Identification of Cardiac-Specific Myosin Light Chain Kinase

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Abstract—Two myosin light chain (MLC) kinase (MLCK) proteins, smooth muscle (encoded by mylk1 gene) and skeletal (encoded by mylk2 gene) MLCK, have been shown to be expressed in mammals. Even though phosphorylation of its putative substrate, MLC2, is recognized as a key regulator of cardiac contraction, a MLCK that is preferentially expressed in cardiac muscle has not yet been identified. In this study, we characterized a new kinase encoded by a gene homologous to mylk1 and -2, named cardiac MLCK, which is specifically expressed in the heart in both atrium and ventricle. In fact, expression of cardiac MLCK is highly regulated by the cardiac homeobox protein Nkx2-5 in neonatal cardiomyocytes. The overall structure of cardiac MLCK protein is conserved with skeletal and smooth muscle MLCK; however, the amino terminus is quite unique, without significant homology to other known proteins, and its catalytic activity does not appear to be regulated by Ca²⁺/calmodulin in vitro. Cardiac MLCK is phosphorylated and the level of phosphorylation is increased by phenylephrine stimulation accompanied by increased level of MLC2v phosphorylation. Both overexpression and knockdown of cardiac MLCK in cultured cardiomyocytes revealed that cardiac MLCK is likely a new regulator of MLC2 phosphorylation, sarcomere organization, and cardiomyocyte contraction. (Circ Res. 2008;102:571-580.)

Key Words: kinase ■ transcription ■ contraction

Phosphorylation of both myosin heavy chain and myosin light chain (MLC) affects motor activity and thick filament assembly.¹ In smooth muscle cells, phosphorylation of MLC2 by smooth muscle MLCK is thought to be responsible for the initiation of contraction.² In skeletal and cardiac muscles, however, initiation of muscle contraction depends on voltage-gated L-type Ca²⁺ channels in the plasma membrane and T-tubules. Increased local Ca²⁺ concentrations allow the sarcoplasmic reticulum to release large amounts of Ca²⁺, which bind to troponin C followed by myosin–actin cross-bridge formation. During this process, MLCK potentiates peak tension in skeletal muscle¹,³ and the force and rate of cross-bridge recruitment in cardiac myocytes.⁴,⁵

To date, smooth muscle (encoded by mylk1 gene) and skeletal (encoded by mylk2 gene) MLCKs have been characterized.³ Mouse skeletal muscle MLCK is predominantly expressed in skeletal muscle, and mouse smooth muscle MLCK is expressed in several tissues but predominantly in smooth muscle.⁶,⁷ Mutations in human skeletal MLCK on human chromosome 20 have been mapped to a disease locus for familial cardiac hypertrophy (Online Mendelian Inheritance in Man no. 606566), suggesting that abnormal function of skeletal MLCK stimulates cardiac hypertrophy.⁸ However, the abundance of skeletal MLCK expression in the heart is controversial,⁸–¹⁰ and gene-targeted mice for skeletal MLCK appear to have normal cardiac function.¹⁰ Short-form (130-kDa) smooth muscle MLCK is expressed in the heart at lower levels than those detected in smooth muscle–rich organs such as gut, uterus, and lung.⁶,⁷ Mice with ablation of long-form smooth muscle MLCK appear to have normal cardiac function,¹¹ and those with short-form ablation remain to be studied. These results suggest that an additional MLCK is preferentially expressed and functional in the heart because MLC2 phosphorylation in cardiac muscle is a key regulator of heart contraction.⁵

In the process of identifying genes regulated by the cardiac homeobox transcription factor Nkx2-5, we identified a gene product highly homologous to the previously characterized skeletal and smooth muscle MLCK. The sequence of this MLCK homolog has been available (National Center for Biotechnology Information [NCBI] UniGene no. Rn.43838.
yet limited information regarding this MLCK gene has sometimes confounded its identity with the previously characterized skeletal and smooth muscle MLCKs. In this study, we report the initial characterization of this MLCK regarding its cardiac-specific expression, intracellular localization, catalytic activity, and potential functions in sarcomere organization and cardiac contraction.

Materials and Methods

The following materials and methods used for this study are described in detail in the online data supplement, available at http://circres.ahajournals.org.

- Cardiomyocyte preparation
- Animal models
- Cloning of cardiac, skeletal, and smooth muscle MLCK
- Production and infection of adenovirus
- Glutathione S-transferase (GST) fusion proteins (cardiac MLCK, MLC2v, MLC2a)
- Production of anti–cardiac MLCK antibody
- Northern and Western blotting
- Immunostaining
- Real-time PCR
- Phosphorylation assays in vitro and in cardiomyocytes
- Two-dimensional gel electrophoresis
- Simultaneous measurements of cell shortening and intracellular free calcium
- Statistical analyses

Results

Identification of a Cardiac-Specific MLCK as a Downstream Target of Cardiac Homeobox Protein, Nkx2-5

In Nkx2-5 knockdown neonatal rat cardiomyocytes (Figure 1A) and inducible Nkx2-5 knockout hearts (Figure 1B), reduced expression of Nkx2-5 dramatically decreased mRNA expression of the MLCK homolog (NCBI UniGene no. Rn.43838 [rat], Mm.32804 [mouse]). Because Nkx2-5 expression is nearly restricted to cardiac myocytes at the postnatal stage,12–14 we tested whether expression of the MLCK homolog is also cardiac-specific. Multitissue Northern blotting readily detected mRNA of the MLCK homolog specifically in the heart in both ventricle and atrium at neonatal and adult stages (Figure 1C, top gels). Increased loading of RNA isolated from adult atrium resulted in higher expression of MLCK in adult atrium vs ventricle (*, lane 4, right). Br indicates brain; Lu, lung; V, ventricle; A, atrium; To, tongue; M(l), leg muscle; M(b), back muscle; Li, liver; Ki, kidney; St, stomach; and In, intestine.
conserved kinase domain at the carboxyl terminus with 58% identity with skeletal MLCK and 44% identity with smooth muscle MLCK; however the amino-terminal domain is quite unique, with no significant homology to other known proteins, including MLCKs. Comparison of protein structure of cardiac, skeletal, and smooth muscle MLCKs, including an alternative gene product, telokin,3 is shown (Figure 2A). The amino acid sequence alignment of carboxyl terminus of cardiac MLCK to skeletal and long-form smooth muscle MLCK is shown in Figure 2B.

