Myosin Regulatory Light Chain Phosphorylation Attenuates Cardiac Hypertrophy*

Jian Huang1, John M. Shelton5, James A. Richardson6, Kristine E. Kamm5, and James T. Stull‡1

From the Departments of 1Physiology, 5Internal Medicine, 6Pathology, and 1Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Hyperphosphorylation of myosin regulatory light chain (RLC) in cardiac muscle is proposed to cause compensatory hypertrophy. We therefore investigated potential mechanisms in genetically modified mice. Transgenic (TG) mice were generated to overexpress Ca2+/calmodulin-dependent myosin light chain kinase specifically in cardiomyocytes. Phosphorylation of sarcomeric cardiac RLC and cytoplasmic nonmuscle RLC increased markedly in hearts from TG mice compared with hearts from wild-type (WT) mice. Quantitative measures of RLC phosphorylation revealed no spatial gradients. No significant hypertrophy or structural abnormalities were observed up to 6 months of age in hearts of TG mice compared with WT animals. Hearts and cardiomyocytes from WT animals subjected to voluntary running exercise and isoproterenol treatment showed hypertrophic cardiac responses, but the responses for TG mice were attenuated. Additional biochemical measurements indicated that overexpression of the Ca2+/calmodulin-binding kinase did not perturb other Ca2+/calmodulin-dependent processes involving Ca2+/calmodulin-dependent protein kinase II or the protein phosphatase calcineurin. Thus, increased myosin RLC phosphorylation per se does not cause cardiac hypertrophy and probably inhibits physiological and pathophysiological hypertrophy by contributing to enhanced contractile performance and efficiency.

Phosphorylation of RLC in striated muscles potentiates the force and speed of contractions that are dependent on Ca2+ binding to troponin on actin-containing thin filaments (1–3). Both skeletal and cardiac muscle RLC phosphorylation in sarcomeres cause a leftward shift in the myofilament force-pCa relationship, showing an increase in Ca2+ sensitivity and an increase in the rate of weakly attached cross-bridges entering the contractile cycle (1, 4–8). In heart muscle, a spatial gradient of cRLC phosphorylation across the ventricle with decreasing cRLC phosphorylation from apex to base may facilitate torsion during contraction (7, 9). Transgenic mice expressing a nonphosphorylatable, ventricular cRLC show suppressed hemodynamic performance (10), consistent with demonstrated effects of RLC phosphorylation in skeletal muscle (2, 11).

Cardiomyocytes also contain a cytoplasmic, nonmuscle myosin IIB with its RLC (nRLC) where phosphorylation increases actin-activated ATPase activity. Myosin IIB is diffusely distributed in the cytoplasm during development and then localizes to Z-lines and intercalated discs after birth (12, 13). Ablation of cytoplasmic myosin II expression results in sarcomere disarray and aberrant development of the heart, leading to heart failure and death (14). RLCs are phosphorylated by dedicated Ca2+/calmodulin-dependent MLCKs (15). skMLCK was reported to be in cardiac muscle where a mutation in the kinase from a young patient with hypertrophic myocardium was associated with an increased Vmax value. Thus, it was suggested that development of compensatory hypertrophy results from an increase in cRLC phosphorylation (7, 9).

In the current study, we tested this hypothesis by generating transgenic mice overexpressing skMLCK in cardiomyocytes. skMLCK is a dedicated protein kinase that phosphorylates RLCs from striated as well as smooth muscle and nonmuscle cells (15). We also examined adaptations to exercise conditioning and β-adrenergic stimulation in these transgenic animals, two stresses that induce cardiac hypertrophy.

