Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo

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Mizuno Y, Isotani E, Huang J, Ding H, Stull JT, Kamm KE. Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo. Am J Physiol Cell Physiol 295: C358–C364, 2008. First published June 4, 2008; doi:10.1152/ajpcell.90645.2007.—Ca2+/calmodulin (CaM)-dependent phosphorylation of myosin regulatory light chain (RLC) in smooth muscle by myosin light chain kinase (MLCK) and dephosphorylation by myosin light chain phosphatase (MLCP) are subject to modulatory cascades that influence the sensitivity of RLC phosphorylation and hence contraction to intracellular Ca2+ concentration ([Ca2+]i). We designed a CaM-sensor MLCK containing smooth muscle MLCK fused to two fluorescent proteins linked by the MLCK CaM-binding sequence to measure kinase activation in vivo and expressed it specifically in mouse smooth muscle. In phasic bladder muscle, there was greater RLC phosphorylation and force relative to MLCK activation and [Ca2+]i with carbachol (CCh) compared with KCl treatment, consistent with agonist-dependent inhibition of MLCP. The dependence of force on MLCK activity was nonlinear such that at higher concentrations of CCh, force increased with no change in the net 20% activation of MLCK. A significant but smaller amount of MLCK activation was found during the sustained contractile phase. MLCP inhibition may occur through RhoA/Rho kinase and/or PKC with phosphorylation of myosin phosphatase targeting subunit-1 (MYPT1) and PKC-potentiated phosphatase inhibitor (CI-17), respectively. CCh treatment, but not KCl, resulted in MYPT1 and CI-17 phosphorylation. Both Y27632 (Rho-kinase inhibitor) and calphostin C (PKC inhibitor) reduced CCh-dependent force, RLC phosphorylation, and phosphorylation of MYPT1 (Thr694) without changing MLCK activation. Calphostin C, but not Y27632, also reduced CCh-induced phosphorylation of CI-17. CCh concentration responses showed that phosphorylation of CI-17 was more sensitive than MYPT1. Thus the onset of agonist-induced contraction in phasic smooth muscle results from the rapid and coordinated activation of MLCK with hierarchical inhibition of MLCP by CI-17 and MYPT1 phosphorylation.

myosin regulatory light chain; calmodulin; bladder; Rho kinase; myosin light chain phosphatase

AN INCREASE in intracellular calcium concentration ([Ca2+]i) initiates smooth muscle contraction through phosphorylation of myosin regulatory light chain (RLC) at Ser19 by Ca2+/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) (15, 16, 37). The phosphorylation of RLC allows actin to activate the myosin ATPase leading to the consequent contraction (34). The Ca2+-dependent activation of MLCK plays an important role in force development in smooth muscle, and RLC phosphorylation is in part regulated by changes in [Ca2+]i. Myosin light chain phosphatase (MLCP) dephosphorylates RLC, resulting in inactivation of actin-activated ATPase. Thus the extent of RLC phosphorylation results from a balance of MLCK and MLCP activities.

The dependence of agonist-induced force and RLC phosphorylation on [Ca2+]i is less than that observed for depolarization in smooth muscle (2, 11) due to Ca2+-sensitization mechanisms (33). It is generally assumed, but yet unmeasured, that force is similarly sensitized to MLCK activation. Mechanisms responsible for Ca2+ sensitization in smooth muscle involve primarily inhibition of MLCP activity by coupling among ligand receptors, G proteins, and guanine nucleotide-binding factors (33). Two major pathways have been identified for inhibition of MLCP activity in smooth muscle. One involves activation of RhoA and Rho kinase through receptor coupling with mainly G12/13 heterotrimeric G proteins (35). Rho-kinase phosphorylates myosin light chain kinase targeting subunit-1 (MYPT1) at Thr694 and/or Thr850 (mouse sequence), leading to inhibition of MLCP activity in smooth muscle cells and tissues (14, 25, 27, 32, 33). This phosphorylation has been reported to be involved in the tonic phase of force development (6, 39); however, the phosphorylation of both sites is not invariably detectable in all tissues (18, 27). A second pathway involves activation of PKC through receptor coupling with Gq11 and phospholipase Cβ (17). CPI-17 is a 17-kDa polypeptide and potential mediator of Ca2+ sensitization where PKC phosphorylates CPI-17 in smooth muscle (7, 17, 19). Phosphorylation of CPI-17 Thr38 enhances its potency for inhibiting MLCP activity.

