Signaling through Myosin Light Chain Kinase in Smooth Muscles[*]

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Background: Myosin light chain kinase is necessary for smooth muscle contraction.
Results: The kinase is limiting for aortic, but not bladder smooth muscle contraction.
Conclusion: Myosin light chain kinase provides dissimilar contributions to signaling responses.
Significance: Haploinsufficiency of myosin light chain kinase may affect aortic smooth muscle cell contractile tone *in vivo*.

Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) phosphorylates smooth muscle myosin regulatory light chain (RLC) to initiate contraction. We used a tamoxifen-activated, smooth muscle-specific inactivation of MLCK expression in adult mice to determine whether MLCK was differentially limiting in distinct smooth muscles. A 50% decrease in MLCK in urinary bladder smooth muscle had no effect on RLC phosphorylation or on contractile responses, whereas an 80% decrease resulted in only a 20% decrease in RLC phosphorylation and contractile responses to the muscarinic agonist carbachol. Phosphorylation of the myosin light chain phosphatase regulatory subunit MYPT1 at Thr-696 and Thr-853 and the inhibitor protein CPI-17 were also stimulated with carbachol. These results are consistent with the previous findings that activation of a small fraction of MLCK by limiting amounts of free Ca^{2+}/calmodulin combined with myosin light chain phosphatase inhibition is sufficient for robust RLC phosphorylation and contractile responses in bladder smooth muscle. In contrast, a 50% decrease in MLCK in aortic smooth muscle resulted in 40% inhibition of RLC phosphorylation and aorta contractile responses, whereas a 90% decrease profoundly inhibited both responses. Thus, MLCK content is limiting for contraction in aortic smooth muscle. Phosphorylation of CPI-17 and MYPT1 at Thr-696 and Thr-853 were also stimulated with phenylephrine but significantly less than in bladder tissue. These results indicate differential contributions of MLCK to signaling. Limiting MLCK activity combined with modest Ca^{2+} sensitization responses provide insights into how haploinsufficiency of MLCK may result in contractile dysfunction *in vivo*, leading to dissections of human thoracic aorta.

All cells in the body contain myosin molecular motors that track on actin filaments to initiate motile events. The myosin II subfamily members, including smooth muscle myosin, are hexamers composed of heavy chain dimers and two pairs of myosin light chains (1, 2). The heavy chain motor domain binds reversibly to actin filaments, hydrolyzes ATP, and thereby converts chemical energy into mechanical force and movement. Smooth and nonmuscle myosins are activated by Ca^{2+}/calmodulin-dependent MLCK[3] that phosphorylates the myosin regulatory light chain subunit RLC (3–5). Myosin light chain phosphatase dephosphorylates RLC to induce relaxation. RLC phosphorylation drives diverse cellular movements such as cell division, cell migration, and cell-matrix adhesion as well as smooth muscle contraction (1, 2, 6, 7).

Neurotransmitters, hormones, and cytokines act on cell surface receptors with assorted signal transduction pathways converging to increase $[Ca^{2+}]_i$. Ca^{2+} binds to calmodulin, which then activates MLCK to phosphorylate RLC and initiate smooth muscle contraction (3–5). Signaling pathways also inhibit myosin light chain phosphatase, thereby increasing RLC phosphorylation without changing elevated $[Ca^{2+}]_i$ (Ca^{2+} sensitization) (8–13). Many studies indicate that agonist-mediated Ca^{2+} sensitization involving decreased myosin light chain phosphatase activity is due to two major signaling pathways, including phosphorylation of the regulatory subunit of the phosphatase, MYPT1, by a Rho kinase pathway (ROCK), and phosphorylation of a small inhibitor protein CPI-17 by PKC (14–17). Myosin light chain phosphatase activity may also be regulated by other kinases, including zipper-interacting protein kinase and integrin-linked kinase (13). The extent of RLC phosphorylation is thus balanced by the relative MLCK and myosin light chain phosphatase activities, each of which are regulated.

The quantitative and integrative relationships among the signaling molecules acting on RLC phosphorylation are not well understood but obviously contribute to cell biological responses. In contrast to tonic vascular smooth muscle cells, phasic smooth cells in the intestine and urinary bladder have

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[3] The abbreviations used are: MLCK, myosin light chain kinase; RLC, myosin regulatory light chain; MYPT1, myosin phosphatase targeting subunit-1; CPI-17, PKC-potentiated phosphatase inhibitor; ROCK, RhoA-associated kinase; SMMHC, smooth muscle myosin heavy chain.

less CPI-17 protein and more myosin light chain phosphatase (18–20). Previous investigations found free Ca^{2+}/calmodulin was limiting for MLCK activation in bladder smooth muscle despite the abundance of total calmodulin (21–23). Free Ca^{2+}/calmodulin is also limiting in nonmuscle cells (24, 25). In bladder smooth muscle, quantitative measurements showed only 20% MLCK activation upon maximal agonist stimulation (17, 23). However, RLC phosphorylation was tightly coupled to the rapid increase in $[Ca^{2+}]_i$ and to kinase activation by Ca^{2+}/calmodulin initiated by nerves releasing neurotransmitters (26). Thus, it appears that most of the MLCK may not be activated to initiate maximal physiological responses dependent on RLC phosphorylation, despite MLCK being essential for smooth muscle contractions (4, 5, 27). However, genetic and functional studies recently indicated that heterozygous loss-of-function mutations in the human MLCK gene, MYLK, are associated with aortic dissections, implying a loss of aortic smooth muscle contractile function (28). Thus, the loss of 50% MLCK activity may be sufficient to compromise contractile function, leading to pathological changes because MLCK may be limiting in smooth muscle cells of the aorta. We have examined this possibility with genetically modified mice to determine the contributions of different amounts of MLCK to signaling responses in bladder and aortic smooth muscles relative to signal transduction mechanisms involving phosphorylation of CPI-17 and MYPT1.

EXPERIMENTAL PROCEDURES

Generation of Genetically Modified Mice—Mice containing floxed Mylk alleles (Mylk$^{f/f}$) (4) were crossed with a SMMHC-CreER^{T2} transgenic mouse line expressing a fusion protein of the Cre recombinase with the modified estrogen receptor binding domain (CreER^{T2}) under the control of the smooth muscle myosin heavy chain (SMMHC) promoter (29). Cre-mediated recombination occurred robustly and exclusively in smooth muscle cells in tissues, but only after tamoxifen treatment (29). Mice were bred and screened as described previously (4, 29). Tamoxifen was injected intraperitoneally for five consecutive days at a dose of 1 mg/day. The tamoxifen (200 mg, Sigma) was dissolved in 2 ml of ethanol followed by 18 ml of sunflower oil at a concentration 10 mg/ml and stored at −20 °C for up to 1 month. Bladder and aortic tissues were harvested 18–20 days after starting tamoxifen treatment from transgenic mice containing Mylk$^{f/f,Cre+}$ and Mylk$^{+/f,Cre+}$ alleles (denoted as MLCK$^{SM-/-}$ and MLCK$^{SM+/-}$, respectively) and wild type mice (MLCK$^{SM+/+}$). All animal protocols were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