Cardiac MLCK Protein Expression

We generated an affinity-purified antibody against the unique amino terminus of cardiac MLCK (amino acids 28 to 463) (Figure 3A, lanes 1 and 2). The specificity of the antibody was confirmed by its reactivity to hemagglutinin (HA)-tagged full-length cardiac MLCK (Figure 3A, lane 3), but not to HA-tagged full-length skeletal MLCK at even 10-fold abundance (Figure 3A, lanes 4 and 5), to which cardiac MLCK has a higher homology compared with smooth muscle MLCK. In neonatal heart lysates, cardiac MLCK protein with an approximate molecular mass of 90 kDa was readily detected in both ventricle and atrium at similar expression levels (Figure 3B), and its protein expression in heart lysates was 0.5 ng/mg (equivalent to 2.3 ng of GST–cardiac MLCK in 5 µg of heart lysates) following densitometric analysis (Figure 3C), which is lower than skeletal MLCK levels in skeletal muscle previously reported (≈5 to 10 ng of skeletal MLCK in 2 µg of skeletal muscle lysates).6 The antibody against the amino terminus of cardiac MLCK does not cross-react with other proteins in skeletal muscle and lung lysates in which skeletal or smooth muscle MLCK is abundantly expressed (Figure

Figure 2. Structure of mouse cardiac MLCK (GenBank accession number EU403565) protein compared with skeletal and smooth muscle MLCKs. A, Schematic of cardiac MLCK protein structure compared with skeletal and long and short forms of smooth muscle MLCK and variant of smooth muscle MLCK, telokin. Protein sequences are retrieved from mouse skeletal MLCK (XP_979674), smooth muscle MLCK (long) (NP647461), smooth muscle MLCK (short),3 and telokin (AAG34169). Ig indicates immunoglobulin C2 like motif. B, Amino acid sequence of cardiac MLCK with alignment among cardiac, skeletal, and smooth muscle (long-form) MLCK at the carboxyl terminus. Identical amino acids between at 2 proteins are shaded. Putative Ca2+/calmodulin binding kinase regulatory domain locating carboxyl terminus to catalytic domain; 2 contiguous serine residues, which are targets of upstream kinases3 and 2 additional autophosphorylation sites29 identified in the smooth muscle MLCK are underlined.
3C, lanes 4 and 5). An additional band migrating around 60 kDa was detected using the anti–cardiac MLCK antibody in heart lysates, which may be an alternatively spliced isoform or a degradation product of cardiac MLCK (see Figure 7). We confirmed the expression of 130-kDa short-form smooth muscle MLCK in the heart (estimated concentration 0.025 to 0.05 μg/mg), skeletal muscle, and more abundantly in the lung (0.5 to 1 μg/mg) (supplemental Figure I). Thus, cardiac MLCK protein expression is ∼10- to 20-fold more abundant than smooth muscle MLCK in the neonatal heart.

Endogenous cardiac MLCK is diffusely localized in the cytoplasm of cardiomyocytes (Figure 3D, green, arrows; arrowheads, noncardiomyocyte); however, in some areas, striated staining of cardiac MLCK was observed. Interestingly, enlarged image of cardiomyocytes coimmunostained to detect actin (Figure 3D, red, localizing at I bands) and MLC2v (Figure 3D, blue, localizing at A bands) demonstrated that striated MLCK staining was colocalized with actin (Figure 3D, green and red) but not with MLC2v (Figure 3D, green and blue). Specificity of immunostaining and additional endogenous cardiac MLCK stainings are shown in supplemental Figure II.

Cardiac MLCK Phosphorylates MLC2 In Vitro and in Cardiomyocytes as Well As Potentially Cardiac MLCK Itself

In vitro kinase assay demonstrated incorporation of 32P to GST-MLC2v and GST-MLC2a fusion proteins using HA-tagged cardiac MLCK expressed in 293 cells in the absence of Ca2+/calmodulin (supplemental Figure III), indicating that ectopically expressed cardiac MLCK is sufficient for phosphorylation of MLC2v and MLC2a without Ca2+/calmodulin. Because catalytic activity of skeletal and smooth muscle MLCK is Ca2+/calmodulin-dependent, this finding was tested in additional kinase assays in a quantitative manner and confirmed that cardiac MLCK phosphorylated MLC2v in the absence of Ca2+/calmodulin (Figure 4A, top, cardiac MLCK, control), and that addition of EGTA (Figure 4A, top, +EGTA), or Ca2+/calmodulin (Figure 4A, top, +Ca2+/calmodulin) had little effect on the catalytic activity of cardiac MLCK. Under the same condition, control experiments using HA-tagged skeletal MLCK performed side by side demonstrated strong Ca2+/calmodulin-dependent kinase activities to MLC2v consistent with previous studies (Figure 4A, bottom, skeletal MLCK). In addition, HA-tagged cardiac as well as skeletal MLCKs purified with proteinase inhibitors appeared as single bands in Western blotting (Figure 4A, bottom, skeletal MLCK). The estimated kinetic constants determined by Lineweaver-Burk plot were as follows: $K_m = 4.3 \pm 1.5$ μmol/L; $V_{max} = 0.26 \pm 0.06$ μmol/min per milligram; $V_{max}/K_m$ ratio, 0.06 (without EGTA and Ca2+/calmodulin) (Figure 4B). The low $K_m$ value of cardiac MLCK indicating high affinity to the substrate is equivalent to skeletal MLCK to skeletal MLC (3.5 μmol/L) and smooth muscle MLCK to smooth muscle MLC (6 to 11 μmol/L). However, an indicator of efficiency of catalysis, $V_{max}/K_m$ ratio of cardiac MLCK, is lower than this ratio for skeletal and smooth muscle MLCK toward their MLC substrates isolated from the same tissue (9.3 and 3.5, respectively). Therefore cardiac MLCK appears to have a high affinity and relatively low catalytic efficiency to MLC2v.

Figure 3. Cardiac MLCK protein expression and intracellular localization. A, Western blotting using anti–cardiac MLCK antibody against GST–cardiac MLCK (amino acids 28 to 463) (lanes 1 and 2) detected HA-tagged full-length cardiac MLCK protein (lane 3) but not HA-tagged full-length skeletal MLCK (lanes 4 and 5). Anti-HA antibody recognizes HA-tagged cardiac and skeletal MLCK (lanes 3 to 5). B, Western blotting using anti–cardiac MLCK antibody detected a similar amount of MLCK protein in protein lysates isolated from mouse neonatal ventricles (lane 1) and atria (lane 2). V indicates ventricle; A, atrium. C, Cardiac MLCK protein expression in heart lysates is equivalent to 2.3 mg of GST–cardiac MLCK in 5 μg of heart lysates. The antibody does not cross-react with other proteins using similar amounts of tissue lysates from skeletal muscle and lung (lanes 4 and 5). D, Coimmunostaining of cardiac MLCK, actin (phalloidin), and MLC2v. Endogenous cardiac MLCK protein is localized diffusely in the cytoplasm with occasional striated patterns (arrows) that overlap with actin but not with striated actin (Figure 3D, green, arrows; arrowheads, noncardiomyocyte); however, in some areas, striated staining of cardiac MLCK was observed. Interestingly, enlarged image of cardiomyocytes coimmunostained to detect actin (Figure 3D, red, localizing at I bands) and MLC2v (Figure 3D, blue, localizing at A bands) demonstrated that striated MLCK staining was colocalized with actin (Figure 3D, green and red) but not with MLC2v (Figure 3D, green and blue). Specificity of immunostaining and additional endogenous cardiac MLCK stainings are shown in supplemental Figure II.

