Nonmuscle myosin is regulated during smooth muscle contraction

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Yuen SL, Ogut O, Brozovich FV. Nonmuscle myosin is regulated during smooth muscle contraction. Am J Physiol Heart Circ Physiol 297: H191–H199, 2009. First published May 8, 2009; doi:10.1152/ajpheart.00132.2009.—The participation of nonmuscle myosin in force maintenance is controversial. Furthermore, its regulation is difficult to examine in a cellular context, as the light chains of smooth muscle and nonmuscle myosin comigrate under native and denaturing electrophoresis techniques. Therefore, the regulatory light chains of smooth muscle myosin (SM-RLC) and nonmuscle myosin (NM-RLC) were purified, and these proteins were resolved by isoelectric focusing. Using this method, intact mouse aortic smooth muscle homogenates demonstrated four distinct RLC isoelectric variants. These spots were identified as phosphorylated NM-RLC (most acidic), nonphosphorylated NM-RLC, phosphorylated SM-RLC, and nonphosphorylated SM-RLC (most basic). During smooth muscle activation, NM-RLC phosphorylation increased. During depolarization, the increase in NM-RLC phosphorylation was unaffected by inhibition of either Rho kinase or PKC. However, inhibition of Rho kinase blocked the angiotensin II-induced increase in NM-RLC phosphorylation. Additionally, force for angiotensin II stimulation of Rho kinase blocked the angiotensin II-induced increase in NM-RLC phosphorylation, and these parameters fall to lower steady-state levels during force maintenance (5, 36). The mechanism for force maintenance is unknown and has been hypothesized to be due to a set of slowly cycling dephosphorylated cross bridges, or latch cross bridges (5, 16, 36). However, the existence of a latch cross bridge is controversial, and therefore a number of other mechanisms have been proposed to explain force maintenance (12, 20, 21, 28).

Recently, a number of groups have provided biochemical (25, 49), as well as mechanical (27, 30, 38), evidence that nonmuscle (NM) myosin can participate in a physiologically relevant pathway for force maintenance in smooth muscle. There are three known classes of NM myosin (15), NM myosin IIA, IIB, and IIC (NMIIA, NMIIB, NMIIC), and NM myosin is the molecular motor that contracts actin-filament networks to drive many cellular processes (2, 24). Similar to smooth muscle myosin, NM myosin is regulated by phosphorylation of its regulatory light chain (NM-RLC); NM-RLC phosphorylation both promotes NM myosin filament assembly (24) and increases the actin-activated ATPase activity of NM myosin, which results in a 10-fold increase in V_{max} (3).

If NM myosin is the elusive latch cross bridge, the enzyme must be activated and/or NM-RLC phosphorylation must increase following stimulation of smooth muscle. However, it is unclear whether NM-RLC phosphorylation increases following activation of smooth muscle, and the signaling pathway(s) that regulates NM myosin is undefined. Furthermore, since SM-RLC and NM-RLC are both 172 amino acids and have >90% sequence identity, resolving these proteins simultaneously to study their phosphorylation is technically difficult. To address this question, we used a two-dimensional electrophoresis method to simultaneously resolve and quantify SM-RLC and NM-RLC phosphorylation. We examined SM-RLC and NM-RLC phosphorylation at rest and during force maintenance (5 min of activation), when SM-RLC phosphorylation has fallen from its peak to a new steady state, consistent with the formation of “latch cross bridges” (5, 36, 38). The data provide evidence that, during both depolarization and agonist activation of smooth muscle, NM myosin is regulated and plays a physiologically relevant role in the mechanism for force maintenance.

EXPERIMENTAL PROCEDURES

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Medical School.

Myosin light chain cloning. Primers for cloning the NM-RLC were designed from the cDNA sequence of the chicken NM-RLC (Genbank P24032). Total RNA was obtained from chicken gizzard using a Qiagen RNeasy Mini Kit, and a specific reverse primer (5'-CTC-GAGTCAGTCATCCTTGTCTTTCGCATGTT-3') was used for the first strand reverse transcription reaction. Then PCR immediately followed using one-fifth of the reverse transcription product and 10 pmol each of forward primer (5'-CATATGTCATCAGGAAAAGGGCAGA-3') and reverse primer. The amplified PCR product was cloned and sequenced in full to verify correct amplification. For protein expression, the full-length NM-RLC cDNA was cloned in frame into the pAE4D prokaryotic expression vector (14). For comparison, a full-length cDNA for chicken SM-RLC was generously provided by Dr. Mitsuo Ikebe. The SM-RLC cDNA was also moved to the pAE4D vector for prokaryotic protein expression.