Preparation of Smooth Muscle Strips from Mice—The isolated urinary bladder was opened, and the urothelium and adventia were removed by blunt dissection to enrich for smooth muscle cells. The smooth muscle layer was dissected into longitudinal strips ($0.5 \times 0.5 \times 8.0$ mm) that were mounted on an isometric force apparatus in physiological salt solution (118.5 mM NaCl, 4.75 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 24.9 mM NaHCO$_3$, 1.6 mM CaCl$_2$, 10.0 mM D-glucose, pregassed with 95% O$_2$, 5% CO$_2$ at 35 °C). Strips were equilibrated and stretched 1.2 times slack length. After mounting, strips were

equilibrated for 30 min and precontracted with 65 mM KCl three times. At the end of the equilibration, 65 mM KCl (pretreated with 0.1 μM atropine for 10 min) or the muscarinic agonist, 10 μM carbachol (Sigma) were added to initiate contractile responses. For Ca^{2+} depletion, the strips were treated with 10 μM carbachol for a period of 30 s and then washed with physiological salt solution containing no added Ca^{2+} plus 1 mM EGTA. This protocol was repeated at 2-min intervals for five to six times. The strips were then treated with 10 μM carbachol for 30 s and 1 μM calyculin A (Enzolite Sciences) for 30 min or 10 μM carbachol for 30 s in the presence or absence of Ca^{2+}. Force measurements were recorded isometrically by a Grass FT03 force transducer connected to Powerlab 8/SP data acquisition unit (AD Instruments, Colorado Springs, CO). Stresses (dynes/cm^2) were calculated to normalize contraction responses to tissue cross-sectional areas. Contractile studies were also performed by similar procedures for ileal smooth muscle.

The aorta was isolated, and endothelial cells were removed by gentle swabbing, and excessive adventia was removed by dissection. Thoracic aortic segmental rings 5 mm long were mounted by triangular wires to the isometric force apparatus. Aortic rings were passively stretched to 1.8–2.0 g and remained quiescent for 60 min before precontraction by 65 mM KCl in Krebs-Ringer solution. Aortic rings were also treated with 10 μM phenylephrine, and force responses were obtained as described above. Force measurements were normalized as grams of developed force per tissue wet weight. Contractile studies were also performed by similar procedures for tracheal rings.

At indicated times after specific treatments, tissues were quick frozen by clamps prechilled in liquid nitrogen for protein phosphorylation measurements. Frozen muscles were processed as described previously (23) by immersion in a frozen slurry of 10% trichloroacetic acid in acetone containing 10 mM dithiothreitol for 30 min and then thawed and transferred to Eppendorf tubes. Tissues were rinsed with ether (three times for 5 min), briefly dried (3 min), and suspended in urea sample buffer containing 8 M urea, 18.5 mM Tris (pH 8.6), 20.4 mM glycine, 10 mM dithiothreitol, 4 mM EDTA, and 5% sucrose. Proteins were then solubilized in a Bullet Blender (Next Advance, Inc, Averill Park, NY) (with 2-mm zirconium oxide beads, four spins $\times$ 3-min each at setting 9). Protein content was determined by Bradford assay, and bromphenol blue was added to 0.004%. Samples were stored at −80 °C.

Western Blot Analysis Following SDS-PAGE—Protein samples solubilized in urea sample buffer were added to 0.2 volumes of SDS sample buffer containing 250 mM Tris (pH 6.8), 10% SDS, 50 mM dithiothreitol, 40% glycerol, and 0.01% bromphenol blue, boiled, and loaded by amount of protein for SDS-PAGE (10–20% acrylamide gradient). Proteins were transferred to a nitrocellulose membrane and visualized by immunoblot staining using antibodies to MLCK (K36, Sigma), integrin-linked kinase (Sigma), ROCK1 (Santa Cruz Biotechnology), CPI-17 total (17), MYPT1 total (Upstate), phospho-MYPT1 (Thr-853) (Upstate), phospho-MYPT1 (Thr-696) (Upstate), phospho-CPI-17 (Santa Cruz Biotechnology), and SMMHC (30). The mouse monoclonal antibody from Sigma (K36) raised to the short MLCK binds the kinase near the N

terminus. Long MLCK is identical to short MLCK except for the extension at the N terminus, so this antibody binds to both forms of the enzyme (31). Because tissues from bladder and aortic smooth muscles were carefully dissected to remove urothelium (bladder) or endothelial cells (aorta) as well as adventia, the tissue strips were highly enriched for smooth muscle cells. We observed no long MLCK in our Western blots (data not shown). GAPDH was stained with anti-GAPDH antibody (Santa Cruz Biotechnology), or actin was stained with Coomassie Brilliant Blue G-250 as a loading control. MLCK, integrin-linked kinase, ROCK1, MYPT1 total, and CPI-17 total amounts were expressed as ratio of relative protein to GAPDH loading control. Additionally, MLCK content was also normalized to the total amount of SMMHC determined by Western blotting with antibodies characterized previously (30). There were no significant differences between normalization with GAPDH or smooth muscle myosin heavy chain, indicating enrichment of smooth muscle cells in the dissected tissues. The phosphorylation of MYPT1 (Thr-853 or Thr-696) was expressed as a ratio of phospho-MYPT1 to actin loading control and then normalized with the average values of MYPT1 phosphorylation response to calyculin A, which caused a maximal increase in MYPT1 phosphorylation. Phosphorylation of CPI-17 was obtained as a ratio of phosphorylated CPI-17 to CPI-17 total and then normalized to the maximal CPI-17 phosphorylation response obtained to phorbol 12,13-dibutyrate, a PKC activator. Quantification of Western blots was performed by quantitative densitometry using the ImageQuant software package (Molecular Dynamics).

Measurement of RLC Phosphorylation—Muscle proteins in 8 M urea sample buffer were subjected to urea/glycerol-PAGE at 400 volts for 80 min to separate nonphosphorylated, monophosphorylated, and diphosphorylated RLC (17, 32). Following electrophoresis, proteins were transferred to nitrocellulose or PVDF membranes and probed with antibodies against smooth muscle RLC. The ratio of monophosphorylated RLC to total RLC (nonphosphorylated plus phosphorylated) was determined by quantitative densitometry and expressed as mol phosphate per mol protein.

Statistical Analyses—All data are presented as mean ± S.E. Statistical comparisons were performed by Student's *t* test for force development and phosphorylation of RLC, CPI-17, and MYPT1. For multiple comparisons, one-way analysis of variance followed by Dunnett's post hoc test or Newman-Keuls post hoc test for multiple comparisons. Data analyses were performed with statistical software (Prism, version 5.0; GraphPad Software, San Diego, CA). *p* values < 0.05 were considered statistically significant.