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Figure 4. Cardiac MLCK phosphorylates MLC2 and potentially MLCK itself. A, Autoradiogram of GST-MLC2v phosphorylation at various concentrations (0.012 to 3 μmol/L) catalyzed by cardiac MLCK (upper gels) and skeletal MLCK (lower gels). Control experiments without EGTA or Ca²⁺/calmodulin (control), with EGTA (+EGTA, 5 mmol/L), or with Ca²⁺/calmodulin (+Ca²⁺/calmodulin, 1 mmol/L Ca²⁺, and 1 μmol/L calmodulin) are shown. B, Kinetic analysis of cardiac MLCK using double reciprocal Lineweaver–Burk plot (0.18 to 3 μmol/L). Calculated kinetic values of cardiac MLCK were as follows: $K_m$ (μmol/L) 4.3±1.5 (without EGTA or Ca²⁺/calmodulin), 2.9±0.8 (with EGTA), and 3.9±1.2 (with Ca²⁺/calmodulin); $V_{max}$ (μmol/min per milligram): 0.26±0.06, (without EGTA or Ca²⁺/calmodulin), 0.18±0.03 (with EGTA), and 0.18±0.03 (with Ca²⁺/calmodulin). Values are expressed as means±SE (n=2). C, MLC2v phosphorylation was examined in neonatal cardiomyocytes with increased expression of cardiac MLCK by infection of Ad-cardiac MLCK (lanes 2, 3 vs lane 1). The relative amounts of phosphorylated to total MLC2v protein are shown below in comparison with control cardiomyocytes. Lane 1: Ad-βgal, 10 multiplicities of infection (mois); lane 2: Ad-cardiac MLCK, 2 mois; lane 3: Ad-cardiac MLCK, 10 mois. The relative values are expressed as means±SE (n=2). D, Decreased expression of cardiac MLCK using 3 different shRNAs (lanes 2 to 4) decreased MLC2v phosphorylation compared with scrambled adenovirus-shRNA (lane 1). The relative values of phosphorylated to total MLC2v protein in comparison with control cardiomyocytes are expressed as means±SE (n=4). E, Increased expression of cardiac MLCK by infection of Ad-cardiac MLCK adenovirus detected by Western blotting with anti–cardiac MLCK antibody. Lane 1: Ad-βgal, 10 mois; lane 2: Ad-cardiac MLCK, 2 mois; lane 3: Ad-cardiac MLCK, 10 mois. F, Reduced expression of cardiac MLCK by 3 different shRNAs is detected by Western blotting with anti–cardiac MLCK antibody. Lane 1, shRNA with scrambled sequence; lanes 2 to 4, shRNA targeting to 3 different sequences. G, Cardiac MLCK immunoprecipitated from neonatal ventricular cardiomyocytes was blotted with anti–phospho-serine or –phospho-tyrosine antibodies. Phospho-serine antibody reacted to cardiac MLCK protein. H, In vitro kinase assay showed 32P-incorporated HA-tagged MLCK expressed in 293 cells. I, Phenylephrine (30 μmol/L for 30 minutes) increased cardiac MLCK phosphorylation in cardiomyocytes infected with Ad-HA cardiac MLCK (1 moi). The relative amounts of phosphorylated cardiac MLCK protein are shown below in comparison with control cardiomyocytes without phenylephrine treatments (mean±SE, n=2). J, Phenylephrine (30 μmol/L, for 30 minutes) increased cardiac MLC2v phosphorylation in rat neonatal cardiomyocytes. The relative values of phosphorylated to total MLC2v protein in comparison with control cardiomyocytes without phenylephrine are shown (mean±SE, n=2).
In cardiomyocytes, overexpression of cardiac MLCK increases MLC2v phosphorylation nearly 2.1-fold in a dose-dependent manner (Figure 4C). Conversely, decreased MLCK expression achieved by infection of 3 MLCK–short hairpin (sh)RNA adenoviruses targeting different MLCK sequences (Figure 4D, RNA interference [RNAi] cardiac MLCK-1, -2, -3) decreased MLC2v phosphorylation levels by 30% to 55%. The levels of cardiac MLCK protein expression in overexpressed (Figure 4E) and knockdown cardiomyocytes (Figure 4F) are shown.

Catalytic activities of smooth muscle and skeletal MLCKs are regulated by phosphorylation by upstream kinases as well as MLCKs themselves.18 Phosphorylation of cardiac MLCK was detected with anti–phospho-serine antibody (Figure 4G, P-ser) but not with anti–phospho-tyrosine antibody (Figure 4G, P-tyr; supplemental Figure IV). We also detected incorporation of 32P to cardiac MLCK itself in an in vitro kinase assay in which immunoprecipitated exogenous cardiac MLCK proteins expressed in 293 cells were used (Figure 4H). This result suggests that cardiac MLCK autophosphorylates MLCK or that MLCK upstream kinases physically interact with cardiac MLCK in 293 cells. The level of cardiac MLCK phosphorylation was increased with phenylephrine stimulation (Figure 4I, lanes 1 and 2 versus lanes 3 and 4), accompanied by an increased MLC2v phosphorylation (Figure 4J, lanes 1 and 2 versus lanes 3 and 4). Potential phosphorylation sites and kinases predicted by amino acid sequence conserved between mouse and rat cardiac MLCK, including protein kinase A, are listed in supplemental Table I.

Cardiac MLCK Promotes Sarcomere Organization and Increases Cardiomyocyte Contractility

We observed that cardiomyocytes in which cardiac MLCK was overexpressed displayed organized sarcomere structures characterized by straight, thick, striated actin bundles, as had been seen with overexpression of skeletal MLCK (Figure 5A, Ad-β-galactosidase [βgal] versus Ad-cardiac MLCK).19 Phalloidin intensity in individual cardiomyocyte was increased in the MLCK-overexpressing cardiomyocytes compared with control βgal-infected cardiomyocytes (Figure 5A and 5B). In MLCK knockdown cardiomyocytes using 3 RNAs showed slight changes in peripheral structure up to 96 hours after adenoviral infection (Figure 5C, RNAi control versus RNAi cardiac MLCK) accompanied by reduced phalloidin intensity in individual cardiomyocyte treated with 3 different shRNAs as shown with the value in control cardiomyocytes defined as 1 (mean±SE).

