to stimulation of the tissues with ACh was reduced by approximately 60% and ACh-induced actin polymerization was completely inhibited; however, RhoA inhibition has only a small effect on the ACh-induced increase in MLC phosphorylation (Zhang, Du, and Gunst 2010) (Fig. 2). The RhoA mediated inhibition of myosin light chain phosphatase contributed slightly to the ACh-induced increase in MLC phosphorylation; however this effect was small in the airway smooth muscle tissues and did not contribute significantly to overall tension development. The inhibition of MLC phosphatase activity using calyculin A did not affect the reduction in actin polymerization or tension development caused by RhoA inactivation (Zhang, Du, and Gunst 2010) (Fig. 2). Thus, while RhoA-mediated processes are clearly important for the regulation of force generation in airway smooth muscle tissues during muscarinic stimulation, the evidence shows that the primary effect of RhoA is on the regulation of pathways that catalyze agonist induced actin polymerization. RhoA-mediated effects on ACh-induced MLC phosphorylation have little impact on airway smooth muscle contractility.

Agonist-induced actin polymerization is mediated by the actin nucleation promoting protein, neuronal Wiskott-Aldrich syndrome protein (N-WASP) in airway smooth muscle tissues (Zhang, Wu, Du, Tang, and Gunst 2005). N-WASP undergoes a change in conformation during its activation that enables it to bind to the actin-related protein complex (Arp2/3 complex). The Arp2/3 complex creates a template for actin polymerization that facilitates the addition of monomeric actin (G-actin) to existing F-actin filaments (Rohatgi *et al.* 1999;Mullins 2000;Higgs and Pollard 2001). N-WASp activation is directly and specifically regulated by the binding of the small GTPase cdc42 to its CRIB (Cdc42- and Rac-interactive binding) domain (Rohatgi *et al.* 2000;Rohatgi, Ma, Miki, Lopez, Kirchhausen, Takenawa, and Kirschner 1999;Higgs and Pollard 2000;Higgs and Pollard 2001;Carlier *et al.* 1999). Neither Rac nor Rho GTPases can bind directly to WASP family proteins, thus RhoA cannot directly regulate N-WASp activity (Suzuki *et al.*2000;Li *et al.* 1999).

In airwaysmooth muscle tissues; cdc42 activation is necessary for N-WASp activation, actin polymerization and active tension development (Tang and Gunst 2004). To determine whether RhoA is an upstream regulator of cdc42 and N-WASp activation, we evaluated its role in regulating cdc42 activation, N-WASp phosphorylation, and the coupling of N-WASP

to the Arp2/3 complex (Zhang, Huang, and Gunst 2012). N-WASP and Arp2/3 complex coupling was assessed using proximity ligation assays (PLA) in freshly dissociated smooth muscle cells and by co-immunoprecipitation assays in airway smooth muscle tissues after RhoA activity had been inhibited (Zhang, Huang, and Gunst 2012) (Fig. 3). The phosphorylation of N-WASP on Tyr 256, an indicator of its activation, was assessed by immunoblot. RhoA inactivation was found to inhibit N-WASp activation and its coupling to the Arp2/3 complex in airway smooth muscle tissues and freshly dissociated cells (Fig. 3A-C). The ACh-induced activation of cdc42 was also inhibited by the inactivation of RhoA (Zhang, Huang, and Gunst 2012)(Fig. 3D). As RhoA cannot directly modulate N-WASp activation, we concluded that it must affect cdc42 and N-WASP activation by regulating upstream cytoskeletal signaling processes that mediate their activation.

Macromolecular protein complexes at integrin adhesion junctions (adhesomes) link extracellular matrix proteins to the actin cytoskeleton and play a critical role in the transduction of extracellular signals to pathways that regulate actin polymerization during airway smooth muscle contraction (Zhang and Gunst 2008a;Zhang and Gunst 2008b). The activation of airway smooth muscle results in the rapid recruitment and assembly of multiple proteins into adhesome complexes (Opazo Saez A., Zhang, Wu, Turner, Tang, and Gunst 2004;Zhang, Wu, Du, Tang, and Gunst 2005;Zhang *et al.* 2007;Zhang, Huang, and Gunst 2012;Zhang and Gunst 2006;Huang *et al.* 2011). The time course of adhesome protein recruitment is rapid and can be detected in dissociated cells andintact airway smooth muscle tissues by immunofluorescence techniquesand in living or fixed dissociated cells expressing GFP constructs of adhesome proteins (Huang, Zhang, and Gunst 2011;Zhang, Wu, Du, Tang, and Gunst 2005;Zhang, Wu, Wu, and Gunst 2007;Zhang and Gunst 2006)(Fig. 4). Among the proteins recruited are paxillin, focal adhesion kinase and vinculin, which undergo phosphorylation and activation after being recruited to membrane adhesomes in response to agonist stimulation (Huang, Zhang, and Gunst 2011;Huang *et al.* 2014;Opazo Saez A., Zhang, Wu, Turner, Tang, and Gunst 2004;Wang *et al.* 1996). The recruitment and phosphorylation of all of these proteins is required for agonist-stimulated actin polymerization and contraction in airway smooth muscle (Huang, Zhang, and Gunst 2011;Huang, Day, and Gunst 2014;Opazo Saez A., Zhang, Wu, Turner, Tang, and Gunst 2004;Tang, Turner, and Gunst 2003;Tang *et al.* 2005;Zhang, Huang, and Gunst 2012).

