Cellular regulation of basal tone in internal anal sphincter smooth muscle by RhoA/ROCK

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Patel CA, Rattan S. Cellular regulation of basal tone in internal anal sphincter smooth muscle by RhoA/ROCK. Am J Physiol Gastrointest Liver Physiol 292: G1747–G1756, 2007. First published March 22, 2007; doi:10.1152/ajpgi.00438.2006.—Sustained contractions of smooth muscle cells (SMC) maintain basal tone in the internal anal sphincter (IAS). To examine the molecular bases for the myogenic tone in the IAS, the present studies focused on the role of RhoA/ROCK in the SMC isolated from the IAS vs. the adjoining phasic tissues of the rectal smooth muscle (RSM) and anococcygeus smooth muscle (ASM) of rat. We also compared cellular distribution of RhoA/ROCK, levels of RhoA-GTP, RhoA-Rho guanine nucleotide dissociation inhibitor (GDI) complex formation, levels of pThr696-MYPT1, and SMC relaxation caused by RhoA inhibition. Levels of RhoA/ROCK were higher at the cell membrane in the IAS SMC compared with those from the RSM and ASM. C3 exoenzyme (RhoA inhibitor) and Y 27632 (ROCK inhibitor) caused a concentration-dependent relaxation of the IAS SMC. In addition, active ROCK-II (primary isoform of ROCK in SMC) caused further shortening in the IAS SMC. In addition, we performed Western immunoblottings to determine the levels of RhoA, ROCK-II, phospho-MYPT1, and phospho-MLC20 in the IAS vs. RSM and ASM SMC. However, the trend was the reverse with the levels of inactive RhoA (GDP-RhoA-RhoGDI complex) and MYPT1. We conclude that RhoA/ROCK play a critical role in maintenance of spontaneous tone in the IAS SMC via inhibition of myosin light chain phosphatase.

Unlike phasic smooth muscles, tonic smooth muscle of the internal anal sphincter (IAS) and other sphincters remains contracted at rest (1, 3, 5, 12, 25). The IAS tone is primarily maintained by the properties of smooth muscle proper. The myogenic tone in the IAS plays a pivotal role in anorectal continence (25, 29). The molecular mechanisms for the basal tone in the IAS are not well understood.


MLCP causes dephosphorylation of MLC20. MLCP present in the smooth muscle is a heterotrimeric enzyme that consists of a catalytic 38-kDa type 1 protein phosphatase δ isoform (PP1cδ) and two regulatory subunits, a 110-kDa myosin phosphatase target subunit 1 (MYPT1) and a 20-kDa small regulatory subunit (M20). ROCK-mediated phosphorylation of MYPT1 at the threonine-696 (Thr⁶⁹⁶) residue is associated with inhibition of MLCP and force (14, 30). ROCK also inhibits the catalytic subunit of MLCP via phosphorylation of CPI-17, an endogenous inhibitory protein of the catalytic subunit of MLCP. Phosphorylated CPI-17 at threonine-38 (Thr³⁸) residue is ~7,000-fold more potent than nonphosphorylated CPI-17 (7). Both ROCK and protein kinase C (PKC) are capable of phosphorylating CPI-17 at Thr³⁸ residue (7, 16, 18). Although the relative contribution of ROCK vs. PKC in the IAS smooth muscle cells (SMC) is not known, we speculate that ROCK-activated inhibition of MLCP in the SMC is primarily responsible for the maintenance of basal tone in the IAS.

In the present study using freshly isolated SMC from the IAS, we determined cellular distribution of RhoA/ROCK, levels of RhoA-GTP and RhoA-RhoGDI complex formation, and SMC relaxation following C3 exoenzyme (RhoA inhibitor). We also determined the effects of Y 27632 (ROCK inhibitor), Gò 6850 (PKC inhibitor), C3 exoenzyme (RhoA inhibitor), active ROCK-II, and active PKC on the cell lengths of SMC. In addition, we performed Western immunoblottings to determine the levels of RhoA, ROCK-II (primary isoform of ROCK in smooth muscle cells), MYPT1, and MLC20 in rat SMC isolated from IAS vs. rectal smooth muscle (RSM) and anococcygeus smooth muscle (ASM).