Figure 5. Effects of cardiac MLCK in sarcomere organization. A, Overexpression of cardiac MLCK by Ad-cardiac MLCK adenovirus (10 mois) promotes sarcomere organization detected by phalloidin compared with cells infected with Ad-βgal (10 mois). Bars=10 μm. B, Relative intensity of phalloidin staining in individual cardiomyocytes overexpressing cardiac MLCK is shown with the value in control cardiomyocytes defined as 1 (mean±SE). C, Reduced expression of cardiac MLCK using 2 different shRNAs, RNAi-1 and RNAi-2, does not disturb sarcomere organization centrally with slight changes in peripheral structure (arrows). Bars=10 μm. D, Relative intensity of phalloidin staining in individual cardiomyocyte treated with 3 different shRNAs is shown with the value in control cardiomyocytes defined as 1 (mean±SE).
transients (Figure 6A through 6C, Ad-βgal versus Ad-cardiac MLCK). On the other hand, MLCK knockdown, targeting 3 specific sequences was without effect on cardiomyocyte contraction at the standard Ca\(^{2+}\) superfusate concentration (1.2 mmol/L) but with significant effect at the higher Ca\(^{2+}\) superfusate concentration (2.5 mmol/L), with the exception of −dL/dT in RNAi-1 (data not shown). These results suggest that alteration in sarcomere organization in MLCK knockdown cardiomyocytes may have an effect on function under increased demand.

Cardiac MLCK Expression and MLC2v Phosphorylation in Mice With Nkx2-5 Knockout, Aging, and Post–Myocardial Infarction Heart Failure

To examine catalytic activities of cardiac MLCK in vivo, we first examined cardiac MLCK expression and MLC2v phosphorylation levels in Nkx2-5 knockout hearts at postnatal day 12 when expression of cardiac MLCK mRNA (Figure 7A) and protein (Figure 7B) were markedly reduced. Skeletal MLCK mRNA expression in Nkx2-5 knockout hearts was below the level of detection by Northern blotting (Figure 7A, skeletal MLCK); however, using quantitative real-time PCR, skeletal (2.55 ± 0.04 fold, n = 2) and smooth muscle MLCK (1.48 ± 0.02 fold, n = 2) expression were increased. Despite this apparent compensatory increase, the level of MLC2v phosphorylation was decreased in hearts from Nkx2-5 knockout mice by nearly 60% compared with age-matched control mice (Figure 7C).

Cardiac MLCK mRNA increased during development from neonatal to adult stages and persisted in the aged hearts (Figure 7D). Of note, greater separation of RNA by agarose gel electrophoresis revealed 2 hybridized bands near 4.7 kb with similar intensities. Cardiac MLCK protein increased in hearts from embryonic day 10.5, neonates and adult, but was decreased in aged hearts (Figure 7E). Consistent with decreased cardiac MLCK protein in aged hearts (18 and 21 months old), MLC2v phosphorylation was decreased in aged hearts compared with postnatal day 12 hearts (Figure 7F).

We next examined cardiac MLCK expression in a post–myocardial infarction mouse model of heart failure 3 weeks after coronary artery ligation. At the mRNA level, cardiac MLCK expression in noninfarcted upper ventricular septal tissue was similar to tissue from sham-operated age-matched mice (Figure 7G, lanes 1 and 2 versus lanes 3 and 4). In contrast, cardiac MLCK protein was decreased in heart failure tissue compared with control tissue (Figure 7H, lanes 1 and 2 versus lanes 3 and 4). Furthermore, levels of MLC2v phosphorylation were decreased in heart failure compared with controls (Figure 7I). The lack of concordance between mRNA versus protein levels in neonatal and aged hearts and in failed hearts suggests altered posttranscriptional regulations of cardiac MLCK in aging and heart failure.

Discussion

In this study, we isolated a homolog of skeletal and smooth muscle MLCK that is preferentially expressed in the heart, herein named cardiac MLCK. Expression of cardiac MLCK mRNA was markedly downregulated shortly after reduction of Nkx2-5 expression by Nkx2-5 knockdown and inducible Nkx2-5 knockout. Nkx2-5 expression is nearly restricted to the heart in the postnatal stage,\(^{12–14}\) and expression of cardiac MLCK, its downstream target (either direct or indirect), was detected only in the heart using multigene Northern blotting.

Cardiac MLCK has a similar overall structure to known skeletal and smooth muscle MLCKs and has a high affinity to MLC2v similar to skeletal MLCK and smooth muscle MLCK.\(^{17}\) However, its catalytic efficiency is lower, and it was not regulated by Ca\(^{2+}\)/calmodulin or EGTA in vitro. Notably, for
Figure 7. Cardiac MLCK expression and MLC2v phosphorylation in mice with Nkx2-5 knockout, aging, and post-myocardial infarction (MI) heart failure. A and B, Decreased expression of cardiac MLCK mRNA and protein in Nkx2-5 knockout hearts at postnatal day 12. Skeletal MLCK mRNA was not detected by Northern blotting. C, Unphosphorylated (left, with higher pI) and phosphorylated (right, with lower pI) MLC2v examined in 2D electrophoresis, followed by Western blotting with anti-MLC antibody. Relative amounts of phosphorylated to total MLC2v are shown (mean±SE, n=2). D, Expression of cardiac MLCK and Nkx2-5 mRNA in neonatal, adult (4 months) and aged hearts (18 months). Relative expression of cardiac MLCK normalized to GAPDH is shown with the value in neonatal heart defined as 1. E, Level of MLC2v phosphorylation in young (PD 12) and aged hearts. Relative amounts of phosphorylated to total MLC2v are shown (mean±SE; young, n=2; old, n=6 from 2 mice at 18 and 21 months). G, Noninfarcted upper septal tissue dissected from mice 3 weeks after coronary ligation (3 month old) was analyzed for cardiac MLCK mRNA expression: 2 sham-operated (lanes 1, 2) and 2 heart failure (lanes 3 and 4) mice. Values of heart weight/body weight are indicated. Additional experimental conditions and parameters of cardiac function have been described previously35 and in Materials and Methods. Relative expression of cardiac MLCK normalized to GAPDH is shown with the value in sample 1 defined as 1. H, Cardiac MLCK protein expression in tissue lysates from the same mice used in G is shown with the value in sample 1 defined as 1. I, Level of MLC2v phosphorylation examined in mice with sham-operated and heart failure. Relative amounts of phosphorylated to total MLC2v are shown (mean±SE; sham, n=4 from 2 mice; heart failure, n=4 from 2 mice).
smooth muscle MLCK, which is also expressed in the heart, the amino acid sequence of substrates appears to be critical for affinity and catalytic activity, particularly an arginine residue in the third-position amino terminus to the phosphorylated serine residue (smooth muscle MLC [Arg-Ala-Thr-Ser]).15,16,20 The catalytic activity of smooth muscle MLCK toward skeletal MLC2, in which the critical Arg residue is replaced with Gly similar to MLC2v (skeletal MLC [Gly-Gly-Ser-Ser], skeletal MLCK [Gly-Gly-Thr-Ser]), was reported as a \( K_m \) value of 94 \( \mu \text{mol/L} \) and a \( V_{\text{max}}/K_m \) ration of 0.03.17 If similar values are applicable to MLC2v, these data imply that cardiac MLC2v may be as good a substrate for cardiac MLCK (\( V_{\text{max}}/K_m \) 0.06) as it is for smooth muscle MLCK but with distinct expression levels in neonatal hearts.