Vinculin binds to paxillin and the two proteins are maintained within a stable inactive complex in the cytoplasm of airway smooth muscle cells (Huang, Day, and Gunst 2014) (Fig. 5). Contractile stimulation of airway smooth muscle stimulates recruitment of the inactive paxillin/vinculin complex to the membrane of airway smooth muscle cells; where vinculin subsequently undergoes phosphorylation at tyrosine 1065 (Huang, Zhang, and Gunst 2011). The phosphorylation of vinculin at this site regulates its conversion to an open ligand binding conformation that enables it to bind to talin and F actin as well as to other proteins involved in cytoskeletal dynamics(Huang, Zhang, and Gunst 2011;Huang, Day, and G unst 2014).

Paxillin also undergoes tyrosine phosphorylation after its recruitment to membrane adhesion complexes in airway smooth muscle. Paxillin is a substrate for FAK (Bellis *et al.* 1995;Schaller and Parsons 1995), and FAK regulates the tyrosine phosphorylation of

paxillin in airway smooth muscle (Tang and Gunst 2001). The recruitment of FAK to adhesome complexes and its activation is required for the tyrosine phosphorylation of paxillin in airway smooth muscle (Zhang, Huang, and Gunst 2012).

After paxillin is recruited to the membrane and undergoes tyrosine phosphorylation, it interacts with protein complexes that contain guanine nucleotide exchange factors (GEFs) that can regulate the activity cdc42 (Zhang, Huang, and Gunst 2012) (Fig. 5). DOCK (Dedicator of Cytokinesis) family proteins have GEF activity towards cdc42 (Sinha and Yang 2008). DOCK180 interacts with Crk, a paxillin-binding adaptor protein that binds to tyrosine phosphorylated paxillin in airway smooth muscle (Petit *et al.* 2000;Tang, Zhang, and Gunst 2005;Zhang, Huang, and Gunst 2012). The PIX (PAK-interacting exchange factor) proteins have GEF activity towards cdc42 and bind to p21-activated kinase (PAK) as well as to GIT proteins (GIT1, G-protein receptor kinase interacting tyrosine phosphorylated) (Rosenberger *et al.* 2003;Sinha and Yang 2008) and GIT2, also called paxillin kinase linker or PKL). GIT proteins are ArfGAPS that also regulate cdc42 (Lynch *et al.* 2006). In airway smooth muscle cells, the binding of GIT molecules to the LD4 domain of paxillin regulates the localization of GIT-Pak-PIX complexes to membrane adhesion sites (Zhang, Huang, and Gunst 2012) (Fig. 5,6). After coupling to paxillin, the PIX-Pak-GIT signaling complex mediates the activation cdc42, which in turn regulates the activation of N-WASp and the Arp 2/3 complex and the polymerization of cortical actin (Zhang, Huang, and Gunst 2012)(Fig. 6.) In airway smooth muscle, the localization and assembly of these complexes of adhesome proteins is a necessary prerequisite to the process of actin polymerization that occurs during contractile stimulation, and the activation of upstream signaling scaffolding/proteins such as vinculin and paxillin depend on their recruitment and localization in membrane adhesion complexes (Huang, Day, and Gunst 2014;Opazo Saez A., Zhang, Wu, Turner, Tang, and Gunst 2004;Zhang, Huang, and Gunst 2012).