To understand how signaling mechanisms involving MLCK activation are integrated with MLCP inhibition in smooth muscle contraction, we developed a genetically encoded sensor for activation of MLCK. The CaM-sensor MLCK contains short smooth muscle MLCK fused to two fluorophores, enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), linked by the MLCK calmodulin-binding sequence. Upon dimerization there is significant fluorescence resonance energy transfer (FRET) from the donor ECFP (480 nm emission) to the acceptor EYFP (525 nm emission) (8, 13). This CaM-sensor MLCK is capable of directly monitoring Ca2+/CaM binding and activation of the kinase, where Ca2+-dependent CaM binding increases kinase activity coincident with a decrease in FRET. The CaM-sensor MLCK is expressed specifically in smooth muscle tissue of transgenic mice to obtain real-time and quantitative information on MLCK activation in vivo. We previously reported that membrane depolarization with KCl induced a greater maximal increase in [Ca2+]i, and MLCK phosphorylation, and isometric force.

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activation than that obtained with the agonist carbachol (CCh) in bladder smooth muscle from the transgenic mice (13). However, the force development and RLC phosphorylation were comparable. In addition, the Rho-kinase inhibitor Y27632 decreased CCh-induced force while not significantly affecting MLCK activation (13). These results were consistent with the hypothesis that Rho-kinase activation inhibits MLCP to enhance the small extent of agonist-induced MLCK activation. However, the mechanism of this apparent increase in sensitivity to [Ca\(^{2+}\)], with CCh was not fully elucidated. Although some reports showed that MLCP inhibition was induced slowly during tonic contraction in response to agonist treatment (35), MLCP inhibition appeared to occur rapidly during phasic contraction elicited by CCh in the bladder (13). Other evidence also suggests that Rho-kinase modulates contraction in phasic smooth muscle (9, 28, 31, 38). It is proposed for intestinal smooth muscle that there is an initial [Ca\(^{2+}\)], transient leading to MLCK activation and then inactivation followed by MLCP inhibition to sustain RLC phosphorylation and contraction (26). Thus activation of MLCK would be temporally distinct from inhibition of MLCP. In the present study, we test the hypothesis that agonist-induced contraction results from rapid Ca\(^{2+}\) sensitization with phosphorylation of CPI-17 and MYPT1 simultaneous with Ca\(^{2+}\)/CaM-dependent activation of MLCK.

MATERIALS AND METHODS

Transgenic mice with CaM-sensor MLCK expression specifically in smooth muscle tissue were bred and screened as previously described (8, 13). Expressed amounts of the MLCK CaM-sensor were <40% the content of endogenous kinase, and stresses generated by bladder strips from wild-type and transgenic animals were comparable (13). Transgenic animals showed no obvious phenotypic differences from wild-type (13). All animal protocols were approved by the University of Texas Southwestern IACUC.