RESULTS

MLCK Content Varies in Different Smooth Muscle Tissues—Quantitation of immunoblots for MLCK in different smooth muscle tissues from wild type mice showed significant variation in content with the greatest amount in bladder smooth muscle relative to other smooth muscles (Fig. 1). Aortic tissue contained <50% MLCK compared with bladder.

We observed no diminishments of MLCK protein in aortas from Mylk[f/f] mice containing the SMMHC-CreER[T2] transgene
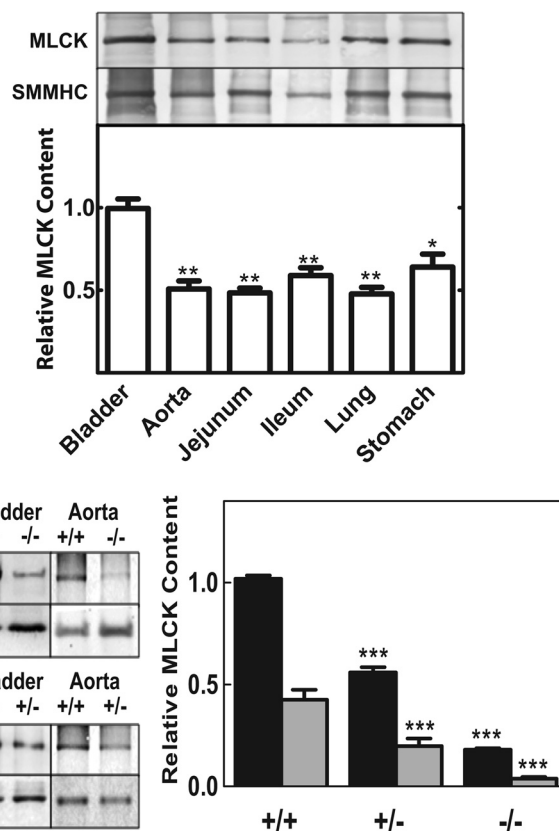

FIGURE 1. **Bladder smooth muscle contains more MLCK than other smooth muscles.** *Top panel*: representative Western blots for MLCK in different tissues with SMMHC as a loading control. *Error bars* represent mean ± S.E., $n \geq 5$. **, $p < 0.01$; *, $p < 0.05$ compared with bladder. *Lower panel*: representative Western blots for MLCK in bladder and aortic tissues from MLCK[SM+/+] (+/+), MLCK[SM+/−] (+/−), and MLCK[SM−/−] (−/−) mice with quantitation of MLCK content relative to bladder +/+. *Black bars*, bladder; *gray bars*, aorta. *Error bars* represent mean ± S.E.; $n \geq 30$ for bladder, $n \geq 6$ for aorta; ***, $p < 0.001$ compared with values for MLCK[SM+/+] mice.

without tamoxifen treatment compared with aortas from wild type mice. Additionally, tamoxifen treatment of Mylk[+/f] mice containing the SMMHC-CreER[T2] transgene predictably reduced bladder and aortic MLCK by 50% (MLCK[SM+/−] mice), consistent with inactivation of the one floxed allele with induced recombination (Fig. 1). Inactivation of both alleles decreased MLCK expression further in both tissues with the amount in bladder reduced 80%, whereas aortic tissues were reduced >90%. Tissues were harvested from these animals before MLCK protein was fully extinguished because of impending death related to the essential role of MLCK in smooth muscle maintenance of homeostasis (4). Because the amount of MLCK in bladder smooth muscle was so much more than that found in aortic smooth muscle, the residual amount of kinase was greater.

The amounts of other proteins relevant to signaling to RLC phosphorylation (MYPT1, CPI-17, ROCK1, and integrin-linked kinase) were not changed in bladder or aortic tissues from MLCK[SM−/−] mice (data not shown). The amounts of actin and myosin were also not reduced (data not shown). Thus, the conditional inactivation of MLCK alleles in adult mice reduces MLCK protein expression but not that of related contractile proteins.
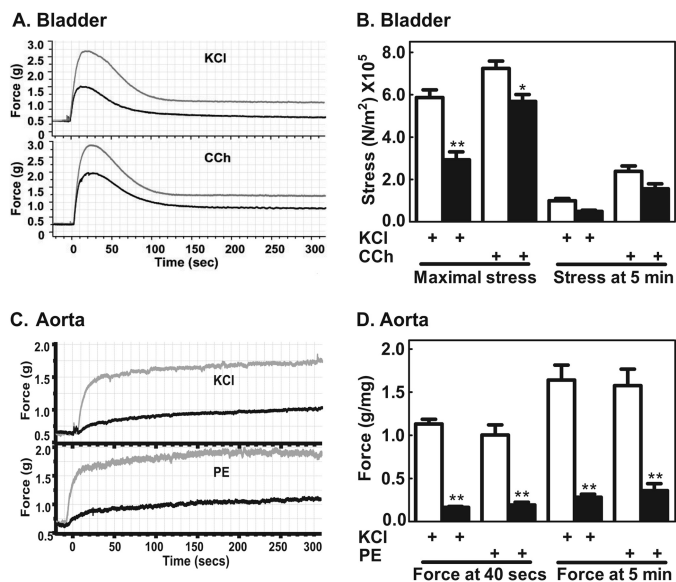
FIGURE 2. **Developed force responses in bladder and aortic smooth muscles from MLCK$^{SM-/-}$ mice are differentially attenuated.** *A*, typical force traces of bladder strips treated with 65 mM KCl or 10 μM carbachol (*CCh*). *Upper gray trace*, MLCK$^{SM+/+}$; *lower black trace*, MLCK$^{SM-/-}$. *B*, calculated contractile stresses of bladder tissue responses for 30 s and 5 min after stimulation. *Open bars*, tissues from MLCK$^{SM+/+}$ mice; *black bars*, tissues from MLCK$^{SM-/-}$ mice. *C*, representative force tracings of aortic tissues treated with 65 mM KCl or 10 μM phenylephrine (*PE*). *Upper gray trace*, MLCK$^{SM+/+}$; *lower black trace*, MLCK$^{SM-/-}$. *D*, contractile aortic force responses for 40 s and 5 min after stimulation. *Open bars*, tissues from MLCK$^{SM+/+}$ mice; *black bars*, tissues from MLCK$^{SM-/-}$ mice. *Error bars* represent mean ± S.E.; $n \geq 10$ for bladder, $n \geq 6$ for aorta. *, $p < 0.05$; **, $p < 0.01$ compared with MLCK$^{SM+/+}$.