Studies of focal adhesion formation during substrate adhesion in cultured cell lines have documented an important role for RhoA GTPase in the assembly and maturation of membrane adhesion complexes (Burridge and Wennerberg 2004;Chrzanowska-Wodnicka and Burridge 1996;Pasapera *et al.* 2010). In these cell lines, RhoA activation in response to a stimulus induces phosphorylation of the light chain of non-muscle myosin II, and this is prerequisite to the formation of focal adhesions and stress fibers (Vicente-Manzanares *et al.* 2009). As adhesome assembly is clearly an essential process in the contractile activation of airway smooth muscle, we evaluated the possibility that RhoA might regulate actin polymerization and contraction by mediating the assembly and activation of critical signaling cascades within membrane adhesion junctions.

We evaluated the effects of RhoA inactivation on adhesome complex assembly in dissociated airway smooth muscle cells using PLA to assess protein interactions that occurred in response to contractile stimulation, and by co-immunoprecipitation analysis of tracheal smooth muscle tissue extracts(Figs. 5,6). The membrane recruitment of paxillin-vinculin complexes and the tyrosine 1065 phosphorylation of vinculin and paxillin were dependent on the activation of RhoA (Huang, Zhang, and Gunst 2011). RhoA inactivation also prevented the membrane recruitment and activation of focal adhesion kinase and its interaction with paxillin at adhesome complexes (Zhang, Huang, and Gunst 2012) (Fig 5B,

Fig. 6). Furthermore, the association of the GIT-Pak-PIX complex with paxillin, which is required for activation of its GEF activity toward cdc42, was also inhibited by RhoA inactivation (Figs.5, 6). These observations demonstrate that RhoA is an upstream regulator of adhesome complex assembly in airway smooth muscle, and that the inactivation of RhoA prevents the assembly and activation of critical signaling cascades that regulateactin cytoskeletal dynamicsand actin polymerization.

In summary, these studies suggest a novel mechanism for the regulation of smooth muscle contraction by the small GTPase RhoA (Fig. 7). The effect of RhoA on airway smooth muscle contractility is clearly due to its role in the regulation of actin dynamics (Zhang, Du, and Gunst 2010;Zhang, Huang, and Gunst 2012). RhoA regulates actin dynamics by catalyzing the assembly of adhesome signaling complexes at the airway smooth muscle membrane that include the actin regulatory proteins paxillin, vinculin and FAK. These proteins undergo activation in response to a contractile stimulus only after recruitment and assembly into membrane associated adhesome complexes. After activated paxillin associates with the GIT-Pak-PIX signaling module, the GEF activity of PIX contributes to the activation of cdc42, which is essential for N-WASP activation and actin polymerization. By regulating the association of paxillin with the GIT-Pak-PIX complex, RhoA regulates the activation cdc42, leading to activation of the actin polymerization catalysts N-WASp and the Arp 2/3 complex and the subsequent polymerization of cortical actin.

These findings suggest that the RhoA-mediated assembly of adhesome signaling complexes is an essential upstream step in the process of excitation-contraction coupling and tension development in airway smooth muscle tissues during agonist-induced contractile activation. Furthermore, the catalytic function of RhoA on adhesome complex assembly appears to be the primary mechanism by which RhoA regulates the contractility of airway smooth muscle during agonist stimulation. The evaluation of other smooth muscle tissues types will provide evidence as to whether Rho-mediated adhesome assembly also plays an important role in regulating their physiologic functions.

Acknowledgments

This work was supported by National Heart, Lung, and Blood Institute Grants HL029289, HL074099; HL109628 and the American Lung Association.