MATERIALS AND METHODS

Preparation of dispersed IAS, RSM, and ASM SMC and cellular lysates collection. Male Sprague-Dawley rats (300–350 g) were killed by decapitation, and ASM and the anal canal with an adjacent region of the rectum were quickly removed and transferred to oxygenated (95% O₂–5% CO₂) Krebs physiological solution (KPS) of the following composition (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl₂, 1.16 MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃, and 11.10 glucose (37°C). Adventitious structures connected to the IAS and adjoining RSM were removed carefully by sharp dissection. Following the removal of the mucosa by sharp dissection, circular smooth muscle strips
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The experimental protocol was approved by the institutional animal care and use committee of Thomas Jefferson University and was in accordance with the recommendations of the American association for the accreditation of laboratory animal care.

SMC were isolated from IAS, RSM, and ASM by sequential enzymatic digestion, filtration, and centrifugation as described previously (6). Briefly, the smooth muscle tissues were cut into 0.2 × 0.2-mm blocks and incubated in KPS containing 0.1% collagenase and 0.01% trypsin inhibitor. The partly digested tissues were washed, and SMC were allowed to disperse spontaneously for 30 min. Cells were harvested by filtration through 500-μm Nitex mesh and centrifuged twice at 350 g for 10 min. SMC were incubated in 10-cm plates in DMEM containing 5% penicillin-streptomycin, 50 μg/ml gentamycin, and 2 μg/ml amphotericin B at 37°C with 5% CO2 for 24 h. Later, DMEM was removed and homogenization buffer (1% SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris, pH 7.4) was added to the SMC. The mixture was homogenized with a homogenizer on ice. The homogenates were centrifuged (14,000 rpm) for 5 min and supernatants were collected. Protein concentration in resultant supernatants was determined by the method of Lowry et al. (20) using BSA as a standard (Pierce, Rockford, IL).

Measurement of cell lengths. At the end of incubation period, culture medium was removed from the plates and SMC were incubated for 5–10 min with 1 ml of 0.05% trypsin-EDTA. SMC were then centrifuged and washed twice with fresh KPS and then resuspended in KPS, and 104 SMC suspended in 100 μl of KPS were treated with 20 μl of a solution containing test agents. The reaction was stopped by 5–10 min by the addition of acrolein (1% final concentration). Lengths of the individual cells were measured before and after the test agent by computerized image microscopy. The average length of cells in the control state or with a test agent was obtained from 50 cells encountered randomly in successive microscopic fields. The experiments were repeated in at least three animals. Changes in SMC lengths were measured following incubation with C3 exoenzyme (1 to 10 μg/ml), Y 27632 (0.1 to 10 μM), G6 6850 (10 μM), b-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP; 100 μM), active ROCK-II (3 to 30 nM), active PKC (30 nM), and bethanechol (100 μM).

Permeabilization of SMC for the introduction of C3 exoenzyme and ROCK-II and PKC. SMC were permeabilized by the method previously used in our laboratory with a few modifications (8). Briefly, SMC were suspended in KPS following 24-h culture as described earlier followed by the centrifugation. KPS was then replaced with cytosolic buffer (20 mM NaCl, 100 mM KCl, 5 mM MgSO4, 0.96 mM NaH2PO4, 25 mM NaHC03, 1 mM EGTA, 0.48 mM CaCl2, and 1% BSA) containing saponin (75 μg/ml) for 3 min. At the end of incubation, saponin-containing cytosolic buffer was replaced with fresh cytosolic buffer with omission of saponin. The procedure was repeated twice to remove the saponin completely. C3 exoenzyme (1 to 10 μg/ml) was then added to the SMC for 30 min. Control SMC received similar treatments except for the omission of C3 exoenzyme. In some SMC, active ROCK-II (3 to 30 nM) or active PKC (30 nM) was added for 10 min. SMC lengths were measured as described earlier.

