Myosin phosphorylation triggers actin polymerization in vascular smooth muscle

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Chen X, Pavlish K, Benoit JN. Myosin phosphorylation triggers actin polymerization in vascular smooth muscle. Am J Physiol Heart Circ Physiol 295: H2172–H2177, 2008.—A variety of contractile stimuli increases actin polymerization, which is essential for smooth muscle contraction. However, the mechanism(s) of actin polymerization associated with smooth muscle contraction is not fully understood. We tested the hypothesis that phosphorylated myosin triggers actin polymerization. The present study was conducted in isolated intact or β-escin-permeabilized rat small mesenteric arteries. Reductions in the 20-kDa myosin regulatory light chain (MLC20) phosphorylation were achieved by inhibiting MLC kinase with ML-7. Increases in MLC20 phosphorylation were achieved by inhibiting myosin light chain phosphatase with microcystin. Isometric force, the degree of actin polymerization as indicated by the F-actin-to-G-actin ratio, and MLC20 phosphorylation were determined. Reductions in MLC20 phosphorylation were associated with a decreased force development and actin polymerization. Increased MLC20 phosphorylation was associated with an increased force generation and actin polymerization. We further found that a heptapeptide that mimics the actin-binding motif of myosin II enhanced microcystin-induced force generation and actin polymerization without affecting MLC20 phosphorylation in β-escin-permeabilized vessels. Collectively, our data demonstrate that MLC20 phosphorylation is capable of triggering actin polymerization. We further suggest that the binding of myosin to actin triggers actin polymerization and enhances the force development in arterial smooth muscle.

isometric force; β-escin; myosin light chain kinase; myosin light chain phosphatase; ML-7; microcystin

INCREASED ACTIN POLYMERIZATION has been demonstrated during smooth muscle contraction (6, 7, 18, 27). As a result, actin polymerization has been deemed essential for smooth muscle contraction (13, 27). However, the dynamic regulation of actin polymerization is still not fully understood. To date, three models describing how filamentous actin (F-actin) is formed have been proposed. The first proposes that the uncapping of existing filament ends allows for the addition of globular actin monomers (G-actin) to the uncapped end of the thin filament (5). The second proposes that the cleavage of existing filaments by cytoplasmic coflin exposes uncapped ends, thereby increasing the number of sites available for filament elongation (5). The third proposes the de novo synthesis of filamentous actin (21, 29, 32). However, none of the aforementioned mechanisms can fully explain the dynamic regulation of actin polymerization during smooth muscle contraction.

One would predict the existence of a common mechanism regulating actin polymerization, especially since the polymerization appears to be independent of the contractile stimulus. In this study, we tested the hypothesis that phosphorylated myosin triggers actin polymerization. Our findings indicate that 20-kDa myosin regulatory light chain (MLC20) phosphorylation is sufficient to trigger actin polymerization and further demonstrate that the binding of phosphorylated myosin to actin increased actin polymerization. We suggest that the phosphorylation of myosin plays a key role in the dynamic regulation of vascular smooth muscle actin polymerization.

MATERIALS AND METHODS

Preparation of isolated vessels. Male Sprague-Dawley rats (~300 g, Charles River, Wilmington, MA) were anesthetized with isoflurane and euthanized. A segment of mid-small intestine and adjacent mesentery was excised and placed in ice-cold physiological salt solution (PSS, pH 7.4). Small mesenteric arteries (diameter, ~150 μm) were dissected free of surrounding tissue. Two adjacent mesenteric artery segments (3 to 4 mm in length) from each animal were used for contractile function studies. The remaining arteries were prepared for biochemical analysis. All animal procedures were approved by the Animal Care and Use Committee of the University of North Dakota.