Myosin light chain protein expression. Expression of NM-RLC and SM-RLC in E. coli BL21 cells was done as previously detailed (14). As SM-RLC and NM-RLC have a high degree of identity (162 of 172 amino acids), the same purification protocol was used for both proteins. Bacteria transformed with expression vectors encoding SM-RLC or NM-RLC were cultured to 600-nm optical density = 0.4, and protein expression was induced with 0.2 M isopropyl-β-D-thiogalactoside for 2 h before centrifugation. The collected bacterial pellets were stored at −80°C overnight and then sonicated in 20 mM Bis-Tris, pH 6.5, 1 mM EDTA, 10 mM dithiothreitol, and 1× Complete Protease Inhibitor (Roche). Following sonication, ammonium sulfate was added to the soluble fraction up to 70% saturation,
and the supernatant from this fractionation contained SM-RLC or NM-RLC. The 70% ammonium sulfate supernatant was dialyzed against 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 1 mM dithiothreitol. The dialyzed sample was then centrifuged to clear insoluble proteins, and the supernatant was resolved by anion exchange chromatography using a Q Sepharose Fast Flow column developed with a linear gradient to 0.5 M NaCl in the equilibration buffer. Fractions containing SM-RLC or NM-RLC were identified by SDS-PAGE, collected, and dialyzed against 50 mM ammonium bicarbonate before lyophilization.

Western blotting. Western blot analyses were done as previously described (14). To verify the identity of the expressed and purified SM-RLC and NM-RLC, a monoclonal antibody against SM-RLC was used (MY-21; Sigma). Smooth muscle myosin heavy chain (MHC) was identified with a specific monoclonal antibody (h-SM-V; Sigma). NM MHC-A or -B were identified with rabbit antisera generated against A- or B-isoform-specific peptides (Sigma). To compare MHC expression levels across various tissues, smooth muscle tissue extracts were resolved by SDS-PAGE using the Bis-Tris buffering system, and proteins were fluorescently stained using Deep Purple® ME Healthcare). Fluorescently stained gels were scanned using a Typhoon scanner, and the actin and myosin bands were quantified across samples using ImageQuant TL software. The NM MHC-A and -B signals from Western blots of smooth muscle extracts were normalized according to total actin and myosin content to make reliable comparisons across fibers.

Determination of myosin RLC phosphorylation. Mouse aorta tissues were cleaned of all connective tissue and the endothelium, and activated by either depolarization (80 mM KCl) or agonist stimulation ([1 μM angiotensin II (ANG II)]. After 5 min in either relaxing solution or the activating solution, the reaction was terminated by 10% TCA in acetone placed in liquid nitrogen. Total protein homogenates from the activated tissues were prepared by extraction with 7 M urea, 2 M thiourea, 2% (wt/vol) 3.5–5 immobilized pH gradient (IPG) buffer, and 1× Roche Complete protease inhibitor. The homogenates were cleared of lipids and extraneous salts using the 2D Clean Up kit (GE Healthcare). Suitable amounts of sample were then added to a rehydration buffer consisting of 7 M urea, 2 M thiourea, 2% (wt/vol) CHAPS, 1% 3,5–5 immobilized pH gradient (IPG) buffer, and 1× Roche Complete protease inhibitor. The homogenates were activated by either depolarization (80 mM KCl) or agonist stimulation (1 μM ANG II, and steady-state force was determined by 10.220.33.6 on June 30, 2017 http://ajpheart.physiology.org/ Downloaded from

RESULTS

SM-RLC and NM-RLC have 94% sequence identity; their predicted molecular ratio and isoelectric point are 19,829 and 4.67 vs. 19,991 and 4.52, respectively. Thus resolving the two proteins is not trivial. To show that SM-RLC and NM-RLC phosphorylation can be determined in smooth muscle tissues, we first cloned, expressed, and purified avian SM-RLC, as well...
as cloned, expressed, and obtained a highly enriched fraction of the NM-RLC (Fig. 1A). The anti-smooth muscle SM-RLC antibody recognized both the NM-RLC and SM-RLC, as expected, based on their high degree of identity. The purified SM-RLC and the enriched fraction of NM-RLC were mixed in a 2:1 ratio and resolved by two-dimensional gel electrophoresis (Fig. 1B). The two-dimensional SDS-PAGE results demonstrate that nonphosphorylated SM-RLC and NM-RLC could be readily resolved. Similar conditions were used to analyze gizzard tissue lysates, and four distinct spots were resolved, presumably representing the non- and monophosphorylated SM-RLC and NM-RLC (Fig. 1C).