Assay for GTP-bound active RhoA. IAS, RSM, and ASM SMC in the basal state and following C3 exoenzyme (1, 5, or 10 μg/ml) treatments were assayed for GTP-bound RhoA. GTP-bound RhoA was assayed by using Rhotekin (Rho binding domain). The GST-tagged fusion protein corresponding to residues 7–89 of mouse RhoA tagged fusion protein corresponding to residues 7–89 of mouse RhoA was solubilized in Laemmli sample buffer (LSB; with final concentrations 62.5 mM Tris, 1% SDS, 15% glycerol, and 0.005% bromophenol blue, and 2% β-mercaptoethanol) and analyzed by 15% SDS-PAGE followed by Western blot analysis and chemiluminescence.

Coomassie precipitation of RhoA-RhoGDI complexes. SMC in the basal state and following C3 exoenzyme treatments were used for the coimmunoprecipitation studies. SMC were homogenized on ice in lysis buffer (composed of 50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM NaVO4, 25 mM NaF). The lysates were centrifuged (14,000 rpm) for 5 min and supernatants were collected. Protein concentration in resultant supernatants was determined as described above. RhoA-RhoGDI complexes were immunoprecipitated using Roche Diagnostics immunoprecipitation kit (Protein G) (Fisher), and 200 μg of lysate in 250 μl were precleared with 25 μl protein G agarose beads. Precleared lysate was incubated with 1 μg of RhoA rabbit polyclonal antibody (Santa Cruz Biotechnology) for 1 h. Then 25 μl of protein G agarose beads were added and further incubated for overnight to immobilize protein complexes. Agarose beads were centrifuged for 20 s at 10,000 g and washed repeatedly with wash buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). Later, 50 μl of LSB were added to the beads and placed in a boiling water bath for 5 min. Protein samples were separated by 15% SDS-PAGE and Western blot was performed using RhoGDI antibody.

Confocal microscopy. SMC were allowed to attach to the bottom of the Lab-Tek II chamber slides (Nulge Nunc International, Naperville, IL) in DMEM with 10% fetal bovine serum, 5% penicillin-streptomycin, 50 μg/ml gentamycin, and 2 μg/ml amphotericin B on at 37°C and 5% CO2 in an incubator with humidity for 24 h. Later, culture medium was removed and SMC were fixed in 4% paraformaldehyde solution in Dulbecco’s phosphate-buffered saline (DPBS) at room temperature for 15 min. SMC were washed three times with DPBS and incubated overnight at room temperature in a humid environment with 1:100 dilution of RhoA or ROCK-II primary antibody (raised in rabbit) (Santa Cruz Biotechnology) in DPBS containing 0.2% Triton X-100 and 0.5% BSA. SMC were washed three times with DPBS and incubated with Texas red (TR)-conjugated anti-rabbit secondary antibody (1:200) (Santa Cruz) and fluorescent isothiocyanate (FITC)-conjugated α-actin monoclonal antibody (1:800) (Sigma) in DPBS with 0.3% Triton X-100 and 2% donkey serum for 1 h. SMC were then washed three times with DPBS and chambers were removed from slides. The slides were air dried and coverslipped with VECTASHIELD mounting medium (Vector Labs, Burlingame, CA).

Flowcytometry. Flowcytometry was analyzed with a Bio-Rad MRC 600 laser scanning confocal microscope (Zeiss Axiovert 100, Overkochen, Germany). TR was excited at 543 nm with a helium-neon laser and FITC was excited at 488 nm with an argon laser. The fluorophores were detected separately and overlay images were generated automatically by the imaging software.

Cytosolic and particulate fraction collections. SMC were homogenized in ice-cold homogenization buffer (10 mmol/l Tris·HCl, pH 7.5, 5 mmol/l MgCl2, 2 mmol/l EDTA, 250 mmol/l sucrose, and 1 mmol/l dithiothreitol). The homogenates were centrifuged at 100,000 g for 30 min at 4°C (Beckman LS–70M Ultracentrifuge, Fullerton, CA). The supernatants were then transferred to a fresh tube and used as the cytosolic fraction. Pellets containing membrane proteins were resuspended in homogenization buffer containing 1% Triton X-100 and homogenized with a homogenizer. The extract was centrifuged at 800 g for 10 min, and the supernatant was collected as the particulate fraction (9). Protein samples were mixed with LSB and analyzed by 15% SDS-PAGE and chemiluminescence.