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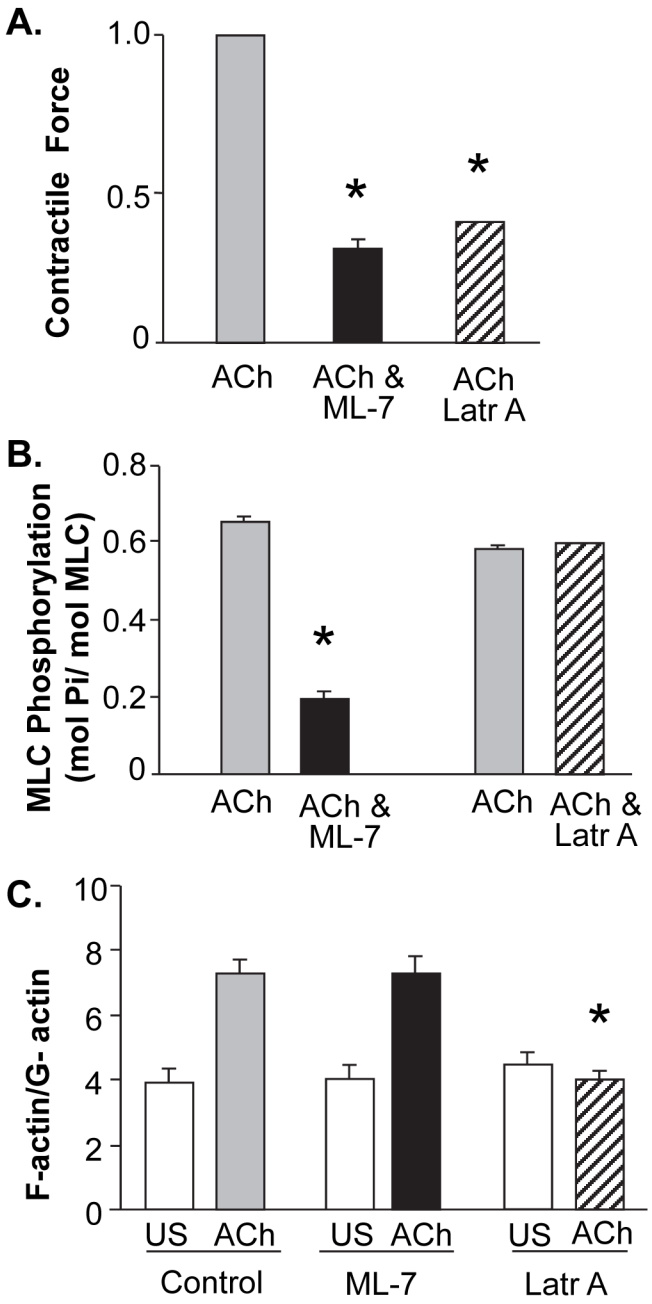

Figure 1. Inhibition of either MLC phosphorylation or actin polymerization prevents ACh induced tension development in tracheal smooth muscle tissues

Contractile tension, actin polymerization and myosin light chain phosphorylation were measured in tracheal smooth muscle tissues that were treated with 30 μM ML-7 to inhibit MLC kinase or 1 μM Latrunculin A (Latr A) to inhibit actin polymerization. **A.** Treatment with ML-7 or Latr A significantly depresses ACh stimulated tension development *(n=8).* **B.** ACh stimulated MLC phosphorylation is significantly inhibited by ML-7, but not by Latr A *(n=5).* **C.** The increase in actin polymerization in response to ACh stimulation is significantly inhibited by Latr A but not by ML-7 *(n=6).* *Significant difference between

ML-7 or Latr A treated tissues and untreated tissues ($P < 0.05$). Values are means ± SE. (Modified from (Zhang, Du, and Gunst 2010) and from (Mehta and Gunst 1999).

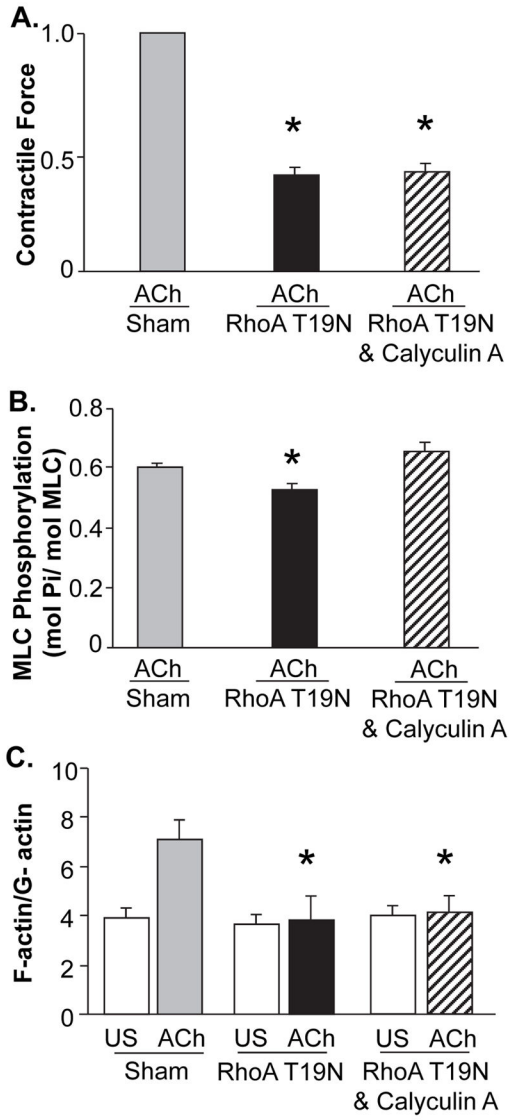

Figure 2. Inhibition of RhoA activity suppresses ACh induced tension development and actin polymerization, but it causes only a small reduction in ACh induced myosin light chain (MLC) phosphorylation