These experiments were repeated with lysates of mouse aorta smooth muscle, reproducibly demonstrating four distinct spots at positions consistent with singly phosphorylated or nonphosphorylated SM-RLC and NM-RLC (Fig. 2A). Identification of these focused proteins (nos. 1–4 in Fig. 2A) by mass spectrometry (Fig. 2B) yielded peptide coverage identifying spots 1 and 2 as NM-RLC and spots 3 and 4 as SM-RLC. This overall sequence coverage is presented in Fig. 3, with >60% coverage for both proteins. Additional analyses revealed that spot 1 was the singly phosphorylated (either Thr 18 or Ser 19) form of NM-RLC, whereas spot 2 was the nonphosphorylated NM-RLC (see data supplement; the online version of this article contains supplemental data). Similarly, spot 3 was the singly phosphorylated form of SM-RLC, and spot 4 was nonphosphorylated SM-RLC. Of the regions of both proteins covered by recovered peptides, mass spectroscopy revealed that there were nine amino acid differences, consistent with the expected sequences for SM-RLC (Genbank no. BAE35452) and NM-RLC (Genbank no. NP080340). In addition to unambiguously identifying each spot, mass spectroscopy recovered peptides that suggested each of the four focused spots contained a single protein species: either phosphorylated or nonphosphorylated NM-RLC (spots 1 and 2; Fig. 2A) or SM-RLC (spots 3 and 4; Fig. 2A). Similarly, mass spectrometry following activation produced identical spectra (compared with relaxed tissue) and demonstrated that each spot remained a unique protein: phosphorylated and nonphosphorylated NM-RLC (spots 1 and 2; Fig. 2B), and phosphorylated and nonphosphorylated SM-RLC (spots 3 and 4; Fig. 2B). To further investigate the phosphorylation status of spots 1–4, tissue was activated with KCl depolarization or ANG II, and the two-dimensional gels were stained with Pro-Q Diamond phosphoprotein stain and then silver stained (Fig. 2C). Pro-Q Diamond stained spots 1, 2, and 3. Spot 4 was not stained by Pro-Q Diamond, consistent with this being nonphosphorylated SM-RLC. Densitometric analysis demonstrated that spot 2 is 5–10% of spot 3 (singly phosphorylated SM-RLC). However, the presence of two phosphates in the diphosphorylated SM-RLC doubles the intensity of the detected protein, and thus diphosphorylated SM-RLC is 3–5% of the total singly phosphorylated SM-RLC signal, or diphosphorylation during KCl or ANG II activation is 1–3%. From the intensities of the corresponding silver stained gels, the diphosphorylated SM-RLC present in spot 2 was calculated as 5% of the total spot intensity, whereas 95% of the spot intensity was nonphosphorylated NM-RLC.

Our data show that, during depolarization (Fig. 4), there was a significant increase in SM-RLC phosphorylation (21 ± 3 vs. 50 ± 3%, n = 14, P < 0.05), and neither inhibition of Rho
kinase nor inhibition of PKC affected the increase in SM-RLC phosphorylation. Similarly, the increase in NM-RLC phosphorylation following depolarization (7 ± 2 vs. 38 ± 5%, n = 8, P < 0.05) was not affected by inhibition of either Rho kinase or PKC. For agonist activation (Fig. 5), SM-RLC phosphorylation increased (19 ± 2 vs. 48 ± 2%, n = 8, P < 0.05), and the increase in SM-RLC phosphorylation was inhibited with both Y-26372 (34 ± 2%, P < 0.05) and the pseudosubstrate PKC inhibitor (37 ± 4%, P < 0.05). Agonist activation increased NM-RLC phosphorylation (8 ± 3 vs. 42 ± 9%, n = 8, P < 0.05), and only the Rho kinase inhibitor (19 ± 6%, P < 0.05), but not the PKC inhibitor (30 ± 7%, P > 0.05), attenuated the ANG II-induced increase in phosphorylation. These results demonstrate that NMIIB is activated during both KCl depolarization and agonist activation and could contribute to force maintenance.