Western blot analysis. Twenty micrograms of proteins in 20 μl of lysate were mixed with 2 × LSB (with final concentrations 62.5 mM Tris, 1% SDS, 15% glycerol, and 0.005% bromophenol blue, and 2%
β-mercaptoethanol) and placed in a boiling water bath for 5 min. Protein samples were separated by SDS-PAGE (7.5% gel for ROCK-II, MYPT1, and pThr696-MYPT1; 15% gel for RhoA, RhoGDI, MLC20, and pThr18/Ser19-MLC20).

The separated proteins were electrophoretically transferred onto either a nitrocellulose membrane for ROCK-II, MYPT1, and pThr696-MYPT1 or a polyvinylidene difluoride membrane for RhoA, RhoGDI, MLC20, and pThr18/Ser19-MLC20 at 100 V for 1 h at 4°C. To block nonspecific antibody binding, the membranes were soaked overnight at 4°C in Tris-buffered saline with Tween (TBS-T; composed of 20 mM Tris pH 7.6, 137 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk. The membranes were then incubated with the specific primary antibodies diluted in TBS-T containing 1% milk (1:1,000 for RhoA, RhoGDI, MYPT1, pThr696-MYPT1, MLC20, and pThr18/Ser19-MLC20; 1:20,000 for α-actin) for 1 h at room temperature.

After being washed with TBS-T three times (10 min each wash), the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody binding, the membranes were soaked overnight at 4°C in Tris-buffered saline with Tween (TBS-T; composed of 20 mM Tris pH 7.6, 137 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk. The membranes were then incubated with the specific primary antibodies diluted in TBS-T containing 1% milk (1:1,000 for RhoA, RhoGDI, MYPT1, pThr696-MYPT1, MLC20, and pThr18/Ser19-MLC20; 1:20,000 for α-actin) for 1 h at room temperature.

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Data analysis. Results are expressed as mean densities ± SE from three independent experiments. For the Western blot analysis, mean density and area for the band were calculated by using Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD). Mean density was multiplied with the area to calculate relative density. The relative density for the protein of interest was normalized over α-actin densities. For the comparison of ASM, RSM, and IAS SMC the relative densities for IAS were adjusted to 1. One-way ANOVA followed by a Bonferroni post hoc test was used (P < 0.05) to calculate statistical significance.
RESULTS

Relaxation of SMC by inhibition of RhoA/ROCK. To determine the role of RhoA/ROCK in the SMC toward basal tone in the IAS, we examined the effect of the RhoA inhibitor C3 exoenzyme. Incubation of permeabilized IAS SMC with C3 exoenzyme caused lengthening of IAS SMC in a concentration-dependent manner. In contrast to the SMC from the smooth muscle of the IAS, SMC from RSM and ASM showed no significant relaxation following RhoA inhibition (n = 4, Fig. 1A). Incubation of the IAS SMC with 10 μg/ml C3 exoenzyme produced 31.1 ± 3.3% relaxation of the SMC (n = 4, Fig. 1A). The SMC relaxation as a result of RhoA inhibition was comparable with that by ROCK inhibitor Y 27632 (10 μM), which produced 31.7 ± 3.2% relaxation of the IAS SMC (n = 4, Fig. 1B). Basal cell lengths of the freshly isolated IAS, RSM, and ASM SMC were 44.8 ± 2.9, 70.9 ± 3.4, and 60.1 ± 2.4 μm, respectively. SMC lengths did not change significantly following 24 h culture or permeabilization.

Contraction of IAS SMC by ROCK-II. To directly examine the role of ROCK in the SMC contraction, we determined the effect of ROCK-II injected directly within the IAS SMC. Active ROCK-II by itself caused a concentration-dependent contraction of the IAS SMC (n = 4, Fig. 2A). A concentration of 30 nM caused maximal contraction (20.4 ± 1.38%) of the SMC. Direct addition of ROCK-II (30 nM for 10 min) restored C3 exoenzyme-induced lengthening of the IAS SMC (n = 4, Fig. 2B).