Simultaneous measurement of fluorescence and CaM-sensor MLCK FRET or [Ca\(^{2+}\)]. Mouse bladder tissues were obtained from 8- to 12-wk-old transgenic mice expressing CaM-sensor MLCK where simultaneous measurements of force and FRET or [Ca\(^{2+}\)], were made as previously described (13). Bladder tissues were dissected into strips (0.5 × 0.5 × 8.0 mm), mounted, and stretched (1.2 × slack length) on a force transducer in a quartz cuvette (180 muscle strips were skinned with 100 phosphate; 0.05 PIPES, 5 magnesium methanesulfonate, 90 potassium methanesulfonate, 1.6 CaCl\(_2\), and 10.0 glucose containing 10 mM indo-1 AM, 0.01% pluronic F-127, and 0.02% indomethacin, pregassed with 95% O\(_2\)-5% CO\(_2\) at 36°C). For FRET measurement, the muscle strips were illuminated with an excitation wavelength of 440 nm, and emission intensity was measured at both 480 and 525 nm to derive the ratio of fluorescence values (R480/525) in strips contracted with 65 mM KCl (KCl replacing equivalent NaCl in PSS) or 10 mM CCh. After incubation in relaxing solution [in mM: 20 PIPES, 5 magnesium methanesulfonate, 90 potassium methanesulfonate, 1.6 CaCl\(_2\), and 10.0 glucose containing 10 μM indomethacin, pregassed with 95% O\(_2\)-5% CO\(_2\) at 36°C]. For FRET measurement, the muscle strips were illuminated with an excitation wavelength of 430 nm, and emission intensity was measured at both 480 and 525 nm to derive the ratio of fluorescence values (R480/525) in strips contracted with 65 mM KCl (KCl replacing equivalent NaCl in PSS) or 10 mM CCh. After incubation in relaxing solution [in mM: 20 PIPES, 5 magnesium methanesulfonate, 90 potassium methanesulfonate, 1.6 CaCl\(_2\), and 10.0 glucose containing 10 μM indomethacin, pregassed with 95% O\(_2\)-5% CO\(_2\) at 36°C]. For FRET measurement, the muscle strips were illuminated with an excitation wavelength of 430 nm, and emission intensity was measured at both 480 and 525 nm to derive the ratio of fluorescence values (R480/525) in strips contracted with 65 mM KCl (KCl replacing equivalent NaCl in PSS) or 10 mM CCh. After incubation in relaxing solution [in mM: 20 PIPES, 5 magnesium methanesulfonate, 90 potassium methanesulfonate, 1.6 CaCl\(_2\), and 10.0 glucose containing 10 μM indomethacin, pregassed with 95% O\(_2\)-5% CO\(_2\) at 36°C]. For FRET measurement, the muscle strips were illuminated with an excitation wavelength of 430 nm, and emission intensity was measured at both 480 and 525 nm to derive the ratio of fluorescence values (R480/525). Statistic. Statistical comparisons were performed by paired Student’s t-test for [Ca\(^{2+}\)], and MLCK activation, independent t-test for force development, and phosphorylation of RLC, CPI-17, and MYPT1. Two-tailed values were used and P values <0.05 were considered significant.

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RESULTS

Time course of responses to depolarization and agonist.
Both KCl and CCh elicit transient force development in the
phasic bladder smooth muscle. Bladder strips were stimulated
for 1 or 5 min with KCl (65 mM) or CCh (10 μM) and force,
RLC phosphorylation, intracellular calcium, and MLCK activa-
tion were measured (Fig. 1). Rates of force development
were similar for KCl and CCh with times to peak of 41 ± 5 s
and 31 ± 7 s, respectively; forces at 1 min were near maximal
at 87 ± 6% and 87 ± 3% (*n = 8, 7), respectively. Agonist-
dependent sensitization of force to [Ca^{2+}], and MLCK activation
was well manifested at 1 min of stimulation where forces
comparable to KCl were obtained by CCh at one-half the value
of MLCK activation (Fig. 1). At 5 min of stimulation, force
was reduced to low but significantly elevated values following
reductions in [Ca^{2+}], and MLCK activation (Fig. 1). However,
both [Ca^{2+}] and MLCK activation remained significantly
elevated above resting values at 5 min. Agonist-dependent
sensitization of force was also observed at 5 min where
comparable forces were achieved with CCh compared with
KCl at about one-half the value of MLCK activation.

Dependence of force on MLCK activation and RLC phosphorylation.
To more clearly define the sensitization of force to
MLCK activation, bladder muscle strips from transgenic mice
were stimulated for 1 min by different concentrations of KCl or
CCh. After each stimulus, strips were washed with PSS and
equilibrated for 15 min. The results illustrate agonist-depen-