Distinct Effects on Developed Contractile Force, RLC Phosphorylation, and Ca^{2+} Sensitization Are Related to Reductions in MLCK Content—When both MLCK alleles were inactivated in MLCK$^{SM-/-}$ mice after tamoxifen treatment, the 80% reduction in MLCK content in bladder smooth muscle tissue produced less reductions in the responses to KCl and the muscarinic agonist carbachol. As shown in Figs. 2 and 3, maximal force development and RLC phosphorylation induced by KCl treatment were reduced 50%, whereas the lower phasic responses at 5 min were similar to those measured in tissues from MLCK$^{SM+/+}$ mice. However, the maximal responses to carbachol were reduced less than those observed with KCl. Thus, the potential G-protein coupled receptor recruitment of Ca^{2+} sensitization via inhibition of myosin light chain phosphatase may blunt the effects of the decrease in MLCK in bladder smooth muscle from MLCK$^{SM-/-}$ mice.

Aortic tissue responses were also affected in MLCK$^{SM-/-}$ animals. The initial force development and extent of RLC phosphorylation in response to treatment with KCl or the α-adrenergic agonist phenylephrine were both greatly attenuated (Figs. 2 and 3). Additionally, sustained tonic responses were also inhibited markedly. Thus, the greater reduction in MLCK content in tonic aortic smooth muscle cells from MLCK$^{SM-/-}$ mice had a more profound effect on developed contractile force and RLC phosphorylation responses.

Ca^{2+} sensitization responses with MYPT1 and CPI-17 phosphorylation were measured in tissues from MLCK$^{SM-/-}$ mice. With the gradient gel system used for these analyses, the different isoforms for MYPT1 and CPI-17 were resolved (33, 34). KCl
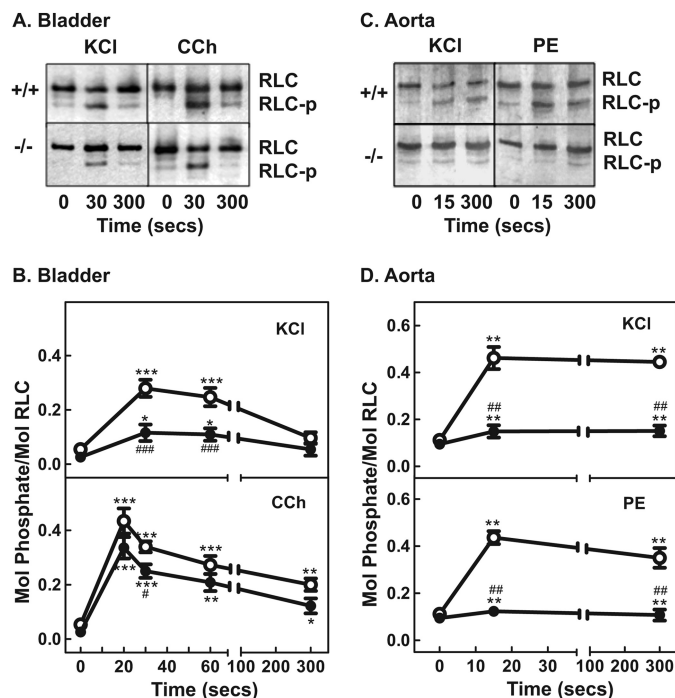

FIGURE 3. **Myosin RLC phosphorylation responses in bladder and aortic smooth muscles from MLCK$^{SM-/-}$ mice are differentially attenuated.** *A*, representative immunoblots following glycerol/urea PAGE for RLC phosphorylation in response to 65 mM KCl or 10 μM carbachol (*CCh*) for bladder tissues. *RLC*, nonphosphorylated; *RLC-p*, monophosphorylated. *B*, time course of RLC phosphorylation for bladder strips in response to 65 mM KCl (*upper panel*) and 10 μM CCh (*lower panel*). *Open circles*, tissues from MLCK$^{SM+/+}$ mice; *closed circles*, tissues from MLCK$^{SM-/-}$ mice. *C*, representative immunoblots following glycerol/urea PAGE for RLC phosphorylation in response to 65 mM KCl or 10 μM phenylephrine (*PE*) for aortic tissues. *RLC*, nonphosphorylated; *RLC-p*, monophosphorylated. *D*, time course of RLC phosphorylation for aortic strips in response to 65 mM KCl (*upper panel*) and 10 μM phenylephrine (*lower panel*). *Open circles*, tissues from MLCK$^{SM+/+}$ mice; *closed circles*, tissues from MLCK$^{SM-/-}$ mice. Values are means ± S.E.; $n \geq 8$ for bladder; $n \geq 4$ for aorta. *, $p < 0.05$; **, $p < 0.01$ when compared with resting strips; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ when compared with control strips at the same time.

treatment of bladder smooth muscles from MLCK$^{SM+/+}$ mice showed modest increases in MYPT1 and CPI-17 phosphorylation (Fig. 4). However, KCl-induced phosphorylation of MYPT1 as well as CPI-17 was enhanced in bladder tissues from MLCK$^{SM-/-}$ mice (Fig. 4). Carbachol treatment showed greater phosphorylation responses for MYPT1 and CPI-17 compared with KCl (Fig. 5). The phosphorylation of MYPT1 was similar in tissues from MLCK$^{SM-/-}$ mice compared with MLCK$^{SM+/+}$ mice. However, CPI-17 phosphorylation was greater in MLCK$^{SM-/-}$ mice. Thus, the enhanced Ca^{2+} sensitization responses elicited with carbachol treatment are consistent with the more modest attenuation of contractile, and RLC phosphorylation responses compared with KCl responses with bladder tissues from MLCK$^{SM-/-}$ mice.