Contribution of ROCK vs. PKC in the spontaneous contractions of IAS SMC. To study the effects of ROCK vs. PKC in the IAS SMC, we compared effects of Y 27632 (10 μM), Gö 6850 (PKC inhibitor; 10 μM), Y 27632 (10 μM) + Gö 6850 (10 μM), and direct injection of active ROCK-II and PKC (30 nM).

Y 27632 produced significantly greater relaxation of the IAS SMC vs. that by Gö 6850 (31.7 ± 3.2 and 16.2 ± 2.3%, respectively) (n = 4; Fig. 3A). The combined effect of Y 27632 and Gö 6850 was not significantly different from that of Y 27632 alone (32.4 ± 3.1%) (P > 0.05; n = 4). PKG activator 8-Br-cGMP (100 μM) produced relaxation of the IAS SMC (32.9 ± 4.1%) that was not significantly different from that by Y 27632 (P > 0.05; n = 4, Fig. 3A).

Shortening of the IAS SMC with ROCK-II was significantly greater than with PKC (P < 0.05; n = 4; Fig. 3B). Y 27632 significantly attenuated the contraction caused by ROCK-II as well as PKC, whereas Gö 6850 only inhibited the effect of PKC (n = 4).

Distribution of RhoA and ROCK-II in the SMC of the IAS vs. the RSM. To determine the cellular distribution of RhoA/ROCK, we performed confocal microscopy studies. The data revealed significantly higher levels of RhoA and ROCK-II toward the periphery of the IAS SMC compared with those of the RSM SMC (Fig. 4, A and B). These findings suggest that RhoA/ROCK are in active form in the basal state of the IAS SMC.

Analysis of RhoA activity in the IAS vs. the RSM and ASM. Active RhoA in the form GTP bound RhoA (RhoA-GTP) was measured to compare RhoA activity in the SMC isolated from IAS vs. RSM and ASM. The highest activity was found in the tonic SMC of IAS. Purely phasic SMC of ASM on the other hand demonstrated almost no activity. RhoA-GTP levels in the RSM lay in between IAS and ASM (n = 3; Fig. 5A).

Inactive RhoA remains associated with RhoGDI. Pull-down assays were performed using RhoA polyclonal antibodies, and RhoGDI associated with RhoA was detected via Western blots using RhoGDI antibody. RhoGDI levels were taken as a measurement for inactive RhoGDI-associated RhoA. In con-
contrast to RhoA-GTP, RhoGDI-bound RhoA was more abundant in the phasic ASM and RSM. The lowest association between RhoA and RhoGDI was found in the tonic SMC of the IAS (n/H11005 3; Fig. 5B).

Expression and cellular distribution of RhoA in SMC of the IAS. The expression of the signal transduction components related to RhoA/ROCK pathway was examined in the cellular protein extracts, by Western blot analysis. Figure 6 illustrates the representative Western blots of RhoA in SMC from functionally diverse tissues.

In addition to the confocal microscopy (as shown above), the cellular distribution of RhoA was further determined by Western blot after separation of cellular homogenates in cytosol and membrane proteins in IAS vs. the RSM and ASM in the basal state. Data revealed higher levels of RhoA in the membrane fraction of the IAS than in the cytosolic fraction. In contrast to IAS, higher levels of RhoA were present in the cytosolic fraction than the particulate fraction in the phasic SMC of RSM and ASM (n = 3; Fig. 6A).

The expression of protein levels in the SMC isolated from different tissues were normalized using α-actin. The latter did not differ significantly among the three different SMC types. The RhoA protein expression was the highest in the tonic SMC of the IAS followed by RSM and the least in the ASM (n = 3; Fig. 6B).

Cellular distribution of ROCK-II in the SMC isolated from IAS vs. the ASM and RSM, in the basal state. Cellular distribution of ROCK-II in the basal state was similar to that of the RhoA, higher levels in the membrane and lower in the cytosol in the tonic IAS. The SMC from the RSM and ASM on the other hand demonstrated higher levels of ROCK-II in the cytosolic fraction (n = 3; Fig. 7A). Western blot analysis of total ROCK-II revealed highest levels in the IAS SMC, followed by the RSM and ASM (n = 3; Fig. 7B).