Fig. 2. Dependence of force on MLCK activation and RLC phosphorylation in
bladder smooth muscle stimulated by KCl or CCh. A: MLCK activation and
force were measured in bladder muscle strips from transgenic mice stimulated
with 30, 40, 50, and 65 mM KCl (●, n = 5) or with 0.3, 1, 3, 10, and 30 μM
CCh (○, n = 8). Measurements were obtained at 1 min. Contraction was
normalized by the 65 mM KCl-induced contraction for each strip. B: force was
measured in bladder muscle strips stimulated with concentrations of KCl (●,
n = 5) or CCh (○, n = 5) as described in A. Strips were frozen at 1 min and
processed for measurement of RLC phosphorylation. Values are means ± SE.
Recent sensitization of force to MLCK activation, with the CCh relation lying to the left of that for KCl (Fig. 2A). In a similar set of experiments, strips stimulated for 1 min were frozen for measurement of RLC phosphorylation. Isometric force was generally linearly dependent on RLC phosphorylation for both CCh and KCl (Fig. 2B). This result is consistent with the hypothesis that the sensitization of force to MLCK activation arises from inhibition of phosphatase activity. Interestingly, the relationship between MLCK activation and force was not linear for either KCl or CCh, with higher concentrations eliciting proportionally greater force relative to MLCK activation. This was more pronounced for CCh and suggests that additional sensitization factors may come into play at higher agonist concentrations.

**Effects of depolarization and agonist on MYPT1 and CPI-17 phosphorylation.** Myosin phosphatase activity is regulated by signaling pathways that target CPI-17 or MYPT1 for phosphorylation. Phosphorylation of these proteins was measured at 65 mM KCl and 10 μM CCh. The stoichiometry of CPI-17 phosphorylation was about 0.25 mol phosphate per mole at rest. KCl stimulation did not significantly change the extent of phosphorylation of CPI-17 or MYPT1 at either 1 or 5 min (Fig. 3), consistent with the action of KCl to depolarize smooth muscle cells without activation of cell surface receptors. CCh treatment significantly increased phosphorylation of CPI-17 at 1 min, whereas MYPT1 phosphorylation was increased at both 1 and 5 min (Fig. 3). Western blot analyses show the two isoforms of MYPT1 with masses of 130 kDa and 110 kDa with both containing the phosphorylation site Thr694 (20, 33).

**Effects of Rho-kinase and PKC inhibitors on CCh signaling to myosin regulatory proteins.** As shown above, CCh led to significant increases in [Ca^{2+}], MLCK activation, RLC phosphorylation, and force after 1-min treatment in bladder strips. Pretreatment with Rho-kinase inhibitor Y27632 or the PKC inhibitor calphostin C significantly inhibited RLC phosphorylation and force responses to CCh without significantly affecting the values of intracellular calcium or the extent of MLCK activation (Fig. 4).

CCh-induced phosphorylation of CPI-17 was inhibited by the PKC inhibitor calphostin C but not by the Rho-kinase inhibitor Y27632 (Fig. 5). Both Y27632 and calphostin C reduced CCh-stimulated phosphorylation of MYPT1. Phosphorylation of either CPI-17 nor MYPT1 was reduced below resting values by the inhibitors. Thus basal phosphorylation of these two proteins does not appear to be mediated by PKC or Rho-kinase.

To test the hypothesis that increased sensitivity of force to MLCK activation with higher concentrations of CCh depends on recruitment of additional phosphatase inhibition, the dependence of CPI-17 and MYPT1 phosphorylation on CCh concentration was measured in a protocol similar to that described for Fig. 2. Results were normalized to compare the CCh sensitivities of CPI-17 and MYPT1 phosphorylation (Fig. 6). CPI-17 was significantly phosphorylated in response to 1 μM CCh, reaching a maximal value at 10 μM. In contrast, phosphorylation of MYPT1 (Thr694) increased significantly at greater concentrations of CCh. Estimated EC_{50} values were 0.7 and 5.0 μM for CPI-17 and MYPT1 phosphorylation, respectively. This differential recruitment may in part contribute to the increased sensitization seen at high concentrations of agonist.