Under physiological conditions, the level of MLC2v phosphorylation is maintained relatively constant by well-balanced phosphorylation and phosphatase-induced dephosphorylation.21 Elevation of cytoplasmic \( [\text{Ca}^{2+}] \) induced by infusion of \( \text{Ca}^{2+} \) did not increase MLC2v phosphorylation consistently.22–24 If \( \text{Ca}^{2+} \)/calmodulin-independent catalytic activity, as well as high-affinity and relatively low catalytic efficiencies of cardiac MLCK toward MLC2v demonstrated in vitro, are applicable to in vivo, these previous studies may reflect functions of cardiac MLCK in the heart.

Increased expression of cardiac MLCK induced sarcomere organization in neonatal cardiomyocytes, as has been observed by overexpression of skeletal MLCK.19 Ser19 phosphorylation of MLC2 leading to potentiation of the force and speed of contraction has been well studied in smooth and skeletal muscle.3,18 Our findings demonstrate that overexpression of skeletal MLCK toward MLC2v demonstrated in vitro, are applicable to in vivo, these previous studies may reflect functions of cardiac MLCK in the heart.

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The amino terminus of cardiac MLCK, lacking homologies to known proteins, may have functions specific for cardiac MLCK. For instance, cardiac MLCK occasionally showed a striated expression pattern not overlapping with MLC2v in A bands but overlapping with actin in I bands. This finding may be interpreted that, locally, the interaction between MLCK and its substrate, MLC2v, may be transient or that cardiac MLCK may have additional functions including phosphorylation of other proteins. Of note, long-form smooth muscle MLCK also colocalizes with actin depending on the actin binding sequence consisting of repeat motifs (DFFRXL) located in the amino terminus,18,26 which was not found in cardiac MLCK.

Cardiac MLCK appeared to be phosphorylated; however, the phosphorylation sites of other MLCKs important for regulating their activities are not conserved in cardiac MLCK. These include 2 contiguous serine residues in the carboxyl terminus of the \( \text{Ca}^{2+} \)/calmodulin binding sequence of smooth muscle MLCK by protein kinase A, protein kinase C, CaMKII (\( \text{Ca}^{2+} \)/calmodulin-dependent protein kinase II) and PAK3 (789R, 790K; in Figure 2B); the autophosphorylation site of skeletal MLCK (amino terminus to the catalytic domain), smooth muscle MLCK (in the calmodulin binding domain and carboxyl terminus to this domain), and dictyostelium MLCK (between the catalytic and calmodulin binding domain).27–30 Phenylephrine stimulation resulted in increased phosphorylation of both cardiac MLCK and MLC2v. Whether increased cardiac MLCK phosphorylation directly increases MLC2v phosphorylation remains to be studied; however, this observation demonstrates 1 pathway for phosphorylation of cardiac MLCK, which indeed has several potential protein kinase A phosphorylation sites.

Cardiac MLCK protein expression appeared to be decreased in aged hearts and in heart failure in mice accompanied by decreased MLC2v phosphorylation. Because previous studies have demonstrated that MLC2v phosphorylation is decreased in patients with heart failure,31,32 and expression of a mutant MLC2v in transgenic mouse hearts that cannot be phosphorylated (Ser14, -15, and 19 to Ala mutations) leads to heart failure,34 it is possible that decreased cardiac MLCK protein expression may contribute to compromised contractile function in aging and in heart failure. Of note, a recent study reported upregulated cardiac MLCK mRNA expression in heart failure.34 In the present study, we found lack of concordance between mRNA and protein levels of cardiac MLCK that was likely attributable to altered posttranscriptional regulation of cardiac MLCK in aging and heart failure in mice.

In summary, we report the initial characterization of cardiac MLCK, which is likely a new regulatory factor for cardiac contraction and sarcomere organization.

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**Disclosures**

None.

**References**


Identification of Cardiac-Specific Myosin Light Chain Kinase
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Materials and Methods:

Cardiomyocyte preparation, adenovirus infection

Neonatal rat ventricular cardiomyocytes were isolated from hearts of 2-day-old Sprague Dawley rats by trypsin digestion followed by Percoll gradient purification according to a protocol described previously with modifications. Infection of adenovirus-shRNA (for MLCK and Nkx2-5, 100 moi) was performed in suspension immediately after purification for 2 hrs followed by plating on plates or laminin-coated glass coverslips for 48 to 96 hrs in medium (DMEM F-12, 5% new born calf serum, 0.5% of Insulin-Transferrin-Selenium) (Gibco-Invitrogen). Adenovirus Adlox-MLCK (2 and 10 moi) and control Adlox-beta-galactosidase (10 moi) were infected for 2 hrs after plating followed by an additional 24 to 48 hr incubation.

Animal models

Floxed-Nkx2-5 mice were bred with transgenic mice carrying the Cre-ER$^\text{TM}$ gene under CMV promoter. Matings generated mice homozygous or heterozygous for the floxed-Nkx2-5 gene with or without being heterozygous for Cre-ER$^\text{TM}$ transgene. Female mice homozygous or heterozygous for floxed-Nkx2-5/Cre-ER$^\text{TM}$(-) were bred with male mice homozygous or heterozygous for floxed-Nkx2-5/Cre-ER$^\text{TM}$ (+) to generate floxed/floxed/Cre-ER$^\text{TM}$ (+), floxed/floxed/Cre-ER$^\text{TM}$ (-) or wild/wild/Cre-ER$^\text{TM}$ (+). To delete the floxed-Nkx2-5 gene, tamoxifen (0.5-1 mg/g body weight, ip) was injected into pregnant mice within 24 hr before delivery. All animal care protocols fully conformed to the Association for the Assessment and Accreditation of Laboratory Animal Care, with approval from the University of Florida Institutional Animal Care and Use Committees. Upper-septum tissues isolated from post-myocardial infarction heart failure mice previously characterized were utilized for Northern and
Western blotting. Values for heart failure in sham-operated and two heart failure mice are as follows (mean ± SE): heart weight/tibial length, 5.83 ± 0.21 vs. 14.6 ± 1.35 mg/mm; LV end-diastolic dimension, 3.46 ± 0.02 vs. 5.72 ± 0 mm; LV end-systolic dimension, 1.89 ± 0.11 vs. 4.24 ± 0.17 mm; LV fractional shortening, 45 ± 3.3 vs. 26 ± 3.0%.