Immunoblots for MYPT1 and MLC20 in the SMC from IAS vs. RSM and ASM. The role of MLCP in RhoA/ROCK signal transduction was examined by monitoring the levels of MYPT1 and MLC20 in the diverse types of SMC isolated from the tonic vs. phasic smooth muscle tissues. Interestingly, in contrast to RhoA/ROCK and all the other downstream signal transduction proteins (as shown below), the basal levels of MYPT1 were found to be lowest in the SMC from the IAS compared with those from RSM and ASM (*P < 0.05; n = 3; Fig. 8A). The basal levels of MLC20 were highest in the SMC from IAS vs. RSM and ASM (*P < 0.05; n = 3; Fig. 8B).
Levels of phosho-MYPT1 and MLC20 followed a trend similar to those of RhoA and ROCK-II, highest in the IAS followed by the RSM and ASM (n = 3; Fig. 8, C and D). The primary sites of MLC20 phosphorylation are Ser19 and Thr18 (17).

**Effects of C3 exoenzyme on RhoA/ROCK activity.** Effects of C3 exoenzyme on RhoA activity in the IAS SMC were analyzed via RhoA-GTP levels and RhoA-RhoGDI complex formation. C3 exoenzyme caused increase in the amount of RhoGDI in the complexes pulled down with RhoA antibody.

**Fig. 7.** A: Western blot analyses compare cellular distribution of ROCK-II in phasic vs. tonic SMC. Note that ROCK-II is primarily present in the particulate fraction in the IAS SMC and in the cytosolic fraction in the ASM SMC followed by that in the RSM and ASM (*P < 0.05) followed by that in the RSM and ASM.

**Fig. 6.** A: particulate and cytosolic fractions collected from SMC and RhoA Western blot analyses compare cellular distribution in phasic vs. tonic SMC. Note that RhoA densities are higher in the particulate vs. cytosolic fractions in the tonic IAS SMC and the distribution pattern is reversed in the phasic ASM SMC. B: Western blot analysis for the relative expression of total RhoA, in the ASM, RSM, and IAS SMC in the basal state. The relative expression of RhoA is calculated in relation to the density of α-actin and the densities in the IAS group adjusted to 1. Data show expression of RhoA in the IAS (*P < 0.05) followed by that in the RSM and ASM.

**Immunoblots for p Thr696-MYPT1 and p Thr18/Ser19-MLC20 in the IAS vs. the ASM and RSM SMC.** MLCP activity in the tonic vs. phasic SMC was evaluated via the basal levels of phosphorylated forms of MYPT1 (a regulatory subunit of MLCP), p Thr696-MYPT1.
RhoA-GTP levels were reduced in the SMC pretreated with C3 exoenzyme compared with control SMC (n = 3, Fig. 9, A and B). ROCK is the immediate downstream target of RhoA. Activity of ROCK leads to decrease in the phosphorylation of MYPT1 at Thr696 residue (pThr696-MYPT1). C3 exoenzyme concentration dependently reduced the levels of pThr696-MYPT1 (n = 3, Fig. 9C).

**DISCUSSION**

The present studies provide the first demonstration that the SMC from the spontaneously tonic smooth muscle are inherently different for the biochemical makeup especially as it relates to RhoA/ROCK signal transduction. Data from studies using selective inhibitors of RhoA and ROCK suggest that RhoA/ROCK play a significant role for the maintenance of contractile state of the SMC in the tonic smooth muscle of the IAS. The studies provide a molecular basis for the genesis of the basal tone in the IAS smooth muscle. A working model to explain the role of RhoA/ROCK as the molecular basis for the basal tone in the IAS smooth muscle been provided in Fig. 10.