Contractile function studies of intact small mesenteric arteries. Isometric tension responses of small mesenteric arteries were evaluated on a small vessel myograph (6). Once the vessels were mounted on the myograph, they were normalized to an internal circumference of 0.9 L100, where L100 is the circumference that the vessels would reach if fully relaxed and under an internal pressure of 100 mmHg. At this internal circumference, the vessels develop near-maximal active wall tension. Normalization was performed by distending the vessel in step increments while measuring force. After normalization, the vessels were primed three times with 10 μM phenylephrine (Sigma). After washout of the phenylephrine with PSS and the return of the vessel to baseline conditions, phenylephrine (10 μM) was added to the bath and, 2 min later, a myosin light chain kinase (MLCK) inhibitor MI-7 (10 μM, Sigma) was added to the bath. Force was recorded throughout the experiment. In some experiments, latrunculin A (0.1 μM, Sigma) was added to the bath after treatment with phenylephrine (10 μM) for 5 min. To prepare vessels for biochemical analysis, the vessels at baseline, phenylephrine (10 μM) stimulation for 2 min, phenylephrine stimulation for 2 min plus MI-7 (10 μM) incubation for 5 min, and phenylephrine stimulation for 7 min were snap frozen and stored in liquid N2.

Contractile function studies of permeabilized small mesenteric arteries. The small mesenteric artery rings were stretched to an optimum length by the normalization procedure described in Contractile function studies of intact small mesenteric arteries. The vessels were then permeabilized by treatment with β-escin according to...
previously described methods (2, 15, 24). Briefly, the small mesenteric arteries were incubated for 20 min in Ca²⁺-free PSS followed by 10 min in a relaxing solution. They were then incubated with β-escin (60 μM) for 30 min at 25°C in a relaxing solution. The relaxing solution contained (in mM) 30 TES, 10 EGTA, 7.5 Na₃ATP, 46.6 potassium methanesulfonate, 7.92 magnesium acetate, and 10.0 creatine phosphate and 100 U/ml creatine kinase (pH 7.0). In addition, the relaxing solution contained 1 mM carbonyl cyanide 4-trifluoromethoxyphenylhydrazone, and 1 mM leupeptin, 1 μM calmodulin, 1 mM leupeptin, 1 μM carbonyl cyanide 4-trifluoromethoxyphenylhydrazone, and 1 mM dithioerythritol. To deplete the sarcoplasmic reticulum of Ca²⁺ and maintain a Ca²⁺-free environment, every vessel was also treated with 10 μM A-23187 for 15 min in the relaxing solution after the β-escin was washed out of the preparation with the relaxing solution. A myosin light chain phosphatase (MLCP) inhibitor, microcystin (3 μM), was then added into the bath and the force recorded for 40 min. In some experiments, latrunculin A (0.1 μM) was added to the bath after treatment with microcystin (3 μM) for 30 min. For biochemical analysis, permeabilized vessels were isolated by fractionation and those with microcystin (3 μM) for 30 min were snap frozen and stored in liquid N₂.

In a separate experiment, permeabilized vessels were incubated with a heptapeptide (50 μM), which is homologous to the actin-binding site of rat myosin II, and a scrambled heptapeptide (50 μM) for 30 min. The sequence for the heptapeptide is NH₂-Ile-Arg-Ile-Cys-Gly-Ile-Arg-Ile-COOH (synthesized for 30 min. In some experiments, latrunculin A (0.1 μM) was then added into the bath and the force recorded for 40 min. In some experiments, latrunculin A (0.1 μM) was added to the bath after treatment with microcystin (3 μM) for 30 min. For biochemical analysis, permeabilized vessels were isolated by fractionation and those with microcystin (3 μM) for 30 min were snap frozen and stored in liquid N₂.

Analysis of F-actin-to-G-actin ratio. F-actin and G-actin in mesenteric arterial smooth muscle were isolated by fractionation and ultracentrifugation and quantified by electrophoresis (6). Briefly, the arteries were homogenized in F-actin stabilization buffer containing 50 mM PIPES (pH 6.9), 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.001% antifoam, 1 mM ATP, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 10 μg/ml benzamidine, and 500 μg/ml tosyl arginine methyl ester. The supernatants of the protein extracts were collected after ultracentrifugation (100,000 g, 1 h at 37°C). The pellets were resuspended in 200 μl ice-cold distilled water plus 1 μM cytochalasin D and then incubated on ice for 1 h to dissociate F-actin. The resuspended pellets were gently mixed every 15 min. The supernatant (G-actin) and pellet (F-actin) fractions were diluted 20 times and analyzed by immunoblotting using mouse anti-α-smooth muscle actin antibody (Sigma). The ratio of F-actin to G-actin was determined by scanning densitometry.