To further define the contribution of NMIIB to force maintenance, we utilized transgenic mice expressing a reduced amount of NMIIB (43, 45). For these experiments, we determined steady-state isometric force for 10 min ANG II stimulation of aortic smooth muscle strips from heterozygous NMIIB KO mice (B/+) and their WT littermates (Fig. 6). Following activation, force rises to a peak in 30–60 s, which is similar (P > 0.05) for WT and NMIIB KO mice. In the WT aorta, force then falls slightly (7 ± 5%, n = 4), while, for the B+/− mice, force falls by 33 ± 10% (n = 4); the sustained steady-state...
However, the 1–3% diphosphorylation of SM-RLC (or in NM-RLC phosphorylation would, in fact, be underestimated a effect of PKC inhibition on SM-RLC and NM-RLC phosphorylation also demonstrates that the presence of a small amount of diphosphorylated SM-RLC does not significantly affect our results for NM-RLC phosphorylation.

Our data demonstrated that, during depolarization, SM-RLC and NM-RLC phosphorylation increased (Fig. 4). Inhibition of neither Rho kinase nor PKC affected the increase in SM-RLC or NM-RLC phosphorylation, which suggests that, during depolarization, a Ca$^{2+}$-calmodulin-mediated increase in MLC kinase (MLCK) activity leads to activation of both smooth muscle and NM myosin. Similarly, ANG II stimulation resulted in a significant increase in both SM-RLC and NM-RLC phosphorylation (Fig. 5). The ANG II-induced increase in SM-RLC phosphorylation was blunted by both Rho kinase and PKC inhibition (Fig. 5). These data are consistent with other state isometric forces were 9.0 ± 0.5 mN/mm² for the WT aorta (n = 4) vs. 6.7 ± 1.0 mN/mm² (n = 4, P < 0.05) for the B$^{+/-}$ strain.

In the B$^{+/-}$ mice, compared with WT littermates, there was no difference (P > 0.05) in the expression of either total MHC or actin (Fig. 7A). Following fluorescent total protein staining, the ratio (B$^{+/-}$/WT) of intensities of MHC was 1.2 ± 0.2 (n = 4), and for actin the ratio was 1.1 ± 0.1 (n = 4). The relative expression of smooth muscle MHC, NMIIA, and NMIIB in the B$^{+/-}$ strain was quantified using Western blots (Fig. 7B), with the intensity of the signal normalized to a loading control (total MHC). For the B$^{+/-}$ strain (Fig. 7C), there was no difference in the expression of either smooth muscle MHC (0.89 ± 0.17, n = 4) or NMIIA (1.1 ± 0.1, n = 4) compared with WT, but there was a significant (P < 0.05) reduction in the expression of NMIIB (0.62 ± 0.01, n = 4).

**DISCUSSION**

Our results demonstrate that we can unambiguously quantify changes in SM-RLC and NM-RLC phosphorylation during smooth muscle activation. Similar to our results, Gaylinn et al. (11) reported that the phosphorylated and nonphosphorylated forms of SM-RLC and NM-RLC could be separated using two-dimensional gel electrophoresis. These investigators screened antisera to NM-RLC and SM-RLC, and all except one displayed equal reactivity to NM-RLC and SM-RLC. They reported that, with maximal levels of stimulation, SM-RLC was diphosphorylated to a small extent, but with lower levels of stimulation, the diphosphorylated form of SM-RLC was not detectable. For our experimental conditions (80 mM KCl depolarization and 1 μM ANG II stimulation), in agreement with Gaylinn’s results, we detected the presence of a small amount of the diphosphorylated form of the SM-RLC comigrating with nonphosphorylated NM-RLC. Mass spectrometry did not recover peptides consistent with diphosphorylated SM-RLC, presumably due to the higher abundance (~20×) of the comigrating NM-RLC.

However, the 1–3% diphosphorylation of SM-RLC (or ~5% of total NM myosin in spot 2, Fig. 2C) would not significantly affect the increase in NM-RLC phosphorylation; the increase in NM-RLC phosphorylation would, in fact, be underestimated by ~2.5% (0.05 × 40%). Furthermore, during ANG II stimulation, both inhibition of Rho kinase and PKC decreased SM-RLC phosphorylation, while only Rho kinase inhibition decreased NM-RLC phosphorylation. This divergence in the