Ca^{2+} sensitization responses involving MYPT1 and CPI-17 phosphorylation were also measured in aortic tissues (Fig. 6). Tissues from MLCK$^{SM+/+}$ mice showed modest phosphorylation responses with phenylephrine treatment. However, the maximal responses obtained were enhanced in MLCK$^{SM-/-}$ mice at 15 s but not 5 min. Despite these enhanced phosphorylation responses, contractile force development and RLC phosphorylation remained greatly impaired (Figs. 2 and 3).
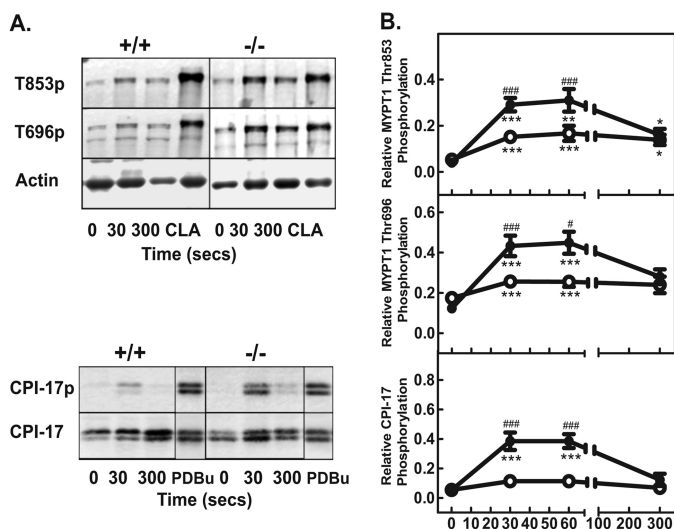
FIGURE 4. MYPT1 and CPI-17 phosphorylation is enhanced in response to KCl in bladder smooth muscle from MLCK$^{SM-/-}$ mice. *A,* representative Western blots for phosphorylation of MYPT1 Thr-853, MYPT1 Thr-696, and CPI-17 in bladder tissues in response to 65 mM KCl. *B, top* and *middle panels:* quantification of MYPT1 Thr-853 and Thr-696 phosphorylation responses to 65 mM KCl normalized to response obtained with calyculin A (*CLA*) treatment. *Bottom panel,* CPI-17 phosphorylation normalized to response obtained with phorbol 12,13-dibutyrate. *Open circles,* MLCK$^{SM+/+}$; *closed circles,* MLCK$^{SM-/-}$. Values are mean ± S.E.; $n \geq 8$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared with resting strips; #, $p < 0.05$; ###, < 0.001 when compared with MLCK$^{SM+/+}$ strips at the same time.
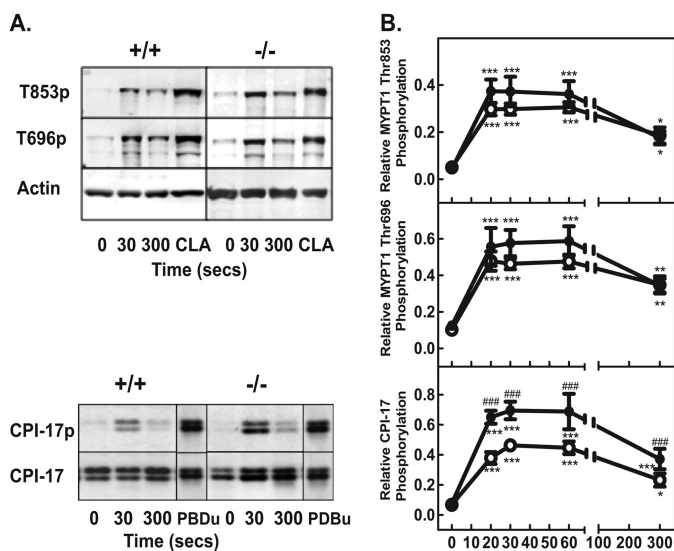

FIGURE 5. Phosphorylation of CPI-17, but not MYPT1 Thr-853 and Thr-696, is enhanced in response to carbachol (*CCh*) in bladder smooth muscle from MLCK$^{SM-/-}$ mice. *A,* representative Western blots for phosphorylation of MYPT1 Thr-853, MYPT1 Thr-696, and CPI-17 in bladder tissues in response to 10 μM CCh. *B, top* and *middle panels:* quantification of MYPT1 Thr-853 and Thr-696 phosphorylation responses to 10 μM carbachol normalized to response obtained with calyculin A (CLA) treatment. *Bottom panel:* CPI-17 phosphorylation normalized to response obtained with phorbol 12,13-dibutyrate. *Open circles,* MLCK$^{SM+/+}$; *closed circles,* MLCK$^{SM-/-}$. Values are mean ± S.E.; $n \geq 8$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared with resting strips; #, $p < 0.05$; ###, < 0.001 when compared with MLCK$^{SM+/+}$ strips at the same time. *CLA,* calyculin A.

Aortic smooth muscle cells do not have robust Ca^{2+} sensitization responses.

We determined the effects of inactivation of a single *Mylk* allele in MLCK$^{SM+/-}$ mice. In bladder tissues, the 50% reduction of MLCK (Fig. 1) resulted in no significant changes in the
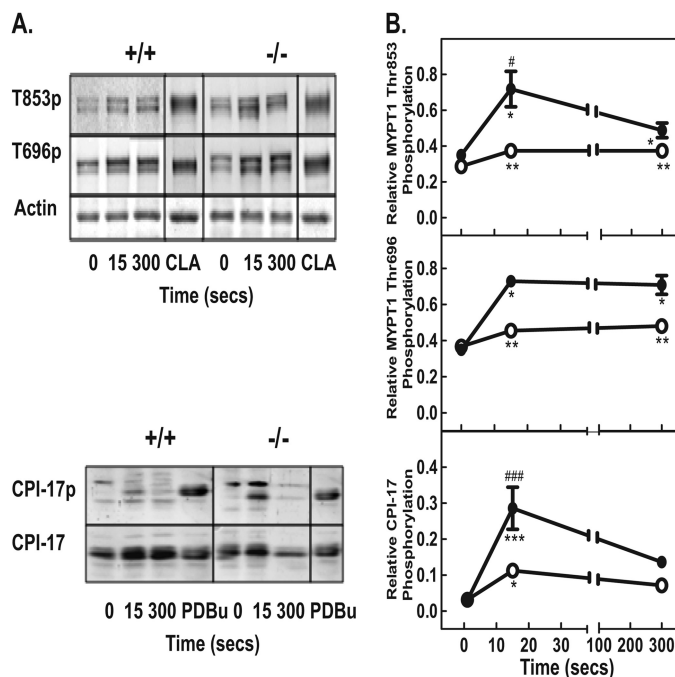

FIGURE 6. Phosphorylation of CPI-17 and MYPT1 is enhanced in response to phenylephrine (*PE*) in aortic smooth muscles from MLCK$^{SM-/-}$ mice. *A,* representative Western blots for phosphorylation of MYPT1 Thr-853, MYPT1 Thr-696, and CPI-17 in aortic tissues in response to 10 μM phenylephrine. *B, top* and *middle panels:* quantification of MYPT1 Thr-853 and Thr-696 phosphorylation responses to 10 μM phenylephrine normalized to response obtained with calyculin A (CLA) treatment. *Bottom panel:* CPI-17 phosphorylation normalized to response obtained with phorbol 12,13-dibutyrate. *Open circles,* MLCK$^{SM+/+}$; *closed circles,* MLCK$^{SM-/-}$. Values are mean ± S.E.; $n \leq 4$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared with resting strips; #, $p < 0.05$; ###, < 0.001 when compared with MLCK$^{SM+/+}$ strips at the same time.

contractile responses to KCl or carbachol (Fig. 7). However, the responses in the aorta were affected with a 44% reduction in initial force development at 15 s with KCl treatment and a smaller reduction in response to phenylephrine treatment (Fig. 7). As predicted from the contractile results, there were no differences in RLC phosphorylation responses in bladder tissues, whereas RLC phosphorylation was reduced in aortic tissues from MLCK$^{SM+/-}$ mice (Fig. 7). In ileal and tracheal smooth muscles from MLCK$^{SM+/-}$ mice, force responses were also significantly reduced. Maximal responses of ileal and tracheal tissues to KCl were 82 ± 6% and 65 ± 8%, respectively, whereas responses to carbachol were 78 ± 7% and 73 ± 7% ($p < 0.05$). MYPT1 and CPI-17 phosphorylation responses were not significantly different in bladder and aortic tissues from MLCK$^{SM+/+}$ and MLCK$^{SM+/-}$ mice (data not shown). Thus, the partial reduction in smooth muscle MLCK content in MLCK$^{SM+/-}$ bladder smooth muscle does not affect contractile responses. Contractile responses are partially reduced in other smooth muscles, particularly in the aorta.