RhoA activation was inhibited by the expression of RhoA T19N in tracheal smooth muscle tissues. **A.** RhoA inhibition significantly depressed ACh-stimulated contractile force, and the depression of contraction was not reversed by treatment with the MLC phosphatase inhibitor, calyculin A ($n = 6$). **B.** RhoA inhibition caused a small inhibition of ACh induced MLC phosphorylation. Treatment with calyculin A prevented the decrease in MLC phosphorylation caused by RhoA inhibition ($n = 9$). **C.** RhoA inhibition inhibited the increase in actin polymerization in response to ACh stimulation. Treatment with calyculin A had no effect on the inhibition of actin polymerization caused by RhoA inhibition ($n = 6$). *Significant difference between sham-treated tissues and tissues treated with RhoA T19N or RhoA T19N plus calyculin A ($P < 0.05$). Values are means ± SE. (Modified from (Zhang, Du, and Gunst 2010).

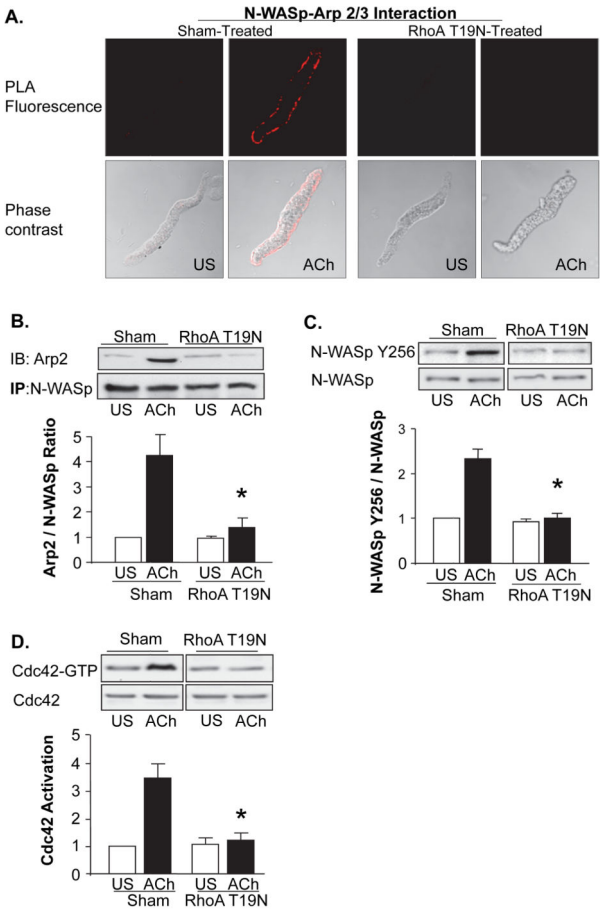

Figure 3. RhoA inactivation inhibits ACh induced N-WASp activation in airway smooth muscle tissues

A. *In situ* proximity ligation assay (PLA) in freshly dissociated differentiated canine tracheal smooth muscle cells stimulated with ACh for 5 min. PLA yields a fluorescent signal when the target proteins are localized within 40 nm of each other. In sham-treated tissues, stimulation with ACh induces the interaction of N-WASp and Arp2 at the membrane. The inactivation of RhoA by the expression of RhoA T19N inhibits the ACh stimulated interaction between N-WASp and the Arp2/3 complex. **B:** ACh induced a significant increase in the co-immunoprecipitation of Arp2 with N-WASp in sham-treated tissues but not in RhoA T19N treated tissues (n =4). *IP* : immunoprecipitate; *IB*, immunoblot. **C:** N-WASp tyrosine 256 phosphorylation, an indicator of N-WASp activation, measured by immunoblot in extracts of muscle tissues. ACh induced N-WASp phosphorylation was significantly inhibited in tissues treated with RhoA T19N (n = 5). **D:** RhoA inactivation inhibits the ACh-induced activation of cdc42 in tracheal smooth muscle tissues. Activated cdc42 (cdc42-GTP) was affinity-precipitated from muscle extracts and the amount of activated cdc42 precipitated from each extract was quantified by immunoblot. Activated cdc42 was significantly higher in extracts from ACh stimulated sham-treated tissues than from ACh stimulated tissues expressing RhoA T19N *(n = 8).* All values are means ± S.E. *, significantly different, $p < 0.05$. (Modified from (Zhang, Huang, and Gunst 2012).