**Fig. 4. Myosin light chain phosphorylation increases during depolarization.**

A: silver-stained two-dimensional gels of representative mouse aortic tissue lysates at rest and following KCl depolarization, as well as KCl depolarization in the presence of the Rho kinase inhibitor, Y-26732, or the PKC inhibitor, myr-psi PKC. B: bar graphs (means ± SE, n = 14) demonstrate that, compared with relaxed SM (Rel), both SM-RLC and NM-RLC phosphorylation increased with depolarization (KCI), and the increases in phosphorylation were unaffected by the inhibition of Rho kinase or PKC. *Statistically significant differences vs. the respective “relaxed” control experiments.
reports and suggest that, during agonist activation of the G proteins, the Ca\(^{2+}\)-independent increase in SM-RLC phosphorylation is mediated by activation of either Rho kinase or PKC-dependent signaling pathways (41). Agonist activation of the G proteins has been demonstrated to produce a Rho kinase-dependent phosphorylation of the myosin targeting subunit (MYPT1) of MLC phosphatase, which inhibits MLC phosphatase activity to increase SM-RLC phosphorylation and force (6–8, 22). In contrast, the ANG II-induced increase in NM-RLC phosphorylation was only reduced by Rho kinase inhibition (Fig. 5). These data suggest that, during ANG II stimulation, a Rho kinase-dependent pathway activates NM myosin.

In NM cells, Rho kinase has been demonstrated to phosphorylate the NM-RLC at Ser 19 to regulate motile events (24, 44). Similarly, inhibition of Rho kinase, but not MLCK or PKC, reduced the contractile forces generated by fibroblasts (10). In epithelial cells, inhibition of both MLCK and Rho-kinase blocks NMIIA NM-RLC phosphorylation (1), and thrombin stimulation, via a Rho kinase-mediated pathway, produces a significant increase in Ser 19 phosphorylation of the NM-RLC of both NMIIA and NMIIB (40). In smooth muscle, agonist activation has been demonstrated to lead to an activation of Rho kinase, PKC, integrin-linked kinase, and Zip kinase (10, 17, 40, 41, 50). Our data suggests that, in smooth muscle during ANG II activation, a Rho kinase signaling pathway leads to the activation of NM myosin. Rho kinase could phosphorylate the NM-RLC, but also could increase phosphorylation of the myosin targeting subunit (MYPT1) of MLC phosphatase (41, 42), or lead to an integrin-linked kinase or Zip kinase-dependent NM-RLC phosphorylation.

Agonist stimulation, compared with depolarization, is well known to result in a Ca\(^{2+}\)-independent force enhancement (4, 23, 41, 46). Force enhancement has been attributed to a G protein-mediated increase in SM-RLC phosphorylation (23). Possible mechanisms include an inhibition of MLC phosphatase activity by a Rho kinase-dependent phosphorylation of MYPT1 (41, 42), as well as a PKC and/or Rho kinase-dependent phosphorylation of CPI-17 and/or PHI-1 (6–8, 22). However, SM-RLC and NM-RLC cannot be resolved by glycerol-based electrophoresis methods often used to determine MLC phosphorylation, nor can commonly used anti-SM-RLC or anti-phospho-SM-RLC antibodies distinguish between SM-RLC and NM-RLC (Fig. 1). These data could suggest that the increase in SM-RLC phosphorylation observed during Ca\(^{2+}\)-independent force enhancement may not always be explained solely by an increase in SM-RLC phosphorylation, but, in some instances, could result from an increase in NM-RLC phosphorylation.

NMIIB’s biochemical properties are consistent with a structure designed to maintain force (25, 39, 49). For NMIIB, the rate of ADP release is near the steady-state ATPase rate, and, unlike other types of class II myosin, the addition of actin does not accelerate ADP release from NMIIB, but instead enhances ADP binding (49). Consequently, NMIIB spends the majority activity to produce an increase in SM-RLC phosphorylation and force (6–8, 22).
of its kinetic cycle attached to actin (39, 49). Sellers’ group has also demonstrated that ADP release from NMIIB is slow and strain dependent; ADP release from NMIIB increases 4-fold with positive strain and decreases 12-fold with negative strain (25). The negative strain, or resistive load, on NMIIB during force maintenance would increase the duty ratio to lengthen the attachment time (25), and both heads of the NMIIB would be attached to actin, which describes the ideal latch cross bridge. Furthermore, load-dependent ADP release, coupled with the rapid binding of ADP to NMIIB, prolongs NMIIB’s attachment to actin at 10–100 μM ADP, even at normal levels of MgATP, which could decrease the ATPase to <0.01 ATP per head second during force maintenance (25). The detachment of smooth muscle myosin from actin has been demonstrated to depend on strain; the duration of the first phase of cross-bridge attachment changes by a factor of 2 when either positive or negative strain is applied to the cross bridge (47). However, compared with smooth muscle myosin, the kinetics of NMIIB are much slower, and the dependence on strain significantly higher, which make NMIIB an attractive candidate for a latch cross bridge.