Detection of MLC2₀ phosphorylation by immunoblotting. The vessels were homogenized with a ground glass homogenizing tube in ice-cold lysis buffer containing 100 mM K₂HPO₄, 1 mM phenylmethyl sulfonyl, and 0.2% Triton X-100, and 0.2% Triton X-100. The homogenates were centrifuged at 14,000 g for 20 min at 4°C, and the supernatants were collected for Bradford protein concentration analysis. An equal amount of proteins (10 μg) were loaded onto 12.5% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane and blocked with 3% BSA for 1 h. The membranes were treated with 1:1,000 anti-phospho-MLC2₀ (direct against Ser19 phosphorylation (Cell Signaling Technology) or 1:10,000 anti-MLC2₀ (a generous gift from Dr. Kristine Kamm, Univ. of Texas Southwestern Medical Center) antibodies overnight at 4°C. After the membranes were washed with Tris-buffered saline plus Tween 20, the blots were exposed to film, scanned, and quantified.

Fig. 1. Effects of myosin light chain (MLC) kinase (MLCK) inhibition on phenylephrine (PE) induced-force development, 20 kDa MLC (MLC₂₀) phosphorylation (MLC₂₀–P), and actin polymerization. A: inhibition of MLCK by ML-7 reduced PE-induced maximal tension back to baseline values (n = 5). B: relative changes in MLC₂₀–P. PE (10 μM, 2 min) significantly increased MLC₂₀–P (PE 2 min). Increased MLC₂₀–P persisted for 7 min after addition of PE (PE 7 min). Increased MLC₂₀–P by PE was reduced back to baseline levels by addition of ML-7 (10 μM) for 5 min (PE + ML-7) (n = 5, *P < 0.05 vs. baseline; #P < 0.05 vs. PE 2 min and PE 7 min). C: PE significantly increased F-actin-to-G-actin (F/G-actin) ratio, which was returned to baseline levels by ML-7 (n = 5, *P < 0.05 vs. baseline; #P < 0.05 vs. PE 2 min). D: maximal force induced by PE persisted for at least 30 min. Maximal force induced by PE was markedly reduced by latrunculin A (0.1 μM).
Statistical analysis. All data were expressed as means ± SE. Comparisons between two groups were performed with an unpaired Student’s t-test. The statistical significance of multiple treatments was determined by ANOVA and Tukey’s post hoc test. P values of <0.05 were considered to be significant.

RESULTS

Effects of MLCK inhibition on phenylephrine-induced force development, MLC20 phosphorylation, and actin polymerization. As shown in Fig. 1, phenylephrine (10 μM) induced a marked increase in force generation in isolated intact small mesenteric arteries, and the maximal tension remained unchanged without the treatment of ML-7. Once the maximal tension was maintained, the inhibition of MLCK by ML-7 (10 μM) reduced the tension back to baseline values (n = 5). The stimulation of the arteries with phenylephrine (10 μM) for 2 min greatly increased MLC20 phosphorylation. This increased MLC20 phosphorylation remained relatively unchanged after 7 min of phenylephrine stimulation, but it was reduced back to baseline levels by MLCK inhibition with ML-7 (10 μM). Phenylephrine also significantly increased the F-actin-to-G-actin (F/G-actin) ratio, which was decreased back to the baseline value by ML-7 (n = 5, P < 0.05). Phenylephrine-induced maximal force persisted for at least 30 min; the adding of an actin depolymerizing agent, latrunculin A (0.1 μM), markedly reduced the maximal force induced by phenylephrine.