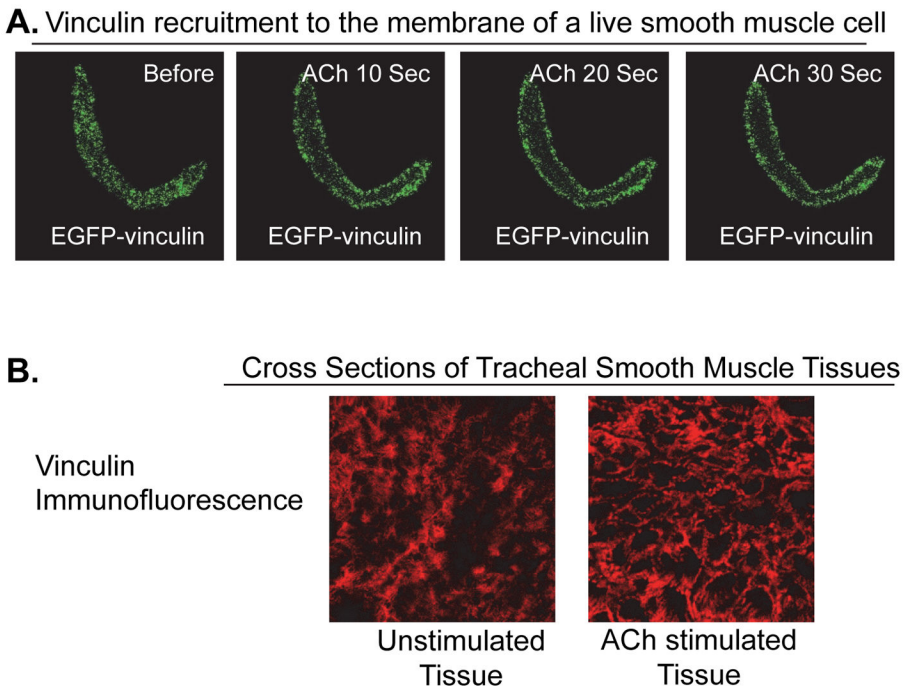

Figure 4.

A. Contractile stimulation with ACh induces the rapid recruitment of recombinant EGFP-vinculin to the membrane of a live airway smooth muscle cell that was enzymatically dissociated from a tracheal smooth muscle tissue expressing EGFP-vinculin. Frames are successive images of the same cell. The localization of EGFP-vinculin was monitored by confocal microscopy during stimulation with 10^{-4} MACh. (Modified from (Huang, Zhang, and Gunst 2011). **B:** Vinculin is diffusively distributed throughout the cytoplasm in the unstimulated tracheal smooth muscle tissue but appears localized to the cell membrane in the ACh-stimulated tissue. Tracheal smooth muscle tissues were stimulated with $10{-}^{4}$ MACh or left unstimulated and quickly frozen and fixed. Tissue cross-sections were analyzed for vinculin immunofluorescence. (Unpublished data).

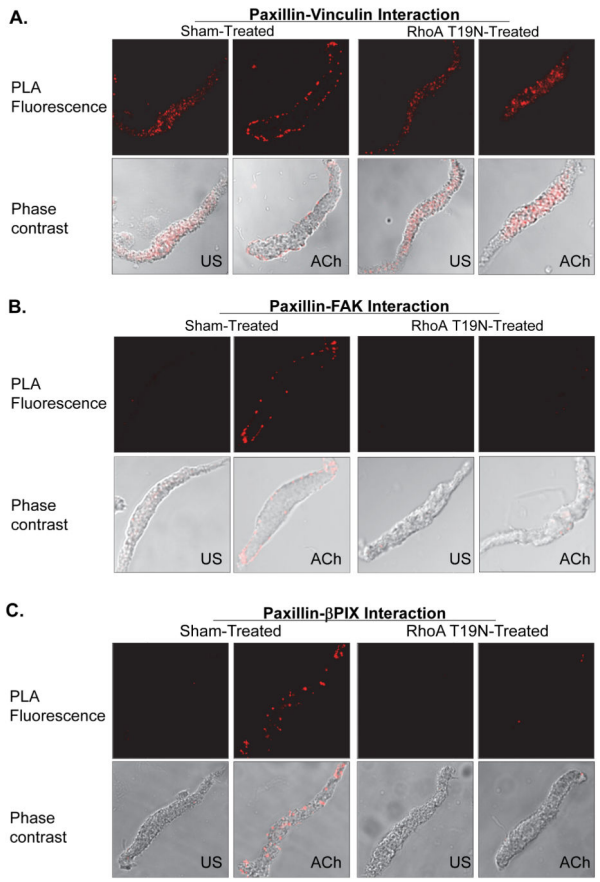

Figure 5. RhoA regulates the recruitment of adhesome proteins to the cell membrane in response to contractile stimulation