**DISCUSSION.**

Previously we reported that CCh induced smaller increases in [Ca^{2+}], and MLCK activation than those induced with membrane depolarization by KCl in bladder smooth muscle, whereas force development and RLC phosphorylation were comparable. These results suggest that CCh enhanced the effect of MLCK activation through Ca^{2+-} sensitization mechanisms (13). One general view is that MLCK activation occurs in the initial phase of a contraction with the sustained phase maintained by inhibition of MLCP (26, 33). In phasic intestinal muscle cells without activation of cell surface receptors. CCh treatment significantly increased phosphorylation of CPI-17 at 1 min, whereas MYPT1 phosphorylation was increased at both 1 and 5 min (Fig. 3). Western blot analyses show the two isoforms of MYPT1 with masses of 130 kDa and 110 kDa with both containing the phosphorylation site Thr694 (20, 33).

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**Fig. 3. Phosphorylation of PKC-potentiated phosphatase inhibitor (CPI-17) and myosin phosphatase targeting subunit-1 (MYPT1) in bladder strips in response to 65 mM KCl or 10 μM CCh.**

**A:** Western blot analysis of tissue extracts subjected to urea-glycerol PAGE and detected with anti-CPI-17 antibody.

**B:** Western blot analysis of tissue extracts subjected to SDS PAGE and detected with anti-MYPT1 (Thr694) antibody.

**C:** Phosphorylation of CPI-17 with KCl and CCh treatment at 1 and 5 min (KCl 1, KCl 5) or CCh treatment at 1 and 5 min (CCh 1, CCh 5). (n = 10–13). Ratio of phosphorylated CPI-17 to total (unphosphorylated and phosphorylated) is expressed as moles of phosphate per mole of protein.

**D:** Phosphorylation of MYPT1 with KCl or CCh treatment for times as indicated in C (n = 7–9). Phosphorylation of MYPT1 is expressed as the ratio relative to that stimulated with CCh in the presence of 5 μM okadaic acid (OA). Values are means ± SE. *P < 0.05 when compared with value at rest; #P < 0.05 when compared with same treatment at 1 min.
smooth muscle it is proposed that a brief [Ca\textsuperscript{2+}]i transient results in MLCK activation, but [Ca\textsuperscript{2+}]i is quickly dissipated leading to MLCK inactivation while RLC phosphorylation and contractile force are maintained (26). By measuring MLCK activation directly in bladder tissue strips, we could evaluate the temporal relationships among these signaling processes. Similar to previous results (13) we find that CCh stimulation results in a fractional activation of MLCK that is smaller than results with KCl. However, in both cases, significant activation is maintained during the sustained phase of contraction.

Agonist-induced Ca\textsuperscript{2+} sensitization in smooth muscle is known to result from inhibition of MLCP activity primarily through signaling pathways that target CPI-17 and/or MYPT1 for phosphorylation (33). In this study, the Rho-kinase inhibitor Y27632 and PKC inhibitor calphostin C diminished RLC phosphorylation and force development without affecting MLCK activation, indicating that agonist-induced RLC phosphorylation and force result from not only MLCK activation but also involve Rho kinase and PKC activation. Elevated phosphorylation of MYPT1 and CPI-17 with CCh at 1 min supports the hypothesis that MLCP activity is inhibited by both MYPT1 and CPI-17 phosphorylation. CPI-17 phosphorylation was attenuated by calphostin C but not by Y27632, which is consistent with PKC-mediated phosphorylation. Interestingly, calphostin C did not reduce CPI-17 phosphorylation below that found in resting muscle, which was a significant amount (~0.25 mol/mol). It has been reported that protein kinases other than PKC can phosphorylate CPI-17 in vitro: ZIP-like kinase (22), protein kinase N (10), integrin-linked kinase (5), p21-activated kinase (40), and Rho kinase (20). However, Y27632 had no effect on CPI-17 phosphorylation, suggesting
that the transient phosphorylation induced by CCh and the extent of phosphorylation in resting muscle do not result from Rho-kinase activity. Furthermore, although CPI-17 phosphorylation occurred rapidly, it was not sustained. Thus CPI-17 phosphorylation would not be involved in Ca\(^{2+}\) sensitization occurred rapidly, it was not sustained. Thus CPI-17 Rho-kinase activity. Furthermore, although CPI-17 phosphorylation induced by CCh and the transient phosphorylation induced by CCh and the \cite{14, 36, 38}, and \cite{33}. Thr694 phosphorylation by Rho kinase has been detected in a variety of cells including smooth muscle (14, 36, 38), and this phosphorylation is inhibited by \cite{27632}. Previous studies show a positive correlation between Thr694 phosphorylation and force in smooth muscle tissue with agonist treatment (14, 29, 32). However, it is not invariably detectable in intact tissues showing Ca\(^{2+}\) sensitization (18, 27, 42). The reasons for this inconsistency are not clear. They may depend partly on the type of cells/tissues, methods of stimulation, or development of phosphorylation of MYPT1 (3). Additional analysis of data in different types of smooth muscle tissue stimulated with agonists that activate different signaling pathways may provide insights.