RLC Phosphorylation Is Ca^{2+}-dependent unless Myosin Light Chain Phosphatase Is Chemically Inhibited—Bladder smooth muscle tissues incubated in Ca^{2+}-free physiological salt solution were not able to contract and lost the RLC phosphorylation response when exposed to carbachol (Fig. 8). Similar responses were obtained with tissues from MLCK$^{SM+/+}$ and MLCK$^{SM-/-}$ mice. Thus, Ca^{2+}-independent kinases such as ROCK, integrin-linked kinase or zipper-interacting protein
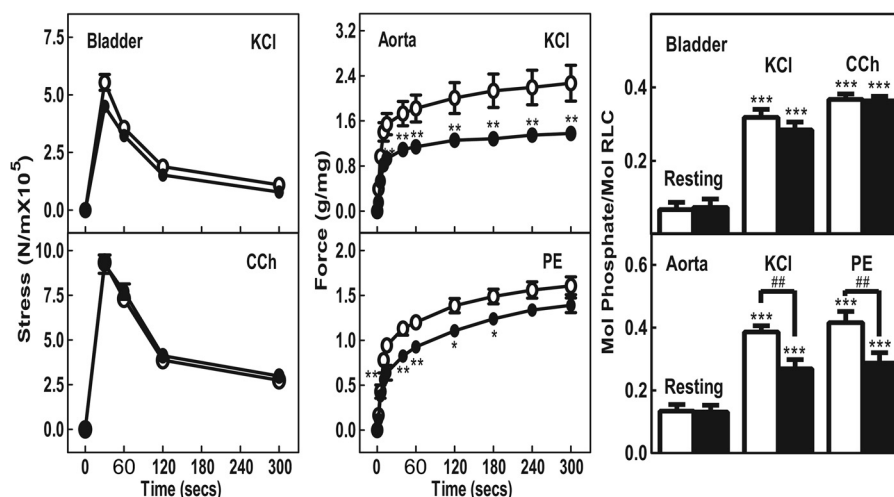
FIGURE 7. **Developed force responses and RLC phosphorylation in bladder and aortic smooth muscles from MLCK[SM+/−] mice are differentially affected.** *Left panels*: bladder tissues treated with 65 mM KCl or 10 μM carbachol (*CCh*). *Middle panels*: aortic strips treated with 65 mM KCl or 10 μM phenylephrine (*PE*). *Open circles*, MLCK[SM+/+]; *closed circles*, MLCK[SM−/−]. Values are mean ± S.E.; $n \geq 40$ for bladder; $n \geq 10$ for aorta. *, $p < 0.05$; **, $p < 0.01$ when compared with MLCK[SM+/+] strips at the same time. *Right panels*: bladder tissues were treated with 10 μM carbachol (*CCh*) or 65 mM KCl for 30 s. Aortic tissues were treated with 10 μM phenylephrine or 65 mM KCl for 15 s. *Open bars*, MLCK[SM+/+]; *closed bars*, MLCK[SM+/−]. Values are mean ± S.E.; $n \geq 8$. *, $p < 0.05$; ***, $p < 0.001$ when compared with resting strips; ##, $p < 0.01$ when compared with MLCK[SM+/+] strips at the same time.
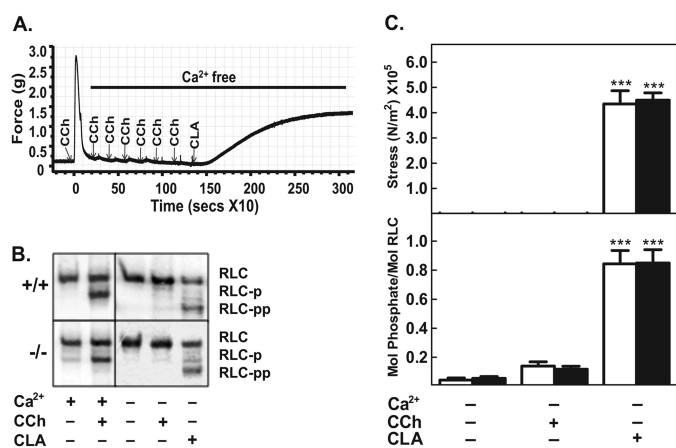

FIGURE 8. **Carbachol-induced but not calyculin A-induced contractions and RLC phosphorylation are Ca²⁺-dependent in bladder tissues from MLCK[SM−/−] mice.** *A*, representative force tracing of bladder strips in the absence of Ca²⁺ and treated with 10 μM carbachol (*CCh*) or 1 μM calyculin A (*CLA*) with carbachol. *B*, representative Western blots for RLC phosphorylation (*RLC-p*, monophosphorylated; *RLC-pp*, diphosphorylated) from MLCK[SM+/+] and MLCK[SM−/−] mice. *C*, *top panel*: calculated contractile stresses of bladder tissue responses. *Bottom panel*: RLC phosphorylation calculated for mono- and diphosphorylated RLC per total RLC. *Open bars*, MLCK[SM+/+]; *closed bars*, MLCK[SM−/−]. *Error bars* show mean ± S.E.; $n \geq 10$. ***, $p < 0.001$ when compared with resting strips.

kinase do not appear to contribute to RLC and contractile force development responses in bladder tissues from these mice. However, if the tissues were incubated with the myosin light chain phosphatase inhibiter calyculin A in the absence of Ca²⁺, RLC phosphorylation slowly increased leading to force development with a significant change in the RLC phosphorylation pattern. Treatment of tissues with KCl or carbachol in the presence of Ca²⁺ resulted in monophosphorylated RLC (Fig. 5). Calyculin A treatment, however, produced diphosphorylated RLC, consistent with phosphorylation by integrin-linked kinase or zipper-interacting protein kinase (Fig. 8) (35, 36).

Similar results on Ca²⁺-dependence of RLC phosphorylation and force development were obtained with aortic tissues (Fig.

9). Responses to phenylephrine were Ca²⁺-dependent in aortic tissues from MLCK[SM+/+] mice. In the absence of Ca²⁺, calyculin A slowly increased RLC phosphorylation and force development. Notably, there was a robust RLC diphosphorylation in contrast to the monophosphorylated RLC observed in the presence of Ca²⁺ with phenylephrine. In aortic tissues from MLCK[SM−/−] mice, the contractile and RLC phosphorylation responses to phenylephrine in the presence of Ca²⁺ were greatly attenuated (Figs. 2 and 3), whereas the responses to calyculin A in the absence of Ca²⁺ were similar to responses with aortic tissues from MLCK[SM+/+] mice (Fig. 9). Collectively, these results show that residual MLCK in the smooth muscle tissues of MLCK[SM−/−] mice contribute to Ca²⁺-dependent RLC phosphorylation responses. The Ca²⁺-independent responses with calyculin A treatment are similar in tissues from MLCK[SM+/+] and MLCK[SM−/−] mice, showing the loss of MLCK, particularly in the aorta, does not impair the contractile potential of smooth muscle cells elicited by small amounts of Ca²⁺-independent kinase activity revealed with chemical inhibition of myosin light chain phosphatase activity.