Our results also demonstrate that, in aortic smooth muscle of the B<sup>+/−</sup> mice, compared with WT aortic smooth muscle strips, the reduction in NMIIB expression was associated with a 25% decrease in force maintenance. The expression of smooth muscle myosin and NMIIA was similar in B<sup>+/−</sup> and WT aortic strips (Fig. 7), which suggests that the 25% reduction in force maintenance is due to the decrease in NMIIB expression. Similarly, Arner’s group has demonstrated that bladder tissue from the smooth muscle MHC KO mouse line produces ~11% of the force of WT bladder (27), and we have demonstrated that inhibition of NM myosin with blebbistatin decreases force maintenance by 8% in the mouse bladder and 24% in the mouse aorta (38). These results are all consistent with NM myosin contributing to a physiologically relevant pathway for force maintenance.

Another approach for determining whether NM myosin can participate in force maintenance is to calculate the isometric force for NM myosin as (F/CB)/H, where F/CB is the force per attached cross bridge, N is the myofilament density, H is the number of myosin heads per half filament, and f is the fraction of attached myosin heads. Presumably in vivo, each SM-RLC associates with a smooth muscle MHC, and each NM-RLC associates with a NM MHC, and thus the ratio of NM myosin to total myosin can be calculated from analysis of one-dimensional gel analysis (Fig. 4 and 5) as the ratio of the density of the NM-RLC/(NM-RLC + SM-RLC). Using this method, NMII expression in the aorta is 10.5 ± 0.1% (n = 15) of total myosin, which agrees with our calculation of 13% determined from analysis of one-dimensional gels, which separate smooth muscle from NM MHC (33), which demonstrates NM-RLC and NM MHCs are expressed at a ratio of 1:1. Similar to our results, others have reported that the NM myosin content of swine carotid media is 14–16% of total myosin (11). If we assume that 1) the force/cross bridge is 4 pN (9, 18, 29); 2) NMII myofilament density is 0.02 × 10<sup>15</sup> m<sup>−2</sup> [10–15% of the density for smooth muscle myosin, which is one-fifth (31) of the 0.51 × 10<sup>15</sup> m<sup>−2</sup> for the density of myosin filament in skeletal muscle (26)]; 3) 20 heads per half filament (48); and 4) 0.36 of NM myosin heads are attached, then the force produced by NM myosin is ~0.6 kN/m² (~0.6 mN/m²), or 7% of the 9 mN/m² produced by WT aortic smooth muscle strips.

This calculated force for NM myosin is about one-fourth of the actual reduction in force maintenance (~2.3 mN/mm²) observed for the B<sup>+/−</sup> strain compared with WT tissues, but of a similar magnitude of that observed in the smooth muscle MHC KO mice (27, 30). The calculated force generated by NM myosin would increase if the force for a single NMIIB were larger than 4 pN. This appears unlikely, as the force produced by an individual cross bridge has been shown to be relatively constant for many different myosin molecules. However, NMIIB could be able to sustain a much greater force for resistive load than what it is able to produce during its force-producing isomerization, reflecting the kinetics of ADP release (25). Additionally, we assumed that only 36% of the NMIIB would attach to actin, since the ANG II-induced increase in NM-RLC phosphorylation is 36%. NMIIB has been demonstrated to have a high affinity for ADP (49), and for resistive loads, both heads of NMIIB would be attached to actin and rarely detach (25). This property could lead to cooperative recruitment of either nonphosphorylated NM or SM myosin heads during force maintenance to significantly increase the fraction of myosin attached to actin. In addition, the ability of NMIIB to bear a resistive load during force maintenance...
would decrease the strain on attached smooth muscle myosin cross bridges. This would decrease detachment of smooth muscle myosin and result in a relative increase in the number of smooth muscle myosin molecules attached to actin during force maintenance, which represents another mechanism to sustain force.

Nonetheless, our results demonstrate that NM myosin is activated during smooth muscle contraction. During depolarization, the phosphorylation of the NM-RLC by Ca\(^{2+}\)-calmodulin-dependent MLCK. However, during ANG II stimulation, the phosphorylation of NM myosin is dependent on a Rho kinase-mediated signaling pathway. Data from trans-stimulation, the phosphorylation of NM myosin is dependent on a decrease in force maintenance. Thus, despite the low levels of expression of NMIIB, its biochemical properties allow it to participate in a physiologically relevant pathway to sustain force in smooth muscle.

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