The role of RhoA/ROCK in the sustained contraction of SMC following various agonists has been known (1, 3, 13, 21, 27, 30), but there has been no information on the SMC isolated from the truly tonic tissues vs. the purely phasic tissues, in the basal state. Present studies demonstrate upregulation of RhoA/ROCK and the related signal transduction components in the truly tonic vs. the phasic SMC. The smooth muscle IAS serves as the prototype of the tonic smooth muscle, since it develops spontaneous tone in the absence of any agonist or stimulus (13, 25). The phasic smooth muscle on the other hand, e.g., ASM, fails to develop tone and exhibits phasic contractions in response to electrical field stimulation (6). RSM was selected for the comparison; similar to colon, RSM displays primarily
phasic activity with a low-grade tone (28). Data are consistent with the concept that activation of RhoA/ROCK maintains high levels of p-MLC20 and basal tone in the smooth muscle by the inhibition of MLCP.

The role of RhoA/ROCK in the SMC was examined by the use of RhoA and ROCK inactivation as well as by the direct introduction of active ROCK-II into the SMC. Kureishi et al. (19) have reported elevation in the levels of p-MLC20 and sustained contraction, independent of the Ca2\(^{2+}\)-calmodulin-dependent myosin light chain kinase pathway, upon introduction of active ROCK into the Triton X-100-permeabilized phasic smooth muscle. Membrane-associated RhoA-GTP activates its downstream target ROCK (30). Activated ROCK inhibits MLCP and PP1c\(^{3'}\) by the increased phosphorylation of MYPT1 (p\(^{Thr696}\)-MYPT1) and CPI-17 (p\(^{Thr38}\)-CPI-17), respectively (31). Inhibition of MLCP reduces MLC20 dephosphorylation, resulting in sustained contraction of the smooth muscle. This concept is supported by the higher levels of total RhoA-Rock in the tonic SMC of the IAS. Also introduction of active ROCK-II causes further shortening of the IAS SMC.

Studies further demonstrate that actual levels of RhoA-GTP are higher in the IAS SMC and the lowest in the purely phasic SMC. In the resting phase of the SMC a majority of RhoA may be trapped in the cytosol by RhoGDI. RhoGDI captures the prenylated COOH terminus of RhoA in its hydrophobic cavity and inhibits nucleotide exchange from GDP to GTP (23). In addition, a high concentration of GDI may extract RhoA-GTP translocated to membrane and reduce agonist-induced smooth muscle contractions (11). Our studies reveal the highest levels of RhoA-RhoGDI complex formation in phasic SMC and the lowest in tonic IAS SMC. We speculate that such complex formation prevents the formation of RhoA-GTP and in turn the activation of ROCK. In addition, data show distinctly higher levels of RhoA/ROCK at the membrane than the cytosol in the IAS SMC vs. ASM and RSM. Confocal microscopy combined with immunocytochemical staining for RhoA and ROCK-II in the IAS further supports this notion.

Studies further demonstrate lowest levels of MYPT1 in the IAS SMC, moderate in RSM and the highest in the purely phasic ASM SMC, and the trend was reversed in the case of

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**Fig. 9.** Effects of C3 exoenzyme (1 to 10 \(\mu\)g/ml) on RhoA-RhoGDI complex formation (analyzed via coimmunoprecipitation of RhoGDI using RhoA antibody). RhoA-GTP and p\(^{Thr696}\)-MYPT1 in the IAS SMC. C3 exoenzyme causes concentration-dependent increase in the RhoA-RhoGDI binding but reduces the levels of RhoA-GTP and p\(^{Thr696}\)-MYPT1 (*\(P < 0.05\)).