**Cloning of cardiac, skeletal and smooth muscle MLCK and plasmid construction**

RNA isolated from neonatal mouse hearts and skeletal muscle were subjected to reverse transcription using random priming followed by PCR using four sets of specific primers to amplify partial cardiac MLCK cDNA: (fragment 1, F, 5’-

CCTTCAGATGTTAGCACACAAAGTG-3’; R, 5’-TGCCGACAGCCCTCACCAG-3’),

(fragment 2, F, 5’-TGCCGACACTCCCCCAACC-3’; R, 5’-

CCAAACCGACCCCCCTCCTAAG-3’), (fragment 3, F, 5’-

AAGAGGAGCAGCAACAAATGGTG-3’; R, 5’-TTTCAGGCACCTGTGGCG-3’), (fragment 4, F, 5’-AGTTGGATGTGCTTTGTCACG-3’; R, 5’-AAAAGGAAGGGTGCGGGG-3’).

Fragments 1-4 were used for cloning of full length MLCK cDNA using PCR. Two sets of specific primers were used for amplifying partial skeletal MLCK cDNA: (fragment 1, F, 5’-

GACTACAGAAAACGGAGCAGTTGAG-3’; R, 5’-TGCCGACAGGCCCTCACCAG-3’),

(fragment 2, F, 5’-AGACACACCAAGGAGAAGATG-3’; R, 5’-

GGCGGTAGCGAGATGGATTC-3’). HA-tagged partial skeletal MLCK was amplified from fragment 1 with the specific primer set (F, 5’-

GGGTACCATGTTACCCATACGATGCCAGATTTACGCTACTACAGAAAACGGAGCAGTT-3’; R, 5’-TGCCGACAGGCCCTCACCAG-3’), followed by insertion of fragment 2 into the appropriate restriction sites to construct full length HA-tagged skeletal MLCK. Three sets of
primers were used for amplifying partial smooth muscle MLCK cDNA for fusion protein productions: (fragment 1, aa 1-337 of short form smooth muscle MLCK, F, 5’-
GAAGCTTCATGGATTTCCGCGCCAACCTTC-3’; R, 5’-
GGTTTCTTTTTTCACAGTTGCGTCG-3’), (fragment 2, aa 274-860, F, 5’-
CCTGAGGACCGAGGTTTGTGA-3’; R, 5’-CGTTTTCTGCCACTTCCTTC-3’), (fragment 3, aa 829-1031, F, 5’-ACCCATGGCTGATGAAAGAC-3’; R, 5’-
TCTGTTTGCGCTTGTCTTCACTC-3’). PCR products were cloned into pCR2.1 vector (Invitrogen) and sequenced.

HA-tagged full length cardiac MLCK was amplified by PCR (F, 5’-
GGTACCAGGTACCCCATACGATGTTCCAGATTACGCTTCAGGAGTTCAGGAGGA
-3’; R, 5’-AAAAGGAAGGGTGCGGGG-3’) and cloned into TOPO-blunt PCR vector. SacI-blunt/KpnI fragment was subcloned into the EcoRI-blunt/KpnI sites of pAdlox shuttle vector5 to generate pAdlox-cardiac MLCK. KpnI/BamHI-blunt fragment of HA-tagged full length skeletal MLCK was subcloned into the BamHI-blunt/KpnI sites of pAdlox shuttle vector to generate pAdlox-skeletal MLCK.

Adenoviral short-hairpin (sh) RNA was generated according to our standard methods.6 Briefly, target sites for RNAi of rat cardiac MLCK were designed using web sites (QIAGEN-http://siRNA.qiagen.com/Index.jsp., and Ambion-http://www.ambion.com/techlib/misc/siRNA_finder.html.m1.). Three specific sites for rat MLCK-RNAi are: (1) CTTAATGTGCTGACTGAGA, (2) CAGATGCAGAGACCATGAA, (3) ATATATGGCTCAGCGTAAA. Rat Nkx2-5-RNAi target sequence is GGCGGTGGAGCTGGACAAA. Scrambled sequences with no homology to known genes were used for control RNAi.
GST-cardiac MLCK(aa 28-463) vector was generated by insertion of NcoI/XbaI fragment of MLCK cDNA into NcoI/XbaI site of pGEX-CD vector. GST-MLC2v vector was generated by insertion of the BamHI/EcoRI fragment of pcDNA3-rat MLC2v into BamHI/EcoRI site of pGEX-CD vector. GST-MLC2a vector was generated by insertion of the HindIII/SalI digested fragment of the PCR products using primers for mouse MLC2A (F, 5’-GAAGCTTCATGGCCAGTAGGAAGGCTGGG-3’; R, 5’- CGTCGACCTACTCCTTTCTCATCCCGTG-3’) amplified from the cDNA (IMAGE Consortium CloneID 30300846, OPEN BIOSYSTEMS). GST-smooth muscle MLCK vectors were generated by insertion of PCR fragments described above into appropriate restriction sites of pGEX-CD vector.

**GST fusion protein and MLCK antibody production**

GST fusion proteins were expressed in *E.coli* BL21(DE3) (Stratagene) with 1 mM of IPTG for 4 hr at 37ºC. Bacteria were lysed by sonication in lysis buffer [20mM HEPES, pH7.5, 100 mM NaCl, 10 mM MgCl2, 1% Triton x-100, proteinase inhibitor cocktail (complete, Roche)]. Lysates were incubated with Glutathione Sepharose (Amersham) and washed with lysis buffer 5 times. For in vitro kinase assays, GST proteins bound to Glutathione Sepharose were further washed with kinase buffer (25 mM HEPES, pH7.6, 200 mM NaCl, 10 mM MgCl2) three times. GST-fusion proteins were eluted with 10 mM of reduced Glutathione (Sigma) in the kinase buffer.