Effects of MLCP inhibition on force development, MLC20 phosphorylation, and actin polymerization. As shown in Fig. 2, the inhibition of MLCP by microcystin (3 μM) induced a twofold increase in tension in β-escin-permeabilized small mesenteric arteries (n = 5). The baseline levels of MLC20 phosphorylation were reduced to below the limits of detection, an observation consistent with a Ca2+-free environment. The treatment of permeabilized vessels with microcystin (3 μM) for 30 min greatly increased MLC20 phosphorylation and significantly increased F/G-actin ratio (n = 5, P < 0.05). Although the maximal tension induced by microcystin (3 μM) persisted for at least 40 min without a marked reduction, the addition of latrunculin A (0.1 μM) markedly decreased maximal tension induced by microcystin.

Effects of actin-binding motif of myosin II on microcystin-induced force development, MLC20 phosphorylation, and actin polymerization. To investigate the effects of actin-binding motif of myosin II on force generation, MLC20 phosphorylation, and actin polymerization, a synthetic heptapeptide mimicking the actin-binding motif of rat myosin II was introduced into permeabilized small mesenteric arteries, with a scrambled heptapeptide as a control (Fig. 3). The treatment with the heptapeptide or scrambled peptide alone did not induce force generation. However, the pretreatment with the heptapeptide significantly enhanced the microcystin-induced maximal tension when compared with the pretreatment of scrambled heptapeptide (n = 4). The pretreatment of scrambled peptide did not affect the microcystin-induced tension development. Neither the heptapeptide nor the scrambled heptapeptide changed the microcystin-induced MLC20 phosphorylation. However, the pretreatment with heptapeptide significantly enhanced the microcystin-induced increases in the F/G-actin ratio when compared with the scrambled heptapeptide pretreatment (n = 4, P < 0.05). The pretreatment with the scrambled heptapeptide did not affect the microcystin-induced increases in the F/G-actin ratio.

DISCUSSION

Phosphorylation of MLC20 is essential for vascular smooth muscle contraction, and myosin phosphorylation is dependent on the balance of the activity of MLCK and MLCP (1, 22). To
phenylephrine was maintained, the inhibition of MLCK by ML-7 (10 μM) reduced the maximal tension back to the baseline values. Interestingly, we found that the inhibition of MLCK by ML-7 returned the phenylephrine-induced increases in the F/G-actin ratio back to the baseline values. These results indicate that the reductions in MLC20 phosphorylation are capable of decreasing actin polymerization in stimulated vessels.

We further evaluated the effects of MLCP inhibition on force development, MLC20 phosphorylation, and actin polymerization (Fig. 2). The inhibition of MLCP would have the opposite effects of MLCK inhibition by shifting the balance of MLCK/MLCP, favoring MLC20 phosphorylation. These experiments were conducted in β-escin-permeabilized small mesenteric arteries under Ca2+-free conditions when only trace levels of Ca2+ would be present. No MLC20 phosphorylation reached a detectable level in our assay under these conditions. This observation is consistent with the fact that Ca2+ plays an important role in the regulation of MLCK activity. MLCP was inhibited by microcystin at the concentration of 3 μM, and this concentration of microcystin has been shown to generate maximal force in permeabilized vessels (31). As expected, microcystin markedly increased MLC20 phosphorylation. Our observation that microcystin markedly increased MLC20 phosphorylation under Ca2+-free conditions indicates that a pool of constitutively active MLCK is present under the conditions when only trace levels of Ca2+ are present (26). The inhibition of MLCP by microcystin (3 μM) also induced a twofold increase in tension development in permeabilized small mesenteric arteries. Importantly, we found that the inhibition of MLCP by microcystin increased the F/G-actin ratio. These results suggest that the increased MLC20 phosphorylation increases actin polymerization. This evidence, coupled with our findings (Fig. 1) that reductions in MLC20 phosphorylation decrease actin polymerization, indicates that MLC20 phosphorylation is sufficient for actin polymerization in vascular smooth muscle. Our findings are consistent with another report that MLC20 phosphorylation is both necessary and sufficient for the assembly of stress fibers and focal adhesions in 3T3 fibroblasts (28). Phosphorylation of MLC20 triggers the binding of type II myosin (the predominant myosin isoform in vascular smooth muscle) to actin filaments (1). Myosin, thus, converts chemical energy (ATP) into mechanical work by cyclic interaction with F-actin (17). With the use of various synthetic oligopeptides, it has been determined that the actin-binding site of the myosin head is near the reactive Cys (SH1)-705 (25). Based on comparisons of the dissociation constants of various analog peptides with F-actin, a heptapeptide, NH2-Ile-Arg-Ile-Cys-Arg-Lys-Gly-COOH, has been assigned as the smallest one, having the highest affinity for F-actin (25). It is interesting to notice that myosin binds two adjacent actin monomers simultaneously (14). Thus myosin might work as a cross-linker and induce actin polymerization by linking the two adjacent actin monomers. Indeed, it has been shown that the myosin head increases actin polymerization in vitro (3, 4, 8, 10, 19, 30).