**Fig. 10.** Model to explain the effect of RhoA/ROCK inhibitors and ROCK into the IAS SMC and specific traits of the IAS SMC that make it tonic in the basal state. In the IAS SMC most of the RhoA exists as active form as RhoA-GTP. C3 exoenzyme causes relaxation of the IAS SMC by the inactivation of RhoA-GTP by its conversion into RhoA-GDP-RhoGDI complex. Y 27632, on the other hand, causes direct inactivation of ROCK. Direct introduction of ROCK within the SMC causes phosphorylation of primarily MYPT1 (donated by thicker lines) and CPI-17. The latter processes result in MLCP inhibition and increase in phospho-MLC20, causing sustained contraction of the IAS SMC responsible for the basal tone in the IAS.
pThr696-MYPT1. We speculate that higher levels of unphosphorylated MYPT1 unleash MLCP in phasic SMC, resulting in low levels of p-MLC20. Conversely, in the IAS SMC higher levels of phospho-MYPT1 (as a result of RhoA/ROCK upregulation) restrict MLCP, resulting in high levels of p-MLC20. ROCK may also inhibit MLCP activity via phosphorylation of CPI-17 (18). It is of interest that CPI-17 by itself inhibits the catalytic subunit of MLCP, but with a lower potency than pThr38-CPI-17 (7). Our preliminary studies reveal higher levels of CPI-17 as well as pThr38-CPI-17 in the IAS SMC vs. those of the ASM. These findings suggest that spontaneous contractions in the IAS SMC are also regulated in part by RhoA/ROCK activation-induced MLCP inhibition via CPI-17.

Further evidence in favor of the RhoA/ROCK pathway in the spontaneous contractions of IAS SMC comes from the use of the RhoA inhibitor C3 exoenzyme. C3 exoenzyme is an ADP-ribose transferase that ribosylates the Asn-41 residue of RhoA, causing inactivation of RhoA and inhibition of Ca2+ sensitization (9). C3 exoenzyme is highly specific and extensively used for inhibiting the RhoA/ROCK pathway upstream (30). ROCK inhibitor Y 27632 reduces pThr696-MYPT1 and completely abolishes the basal tone in the IAS smooth muscle (24). Inhibition of RhoA/ROCK may unleash MLCP, causing rapid dephosphorylation of MLC20, preventing spontaneous contraction in the IAS SMC. In agreement with this concept, C3 exoenzyme causes concentration-dependent increase in the RhoA-RhoGDI binding and decrease in the levels of RhoA-GTP. This results in reduced levels of pThr696-MYPT1 and relaxation of spontaneously contracted IAS SMC that is comparable to the relaxation by ROCK inhibitor (Y 27632). On the other hand, introduction of active ROCK-II into IAS SMC restores spontaneous contractions. In sharp contrast to tonic IAS SMC, RhoA/ROCK inhibitors are relatively ineffective in cells from phasic smooth muscles of RSM and ASM.

Relaxation of the IAS SMC by ROCK inhibitor is significantly greater compared with the PKC inhibitor. Direct introduction of active PKC similar to the earlier studies causes contraction of the IAS SMC (2). The ROCK inhibitor attenuates PKC-induced contraction in the IAS SMC whereas ROCK-II-mediated contraction of the SMC is not affected by the PKC inhibitor. Interestingly, in the vascular smooth muscle it has been shown that contraction caused by PKC is mediated primarily via ROCK activation (15). In addition, Y 27632 blocks active PKC-induced increase in the pThr38-CPI-17 (data not shown). Present results in the SMC suggest that both ROCK and PKC are working on the same signaling pathway but the former may play a larger and direct role in basal IAS tone. The effects of PKC on the other hand, at least in part may be dependent on ROCK activation.

Smooth muscles of the gut represent functionally diverse organ systems characterized either with true tone, e.g., the sphincters, or with the phasic contractions of nonspincteric regions. Woodsome et al. (32) reported lower levels of MYPT1 and higher levels of CPI-17 in tonic smooth muscle of the femoral artery in contrast to the phasic smooth muscle of the vas deferens. Systematic studies to compare the levels of RhoA/ROCK and related intracellular machinery in functionally diverse SMC of gut (ranging from truly tonic to the phasic types) in the basal state have not been reported before the present study.

In summary, upregulation of RhoA/ROCK in the SMC play a significant role in the maintenance of spontaneous tone in the IAS and PKC in part mediates its effects via ROCK. Whether this concept also holds for the other sphincteric regions of the GI tract remains to be determined. These data provide important insights into the role of RhoA/ROCK in the pathophysiology and therapeutic approaches in the rectoanal motility disorders especially affected with hypertensive IAS.

REFERENCES