GST-cardiac MLCK proteins bound to Glutathione Sepharose were washed with PBS, followed by cleavage of GST protein using thrombin (20 U/ml) for 3 hrs. After addition of Tris-HCl (final concentration 50 mM) and NaCl (final concentration 0.5 M) thrombin was removed by Benzamidine Sepharose (Amersham). MLCK proteins were further purified by SDS-PAGE
followed by electro-elution. The purified proteins (150 μg) were emulsified with complete Freund’s adjuvant (day 1) or incomplete Freund’s adjuvant (days 14, 28, and 42) to immunize New Zealand White rabbits for polyclonal antibody production. Affinity purification of antibodies was performed using GST-cardiac MLCK(aa 28-463) covalently bound to agarose beads using AminoLink Plus Immobilization Kit (PIERCE).

Northern and Western blotting, immunostaining and real time PCR

Northern blotting was performed using the following probes: cardiac MLCK RT-PCR product (1266 bp, F, 5’-TGGCAGCCTCCCCCAACC-3’; R, 5’-CCAAACCGACCCCTCCTTAAG-3’); mouse skeletal MLCK RT-PCR product (552 bp, F, 5’-GACTACAGAAACGGAGCAGGTGAG-3’; R, 5’-GGGTACAGGGGTACAGACACC-3’); Nkx2-5 probe, PflMI-EcoRI fragment of mouse Nkx2-5 cDNA, and GAPDH probe described previously.8

Western blot analyses and immunostaining were performed with the following antibodies: anti-HA (clone 3F10, Roche), anti-MLC2 (F109.3E1, BioCytex, Marseille, France), anti-cardiac MLCK pAb described above, anti-smooth muscle MLCK (clone K36, M7905, SIGMA), anti-phospho-serine (29675, AnaSpec), anti-phospho-tyrosine (4G10, Upstate), anti-troponin T (T6277, SIGMA) and anti-GAPDH (Research Diagnostics Inc.).

Phallodin-TRITC (77418, Sigma) was utilized for detecting polymeric F-actin. Fluorescent microscopic images were obtained using ZEISS Axiovert200M with or without Apotome. Intensity of phalloidin signal to quantitate F-actin staining was measured side by side in control vs. cardiac MLCK overexpressed cardiomyocytes or control vs. cardiac MLCK knockdown cardiomyocytes, using the same cardiomyocyte preparation, cell fixation, phalloidin
staining and imaging with the same exposure time below the level of saturation using ZEISS Axiovert200M without Apotome. Individual cells were traced and signal intensity from the traced area was measured in Image J.

Real-time PCR was performed using the following Taqman Gene Expression Assays (Applied Biosystems): skeletal MLCK Mm01251292_m1 and smooth muscle MLCK Mm00653039_m1. RNA isolated from control and Nkx2-5 knockout hearts was subjected to reverse transcription followed by real-time PCR (final cRNA concentration 0.045 μg/μl, StepOne, Applied Biosystems) and quantified against standard curves (4 points, 10 fold serially dilution, duplicated) and normalized to 18S rRNA (4310893E, Applied Biosystems). Duplicated experiments were averaged.

**Phosphorylation assays**

Phosphorylation assays were performed according to protocols described previously with some modifications.\(^9\) Briefly, in vitro phosphorylation reactions were performed using recombinant HA-tagged full-length MLCK proteins expressed in 293 cells. Immunoprecipitated MLCK proteins using anti-HA antibody (3F10, Roche) bound to protein G sepharose (Sigma) were washed three times with kinase buffer, mixed with GST, GST-MLC2v or GST-MLC2a fusion protein (approximately 2 μg) in the presence of 1 μl of \([132P]ATP (0.25 \text{ mCi/ml})\) in 20 μl of kinase buffer at 37°C for 30 min. Immunoprecipitants without anti-HA antibodies were used as negative controls. 10 μl of each sample were boiled in SDS-PAGE sample buffer and subjected to SDS-PAGE, stained with Coomassie blue and autoradiographed.

HA tagged-cardiac MLCK and HA-tagged skeletal MLCK expressed in 293 cells were purified by HA-affinity columns and eluted with HA-peptides (Roche). GST-MLC2v and HA-
tagged cardiac and skeletal MLCK proteins were dialyzed against protein lysis buffer (25 mM HEPES, pH 7.6, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% triton, 10% glycerol, protease inhibitor cocktail). After measurement of protein content using BCA™ Protein Assay Kit (PIERCE), a 1:10 dilution of proteins was utilized in the kinase reaction (approximately cardiac MLCK 0.15 μg/ml, 1.7 nM; skeletal MLCK 0.15 μg/ml, 2 nM; GST-MLC2v 0.14 mg/ml, 3 mM with 2 fold serial dilution) in 25 μl of kinase buffer (final concentration 25 mM HEPES, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 20 mM NaCl, 0.2% triton, 2% glycerol, 0.5 mg/ml BSA, 0.5 mM [γ-³²P]ATP at 267 cpm/pmol) at 30°C for 15 min. Either 1 mM Ca²⁺ and 1 μM calmodulin (Sigma P2277) or 5 mM EGTA were added in the kinase reaction to examine Ca²⁺/calmodulin dependence. After termination of the kinase reaction by the addition of SDS-sample buffer, the samples were separated by 15% SDS-PAGE gel, stained with Coomassie blue and autoradiographed. Phosphorylated MLC2v proteins were excised from the gel and their radioactivity was measured using a liquid scintillation counter. Km and Vmax of cardiac MLCK were determined using Lineweaver-Burk analysis (Prism 4, GraphPad Software, Inc.).

For detection of ³²P-labeled MLC2v and cardiac MLCK proteins, neonatal rat ventricular cardiomyocytes (2x10⁵ cells/3 cm plate) with or without infection of adenovirus were labeled with [³²P]-orthophosphate at 0.1 mCi/ml in phosphate-free DMEM containing 10% dialyzed FBS (GIBCO/Invitrogen) for 1 hr. Cells were lysed with 0.6 ml of RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP40, 2 mM EDTA, 1% deoxycholate, protease inhibitor cocktail, 10 mM NaF, 2 mM Na₃VO₄, pH 7.5). 0.25 ml of the lysates was pre-cleared with protein G or A, and then immunoprecipitated with anti-MLC2 antibody, anti-cardiac MLCK or anti-HA antibodies coupled to protein G or A. Immunoprecipitants without antibodies were used as negative controls. After washing with RIPA buffer (5 times), samples were transferred to PVDF
membrane (Millipore) after SDS-PAGE and autoradiographed. The membrane was then immunoblotted with anti-MLC2, anti-cardiac MLCK, anti-phospho-serine, or anti-phospho-tyrosine antibodies. The relative amount of phosphorylated and total MLC2v or phosphorylated and total cardiac MLCK was determined by densitometric analyses.