DISCUSSION

Chemical messengers binding to cell surface receptors on smooth muscle cells stimulate a variety of membrane signaling events to initiate a contractile response. Primary responses include depolarization of the sarcolemma with opening of voltage-gated Ca²⁺ channels, modulation of a cytosolic Ca²⁺ oscillator, release from internal stores, or changes in membrane potential via gap junctions formed with pacemaker cells (interstitial cells of Cajal) (37, 38). A common end result is to increase global $[Ca^{2+}]_i$, which leads to the contractile response. These chemical messengers may also activate specific G-protein-coupled receptors distinct for different smooth muscle cells, leading to the additional activation of ROCK/PKC signaling pathways to modulate the contractile response to $[Ca^{2+}]_i$ (39).

The increase in global $[Ca^{2+}]_i$ results in Ca²⁺binding to calmodulin, which then binds and activates MLCK to phosphoryl-
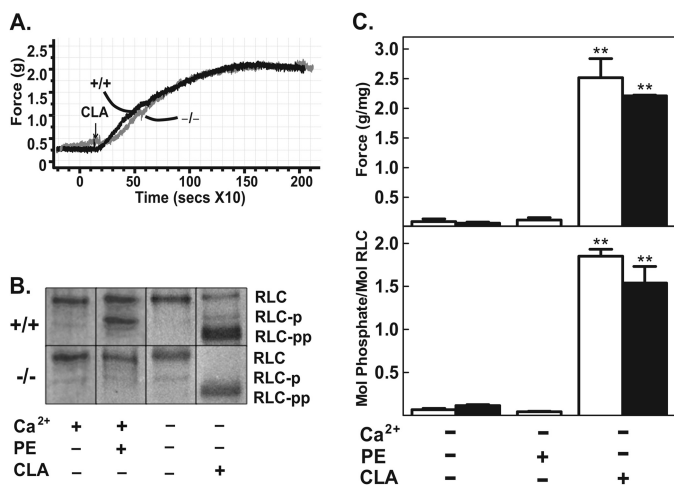
A.

B.

C.

FIGURE 9. **Phenylephrine-induced but not calyculin A-induced contractions and RLC phosphorylation are Ca^{2+}-dependent in aortic tissues from MLCK$^{SM-/-}$ mice.** *A*, representative force tracing of aortic strips in the absence of Ca^{2+} treated with 1 μM calyculin A (*CLA*) in MLCK$^{SM+/+}$(+/+), and MLCK$^{SM-/-}$(−/−) mice. *B*, representative Western blots for RLC phosphorylation (*RLC-p*, monophosphorylated; *RLC-pp*, diphosphorylated) from MLCK$^{SM+/+}$ and MLCK$^{SM-/-}$ mice. *PE*, phenylephrine. *C*, force development for aortic tissue responses. *Bottom panel*: RLC phosphorylation calculated for mono- and diphosphorylated RLC per total RLC. *Open bars*, MLCK$^{SM+/+}$; *closed bars*, MLCK$^{SM-/-}$. *Error bars* show mean ± S.E.; $n \geq 4$. **, $p < 0.01$ when compared with resting strips.

ate RLC for the physiological contractile response in smooth muscle cells (3–6, 27, 40, 41). Although biochemical studies show integrin-linked kinase and zipper-interacting protein kinase are Ca^{2+}-independent kinases potentially capable of phosphorylating RLC in smooth muscle tissues, their catalysis results in simultaneous diphosphorylation at Thr-18 and Ser-19 (35, 42). MLCK can also diphosphorylate RLC at Thr-18 and Ser-19, but the biochemical rate of phosphorylation of Thr-18 is much slower than phosphorylation at Ser-19 *in vitro* (43). Stimuli that lead to physiological contractions result predominantly in RLC monophosphorylation and the knock-out of MLCK in adult smooth muscle tissues eliminates RLC phosphorylation as shown in this study and previous publications (4, 5, 10, 40, 44). However, pathological situations may lead to significant RLC diphosphorylation by integrin-linked kinase and ZIP kinase in smooth muscle tissues (45). Thus, the Ca^{2+}/calmodulin-dependent MLCK appears to be the only kinase that phosphorylates RLC physiologically in adult smooth muscle tissues.

Interestingly, isolated blood vessels from embryonic MLCK-null mice contract, presumably due to RLC phosphorylation (46). These results are consistent with another observation that embryonic fibroblasts contain no MLCK, and RLC is phosphorylated by ROCK (47). Thus, MLCK is not the only kinase that can phosphorylate RLC in embryonic cells. Additionally, ROCK may serve as the primary kinase for phosphorylation of nonmuscle myosin in other kinds of cells (48–50).

The amount of cellular MLCK is an important consideration regarding signaling to RLC phosphorylation in muscle cells. There are significant differences in MLCK activities in smooth, skeletal, and cardiac muscle consistent with physiological functions (3, 51, 52). The greater amount of MLCK activity is found in smooth muscle tissues and supports the rapid rate of RLC

phosphorylation necessary to initiate contraction. In fast-twitch skeletal muscle fibers, the rates of phosphorylation-dephosphorylation are slower where sustained RLC phosphorylation enhances contractile force with repeated contractions that occur during exercise (postactivation potentiation). RLC phosphorylation-dephosphorylation is much slower in cardiac muscle where MLCK maintains the extent of RLC phosphorylation at 45% in the constantly beating heart. This phosphorylation is physiologically important for enhancing myofilament contractile force (52).

Differences in MLCK content may vary in different smooth muscles. Measurements of MLCK activity in tissue homogenates showed a 3-fold difference between the phasic ileum and tonic femoral artery smooth muscle tissues (19). Using a highly specific monoclonal antibody for MLCK for Western blotting, we find more modest differences among different phasic and tonic smooth muscles. Although heavy meromyosin was used as a selective substrate in the previous study (19), the possibility remains that another kinase(s) contributed to its phosphorylation. The greatest difference we observed was the 2-fold greater amount of MLCK content in bladder smooth muscle compared with aortic smooth muscle.