Proximity ligation assays (PLA) were used to evaluate protein interactions in cells dissociated from tracheal smooth muscle tissues with or without RhoA T19N to inhibit RhoA activation. Fluorescence spots indicate target proteins are localized within 40 nm of each other. **A**. PLA was used to evaluate the interaction of paxillin and vinculin in freshly dissociated cells stimulated with 10^{-4} M ACh for 5 min or left unstimulated. In sham treated tissues, fluorescent spots were observed throughout the cytoplasm of unstimulated cells but primarily at the cell membrane of ACh stimulated cells. In cells from RhoA T19N-treated tissues, PLA spots were distributed throughout the cytoplasm of both unstimulated and ACh-stimulated cells. **B.** PLA reveals interaction between paxillin and FAK at the membrane of muscle cells after contractile stimulation with ACh, whereas interactions between paxillin and FAK are not observed in unstimulated muscle cells. RhoA T19N inhibited the formation of paxillin/FAK complexes in response to ACh stimulation. **C.** β-PIX and paxillin interact at the membrane of smooth muscle cells after contractile stimulation, but they do not interact in unstimulated muscle cells. Expression of RhoA T19N inhibited the formation of paxillin/β-PIX complexes. Modified from (Zhang, Du, and Gunst 2010)and (Zhang, Huang, and Gunst 2012).