Phenylephrine (30 μM) was added 30 min after 32P labeling and incubated additional 30 min (total 1 hour of 32P labeling). Effects of phenylephrine on cardiac MLCK phosphorylation was examined in cardiomyocytes infected with Ad-cardiac MLCK (1 moi, 36 hrs); and its effects on MLC2v phosphorylation was examined in cardiomyocytes without Ad-cardiac MLCK infection.

**Two-dimensional gel electrophoresis**

Heart tissues or cardiomyocytes were homogenized in acetone containing 10% trichoroacetic acid (TCA) and 10 mM DTT to fix the phosphorylation status of MLC2v, centrifuged after 1 h incubation at -20°C, and washed three times with acetone. Two-dimensional gel electrophoresis was performed as recommended (BIO-RAD). Samples (approximately 5 μg) were dissolved in first-dimensional sample buffer (8 M urea, 2% Triton X-100, 5% -mercaptoethanol, 2% Bio-Lyte 4/6) and loaded on first dimensional gel (8 M urea, 4% acrylamide, 2% Triton X-100, 2% Bio-Lyte 4/6, 0.01% ammonium persulfate, 0.1% TEMED). After first-dimensional separation, gel strips were separated in SDS-PAGE gel, transferred to PVDF membrane and autoradiographed. The membrane was then immunoblotted with anti-MLC2. To confirm that negatively charged MLC2v is phosphorylated, cultured mouse neonatal ventricular cardiomyocytes (2x10^5 cells/3cm) labeled with [32P]-orthophosphate 0.1
mCi/ml for 4 hrs were utilized for two-dimensional gel electrophoresis for detection of phosphorylated MLC2v (data not shown).

**Simultaneous measurements of cell shortening and intracellular free calcium**

Simultaneous measurements of cell shortening and intracellular free calcium were performed as described with modifications. Myocytes were loaded with the acetoxyethyl ester of fura-2 (0.1 μmol/L, Molecular Probes/Invitrogen) in Tyrode’s solution (mmol/L: NaCl 137, KCl 3.7, NaH₂PO₄ 1.2, CaCl₂ 1.2, HEPES 20, MgSO₄ 1.2, glucose 15; and 0.0005% Pluronic F-127, pH 7.4) for 10 minutes at room temperature. Myocytes were rinsed with Tyrode’s solution and maintained for 20 minutes at room temperature to allow de-esterification of the dye, followed by superfusion with modified Tyrode’s solution (mmol/L: NaCl 137, KCl 3.7, NaH₂PO₄ 1.2, CaCl₂ 1.2, HEPES 4, MgCl₂ 0.5, glucose 15, probenecid 1.0, pH 7.4) on a temperature-controlled chamber (32°C) mounted on an Olympus inverted microscope. When studied at two Ca²⁺ concentrations, myocytes were sequentially superfused with 1.2 mM Ca²⁺ followed by 2.5 mM Ca²⁺ for 30 minutes before measurement. A dual excitation spectrofluorometer was used to record fluorescence emissions (505 nm) elicited from excitation wavelengths at 340 and 380 nm. Myocytes were imaged with a CCD video camera attached to the microscope and motion was quantified by video motion detection (IonOptix). To provide high-contrast spots for tracking cell motion of neonatal cardiomyocytes, glass beads 2 ± 0.5 μm (Duke Scientific Corp) were added as described previously. These experiments were performed using the same cardiomyocyte preparations plated at the same density for treatment with adenovirus Ad-MLCK and Ad-beta-galatosidase to reduce experimental variability.
Statistical analyses

Results between groups were compared using ANOVA and Fisher PLSD post-hoc test (StatView version 5.0). $p < 0.05$ was considered significant.
References:


Table S1. Predicted phosphorylation sites and kinases commonly identified in mouse and rat cardiac MLCK

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Analyses were performed using NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/). Prediction score higher than 0.8 (orange) and 0.7 (yellow) are shown.
Figure S1. Smooth muscle MLCK expression in the heart, skeletal muscle and lung.

Anti-smooth muscle MLCK monoclonal antibody clone K36\textsuperscript{14,15} recognized GST-smooth muscle MLCK(aa 1-337 of short form MLCK) (lanes 1-3), but not two other GST-fused carboxyl-termini of smooth muscle MLCK(aa 274-860 and 829-1031) (lanes 4, 5). Expression of 130-kDa short-form smooth muscle MLCK protein is detected in the heart (estimated concentration 0.025-0.05 \( \mu \text{g/mg} \)), skeletal muscle, and more abundantly in the lung (estimated concentration 0.5-1 \( \mu \text{g/mg} \)) (lanes 6-8, arrowhead). Additional proteins with approximate MW 60 kDa are also recognized (* in lanes 6-8).
Figure S2. Intracellular localization of cardiac MLCK.

(A) HA-tagged MLCK proteins overexpressed by adenovirus (Ad-HA cardiac MLCK) were co-immunostained with anti-MLCK and anti-HA antibody. Punctate MLCK was diffusely localized in the cytoplasm. The merged image demonstrates colocalization of the anti-HA-tag and the anti-MLCK antibodies. Of note, overexpressed cardiac MLCK is detected in the cytoplasm as well as nuclei. (B) Endogenous cardiac MLCK protein is localized diffusely in the cytoplasm with occasional formation of a striated pattern. Bars 10 μM.
Figure S3. In vitro cardiac MLCK phosphorylation of MLC2v and 2a.

HA-tagged cardiac MLCK expressed in 293 cells was immunoprecipitated with anti-HA antibody, then mixed with GST (lane 1), GST-MLC2v (lane 2), or GST-MLC2a (lane 3) in the presence of \[^{32}\text{P} \cdot \text{ATP}\]. Control experiments in the absence of anti-HA antibody are shown in lanes 4-6. Autoradiogram (top panel) of the Coomassie stained gel (bottom panel) is shown.
Figure S4. Anti-phosphotyrosine antibody (clone 4G10) did not detect endogenous and overexpressed cardiac MLCK in rat neonatal cardiomyocytes.

Endogenous cardiac MLCK (lane 2) or overexpressed HA-tagged cardiac MLCK (lane 6) in neonatal rat cardiomyocytes were immunoprecipitated, separated in SDS-PAGE and blotted to PVDF membrane. Control experiments in the absence of antibodies are shown in lanes 1 and 5. Anti-phosphotyrosine antibody (clone 4G10, Upstate) did not recognize cardiac MLCK proteins (lanes 2 and 6). A431 cell lysates (10 μg, Upstate) without EGF stimulation (lanes 3, 7) or with EGF stimulation (lanes 4, 8) were utilized as positive controls.