The in vitro evidence that the myosin head increases F-actin formation prompted us to further evaluate the effects of the actin-binding motif of myosin II on microcystin-induced force development, MLC20 phosphorylation, and actin polymerization (Fig. 3). In our study, a synthetic heptapeptide mimicking
the actin-binding motif of rat myosin II was introduced into β-escin-permeabilized small mesenteric arteries, with a scrambled heptapeptide as a control. We found that neither the heptapeptide nor scrambled heptapeptide alone induced force generation. However, the pretreatment of the heptapeptide (50 μM) but not the scrambled heptapeptide (50 μM) significantly enhanced microcystin-induced maximal force development in permeabilized small mesenteric arteries.

At first glance, these results seem contradictory to the reasoning that the introduction of the actin-binding motif into vascular smooth muscle would block the myosin-binding sites on actin filament, thus decreasing the force development. However, by further examining the machinery of vascular smooth muscle, one will notice that the efficiency of the force generation is also dependent on actin cytoskeleton remodeling and that the remodeling of the actin cytoskeleton enhances the efficiency of force generation in smooth muscle (11–13). In supporting our results, a similar synthetic peptide with an amino acid sequence in the vicinity of the actin-binding site on the myosin head has been shown to increase the force generation in both permeabilized skeletal muscle and cardiac muscle (14, 23). It has long been noticed that the myosin head accelerates the polymerization of actin (3, 4, 8, 10, 19, 30), and a heptapeptide similar to that used in the present study has been shown to stimulate actin polymerization in vitro (9). Consistent with these reports, the present study demonstrates that the introduction of the heptapeptide (actin-binding motif of myosin II) into permeabilized vessels significantly enhanced microcystin-induced MLC20 phosphorylation (Fig. 3). These results indicate that the binding of phosphorylated myosin to actin enhances the microcystin-induced actin polymerization and force development in permeabilized small mesenteric arteries.

In the present study, we also found that depolymerizing actin filaments with latrunculin A markedly decreased phenylephrine- and microcystin-induced maximal force development (Figs. 1 and 2). The concentration of latrunculin A used in the present study has been previously shown by us to have no effect on MLC20 phosphorylation (6). This observation, coupled with our previous finding that the maximal force development is directly related to the degree of actin polymerization (6), indicates that actin polymerization plays a key role in the force development of vascular smooth muscle. Our results suggest that smooth muscle contraction requires the synchronized and choreographed work of both myosin phosphorylation and the dynamics of actin polymerization. We further hypothesize that an increased actin polymerization is necessary to maintain the maximal force development and that the maximal force will persist as long as the actin filament is polymerized.

In summary, our results indicate that MLC20 phosphorylation is sufficient to trigger actin polymerization and that the binding of phosphorylated myosin to actin further increases actin polymerization and force development. We suggest that the phosphorylation of myosin plays a key role in the dynamic regulation of actin polymerization in vascular smooth muscle. Our finding that phosphorylated myosin triggers actin polymerization in smooth muscle provides new insights into the understanding of contractile function of smooth muscle.

**REFERENCES**


**GRANTS**

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