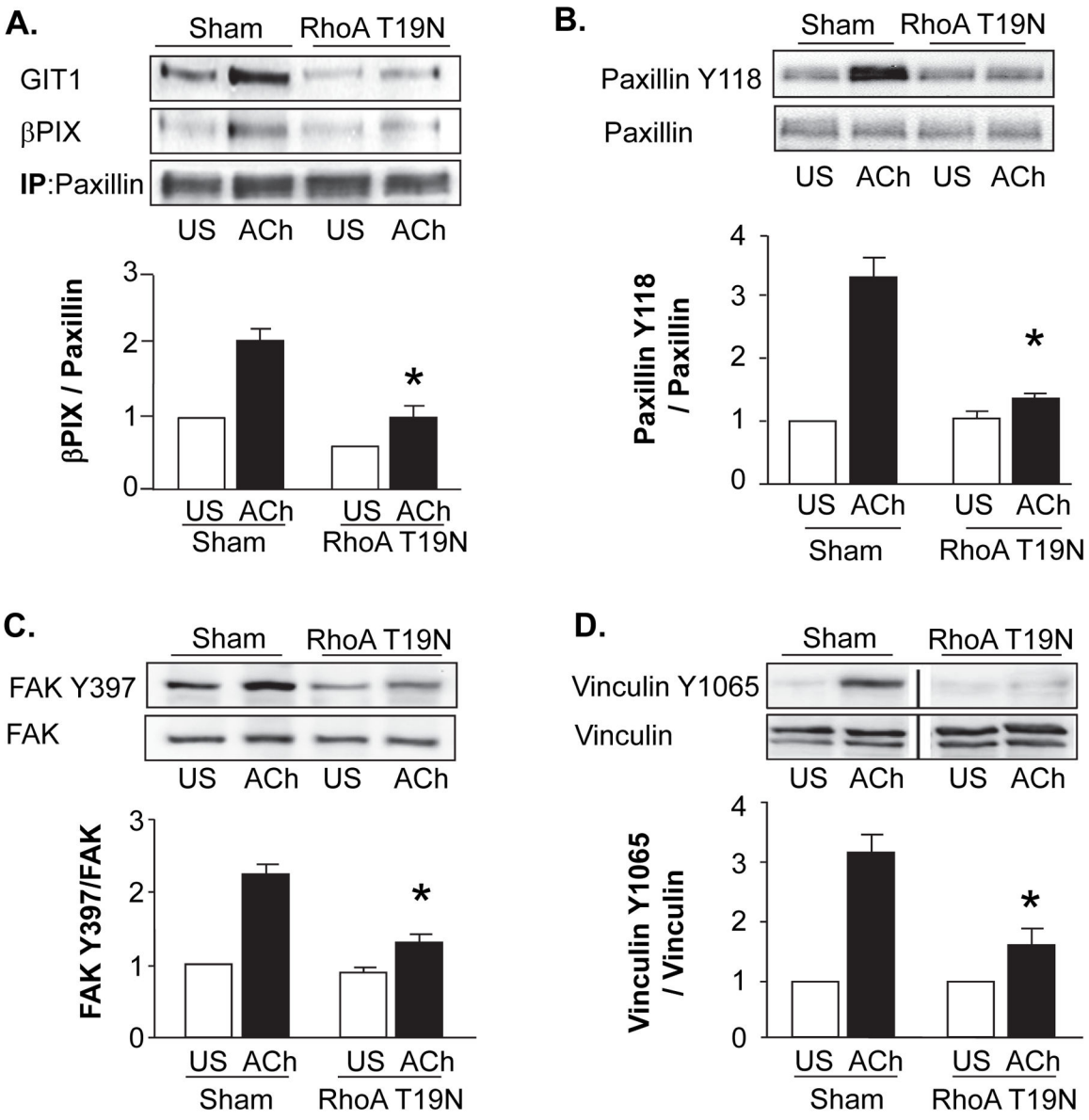

Figure 6. RhoA regulates interaction of paxillin with the GIT-Pak-PIX complex and the phosphorylation of paxillin, FAK and vinculin

A. Co-immunoprecipitation of GIT1 and β-PIX with paxillin increased in ACh-stimulated tissues and was significantly inhibited in tissues expressing RhoA T19N (n = 4).**B.** Paxillin phosphorylation at tyrosine 118 was significantly increased by ACh stimulation in sham-treated tissues but not RhoA T19N-inhibited tissues (n = 10). **C.** FAK phosphorylation at tyrosine 397 was significantly increased by ACh in sham-treated tissues but not in RhoA T19N-inhibited tissues (n = 11). **D,** Vinculin phosphorylation at tyrosine 1065 was significantly increased by ACh stimulation in sham-treated tissues but not in RhoA T19N inhibited tissues (n= 5). All values are means ± S.E. *, significantly different, $p < 0.05$. (Modified from (Zhang, Huang, and Gunst 2012)).

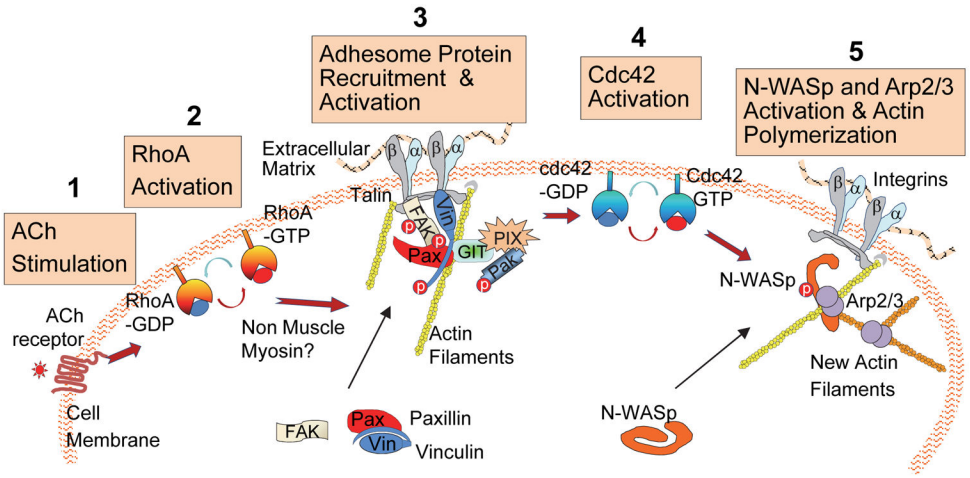

Figure 7. Model for molecular mechanism by which RhoA regulates actin polymerization and contraction in airway smooth muscle

1–2, ACh stimulation activates RhoA. **3,** Activation of RhoA induces the independent recruitment of paxillin-vinculin complexes and FAK to cell adhesomes. FAK and paxillin interact at the membrane and activated FAK catalyzes the phosphorylation of paxillin, which is bound to activated vinculin. P hosphorylation of paxillin facilitates the formation of a complex containing paxillin, GIT, PAK and PIX. **4.** The GIT-Pak-PIX complex induces the activation of cdc42 through the GEF activity of PIX. **5.** cdc42 activation catalyzes the activation of N-WASp, which interacts with the Arp2/3 complex to induce actin polymerization in the cortical region of the smooth muscle cell. This enables the transmission of tension generated by the smooth muscle actomyosin crossbridge cycling within the contractile apparatus.