We had previously determined that free Ca^{2+}/calmodulin was limiting for MLCK activation in bladder smooth muscle, and thus, only a fraction of the kinase was activated in responses to carbachol or KCl (17, 23). Ca^{2+} binds to calmodulin as a diffusion limited, bimolecular association to induce rapid activation of MLCK by displacement of the kinase autoinhibitory segment from its catalytic cleft (17, 23, 40, 52–55). MLCK has a high affinity for Ca^{2+}/calmodulin with an apparent K_D value of 1 nM (43). However, the kinase is competing for Ca^{2+}/calmodulin with a large number of other proteins with varied types of binding modalities (56). These biochemical and biophysical results are consistent with bladder RLC phosphorylation and contractile responses in MLCK$^{SM+/-}$ and MLCK$^{SM-/-}$ mice. The 50% MLCK reduction in bladder smooth muscle from MLCK$^{SM+/-}$ mice had no significant effect on RLC phosphorylation or contraction. Thus, the remaining 50% MLCK is sufficient relative to limiting Ca^{2+}/calmodulin. Both MYPT1 Thr-696 and Thr-853 were phosphorylated with greater phosphorylation noted with carbachol treatment compared with KCl. In bladder smooth muscle from MLCK$^{SM-/-}$ mice the amount of MLCK was reduced 80%, but the remaining amount still supported RLC phosphorylation and contraction with greater responses obtained with carbachol compared with KCl due to recruitment of Ca^{2+} sensitization responses with activation of the muscarinic receptor (10). Both MYPT1 and CPI-17 phosphorylation responses were enhanced in tissues from MLCK$^{SM-/-}$ mice relative to responses obtained with tissues from MLCK$^{SM+/-}$ and MLCK$^{SM+/+}$ mice. The reason for this compensatory enhancement is not clear. The enhanced phosphorylation of both MYPT1 and CPI-17 could provide greater support for RLC phosphorylation by inhibiting myosin light chain phosphatase activity when the MLCK amount was greatly diminished in MLCK$^{SM-/-}$ mice. Collectively, the reduction of MLCK in bladders from MLCK$^{SM+/-}$ and MLCK$^{SM-/-}$ mice is consistent with biophysical measure-

ments that <50% of the kinase is normally activated with carbachol or KCl treatments (17, 23).

Reduction of MLCK in aortic smooth muscle had a more pronounced effect. A 50% reduction in MLCK content decreased force development and RLC phosphorylation in response to both KCl and phenylephrine treatments. The decreases were smaller with phenylephrine suggesting activation of Ca^{2+} sensitization pathways, which were confirmed with the measurements of phosphorylation of MYPT1 Thr-696 and Thr-853 as well as CPI-17. However, the phosphorylation responses of these proteins were modest compared with the responses observed in bladder tissues. Similar to bladder smooth muscles from MLCK$^{SM-/-}$ mice, phosphorylation of MYPT1 and CPI-17 was enhanced in aortic tissues from MLCK$^{SM-/-}$ mice, which had a >90% decrease in MLCK. However, both RLC phosphorylation and force development were still profoundly attenuated. Thus, aortic tissue appears to be highly dependent on MLCK content without robust Ca^{2+} sensitization responses. This conclusion is consistent with the recent analysis of heterogeneity of Ca^{2+} sensitization responses in arteries of different sizes, including the aorta (57).

Rather than comparing MYPT1 and CPI-17 phosphorylation responses relative to resting values or normalizing to the maximal phosphorylation responses, we measured their phosphorylation relative to maximal responses obtained to calyculin A or phorbol 12,13-dibutyrate, respectively. Quantitative measurements show 10% CPI-17 phosphorylation in response to phenylephrine in aortic tissue from wild type animals. This represents a 3-fold increase over basal phosphorylation. However, the more quantitative measurement shows how small the CPI-17 phosphorylation response is, consistent with its minimal contribution to Ca^{2+} sensitization in aortic tissues (57). Similarly, basal MYPT1 phosphorylation was high and increased modestly with phenylephrine treatment, consistent with recent observations (57).

This perspective on limiting MLCK combined with weak Ca^{2+} sensitization responses in aortic smooth muscle cells may provide some insight into heterozygous loss-of-function mutations in *MYLK* that cause dissections of human thoracic aorta (28). The absence of other smooth muscle phenotypic manifestations suggests that half of normal MLCK activity does not disrupt myosin activity in the majority of smooth muscle tissues to an extent that a phenotype is manifested, including other vascular tissues. The haploinsufficiency of MLCK specifically involves the ascending thoracic aorta exposed to the highest biomechanical force from pulsatile blood ejection from the heart where smooth muscle contractile tone may be compromised.

We have also obtained insights in the relative roles of MLCK and Ca^{2+}-independent kinases in smooth muscle contraction. Consistent with the Ca^{2+}/calmodulin dependence of MLCK activity, RLC phosphorylation was completely inhibited with Ca^{2+} depletion in bladder tissues from MLCK$^{SM-/-}$ mice, which contained 20% MLCK. Under conditions of Ca^{2+} depletion, the tissues from both MLCK$^{SM-/-}$ and MLCK$^{SM+/+}$ mice contracted similarly when treated with the phosphatase inhibitor calyculin A. Similar results were obtained in aortic tissues with Ca^{2+} depletion. Treatment with calyculin A

resulted in RLC diphosphorylation in both bladder and aortic tissues, suggesting candidate protein kinases integrin-linked kinase and ZIP kinase. Although calyculin A inhibits both protein phosphatase 1 and 2A (58, 59), protein phosphatase 2A does not dephosphorylate RLC bound to myosin heavy chain (60). Thus, the actions of calyculin A related to RLC phosphorylation will be directed to myosin light chain phosphatase containing the protein phosphatase 1 catalytic subunit. Calyculin A has a high affinity for protein phosphatase 1 catalytic subunit (1 nM); thus, its activity is predicted to be highly inhibited with the addition of 1 μM calyculin A to the smooth muscle tissues in the absence of Ca^{2+}. Under these conditions, very low kinase activities directed to RLC were revealed, similar to previous reports on the effects of chemical inhibitors of myosin light chain phosphatase in various smooth muscle tissues (10, 19, 40, 61). The activity of these kinases is probably too low to be physiologically significant, particularly when it is considered that significant RLC diphosphorylation is normally not observed with physiological increases in cytosolic Ca^{2+} in adult smooth muscle tissues.

In summary, activation of a fraction of MLCK by limiting amounts of free Ca^{2+}/calmodulin combined with myosin light chain phosphatase inhibition via Ca^{2+} sensitization mechanisms appears to be sufficient for robust RLC phosphorylation and contractile responses in bladder smooth muscle. In contrast, limiting MLCK combined with weak Ca^{2+} sensitization responses are primary effectors for RLC phosphorylation and contraction in aortic smooth muscle cells. These results indicate differential contributions of MLCK to signaling responses relative to mechanisms involving inhibition of myosin light chain phosphatase activity by Ca^{2+} sensitization signaling. Importantly, the limiting amount of MLCK activity in aortic smooth muscle cells may compromise contractile tone under biomechanical stress in the thoracic aorta, thereby predisposing the tissue to dissection with loss-of-function mutations in *MYLK* (28).

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