Depolarization by KCl did not lead to changes in phosphorylation of either Thr694 on MYPT1 or CPI-17 in the mouse bladder. However, KCl-dependent phosphorylation of MYPT1 or CPI-17 has been observed in vascular smooth muscle (6, 41). Preliminary data with the bladder indicate that, like Thr694, phosphorylation of Thr850 on MYPT1 was not changed with KCl treatment (0.25 ± 0.03 and 0.31 ± 0.05, control and stimulated 1 min, respectively). The dependence of force on MLCK activation with KCl is not entirely linear (Fig. 2A), suggesting that sensitization may occur at higher concentrations. Unlike the case for CCh, this effect does not appear to arise from inhibition of myosin phosphatase activity as neither MYPT1 nor CPI-17 phosphorylations are affected, and force is significantly elevated with no change in RLC phosphorylation between 50 and 65 mM KCl (Fig. 2B). Whereas this observation is tangential to the present study, it warrants speculation as to whether the effect reveals minor forms of regulation possibly mediated through actin-binding proteins caldesmon or calponin, though the physiological importance of thin filament regulation remains unclear (33). It is also worth noting a second aspect of the phasic contraction in mouse bladder, which is the apparent desensitization of contraction to calcium, as defined by the greater reduction in force than [Ca\(^{2+}\)]\(_{i}\), between 1 and 5 min, particularly evident with KCl. This phenomenon has been observed in skinned fibers of phasic smooth muscles as well and is shown to be calcium dependent, although the mechanism remains to be defined (24).

It is evident that the relative contributions of CPI-17 and MYPT1 in regulating MLCP activity differ depending on the time, type, and intensity of stimulation for a specific smooth muscle. In this study of phasic urinary bladder muscle, results suggest that both CPI-17 and MYPT1 participate in the early phase of sensitization, whereas only MYPT1 participates in the latter phase. This contrasts with tonic muscle, where only CPI-17 is phosphorylated in the early phase, and both CPI-17 and MYPT1 are phosphorylated in the sustained phase of contraction (6). Tonic, compared with phasic, smooth muscle contains relatively high ratios of CPI-17 to MYPT1, which Rho kinase is activated by PKC remains elusive. It is possible that the decrease of MYPT1 phosphorylation by calphostin C results in part from inhibition of RhoA/Rho-kinase pathway via PKC activation.

The phosphorylation site of MYPT1 in smooth muscle stimulated with agonist needs to be considered. There are two major phosphorylation sites on MYPT1, Thr694 and Thr850, and several minor sites mediated by Rho kinase or integrin-linked kinase (12). Recent studies reported direct or indirect interactions of PKC with RhoA in different cell types (1, 28, 30, 32), although the exact mechanism by which Rho kinase is activated by PKC remains elusive. It is possible that the decrease of MYPT1 phosphorylation by calphostin C results in part from inhibition of RhoA/Rho-kinase pathway via PKC activation.

In conclusion, our genetically encoded CaM-sensor MLCK provides opportunities for investigations on physiological processes involved in smooth muscle contraction. Results from these studies in bladder smooth muscle suggest that agonist-induced contraction results from the rapid and coordinated activation of Ca\(^{2+}\)/CaM-dependent MLCK that is significantly sustained as well as inhibition of MLCP through phosphorylation of CPI-17 and MYPT1.

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DISCLOSURES

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