The availability of free Ca2+/calmodulin for activation of its cellular targets may be limiting in cells. Total calmodulin ranges from 5 to 40 μM in different kinds of cells where it affects protein structures and enzyme activities as well as gene expression (16). The free Ca2+/calmodulin concentration is typically <1% of the total cellular calmodulin (16–19). The free Ca2+/calmodulin in smooth, but not skeletal, muscle is limiting for full activation of a high affinity target such as MLCK (11, 20). In cardiac muscle, free Ca2+/calmodulin also appears to be only 1% (50–75 nM) of the total calmodulin, suggesting intense competition among its targets (21). Therefore, by overexpressing Ca2+/calmodulin-binding MLCK in cardiomyocytes, we could...
determine whether Ca\(^{2+}\)/calmodulin is functionally limiting for activation of target enzymes such as CaM kinase II and the protein phosphatase calcineurin.

**EXPERIMENTAL PROCEDURES**

**Production of Transgenic and Knock-out Mice**—The coding region of rabbit skMLCK cDNA (1.8 kb) was subcloned into pCMV5 using the EcoRI restriction sites in pCMV5 to generate pCMV5 skMLCK. The enhanced yellow fluorescent protein-enhanced cyan fluorescent protein biosensor containing the Ca\(^{2+}\)/calmodulin-binding sequence (16) was subcloned immediately downstream of the rabbit skMLCK cDNA using BamHI and XhoI restriction sites similar to the construction of the biosensor smooth muscle MLCK (20). The addition of the calmodulin sensor to skMLCK allows us to monitor expression as well as to study calmodulin activation properties in cardiomyocytes in the future. Expression and Ca\(^{2+}\)/calmodulin-dependent activity of skMLCK from pCMV-41M-skKCS was determined in transfected COS cell lysates (11). Mouse α-myosin heavy chain promoter in the Bluescript vector (pMHC/BS) was a generous gift from Dr. Robbins (22). The skMLCK biosensor cDNA was isolated from CMV-41M-skKCS using EcoRI and SmaI restriction enzymes to produce a 3.6-kb fragment. The ends were filled using Klenow enzyme and ligated into pMHC/BS, which had been linearized immediately downstream of the MHC promoter using Sall and HindIII restriction enzymes and subsequently filled in with Klenow enzyme. The linearized MHC-41M-skKCS construct DNA was microinjected into fertilized oocytes (ICR strain) for production of transgenic mice by the Transgenic Mouse Facility at the University of Texas Southwestern Medical Center at Dallas. All protocols involving mice in this study were approved by the University Institutional Animal Care and Use Committee. Five founder animals containing the MHC-41M-skKCS transgenes were identified by Southern analysis and used to establish homozygous transgenic mouse lines. Founder animals containing the MHC-41M-skKCS transgene were identified by Southern blot analysis of tail genomic DNA by using a \(^{32}\)P-labeled 750-bp cDNA encoding enhanced yellow fluorescent protein as a probe. Reverse transcription-PCR was also used to identify transgenic mice. A forward/sense primer (5′-ATCCATCCAGCAGCAAGAAAG-3′) and a reverse/antisense primer (5′-CTCGGTCTTCCT-TGTCCTCT-3′) were derived from rabbit skMLCK cDNA sequence. These produced a 492-base pair fragment in mice harboring the transgene. Another pair of forward/sense primer (5′-GGTGCCCTAGTCTCAATTTC-3′) and reverse/antisense primer (5′-GAAGAGACTGGAGCCAGGGA-3′) derived from mouse α-MHC promoter produced a 256-base pair fragment for control. Positive founders were used to establish transgenic mouse lines. Mice from two lines expressing different amounts of skMLCK with no obvious phenotype were characterized as described below. Samples of mouse ventricles were excised from transgenic mice and quick-frozen in liquid nitrogen. Tissues were analyzed by Western blotting following SDS-PAGE to determine expression of skMLCK (2, 20).

**Western Blotting for Protein Phosphorylation**—The extent of RLC phosphorylation was measured for both cRLC and nRLC in cardiac tissues with procedures routinely used for smooth and skeletal muscles and quantitated on a PhosphorImager with ImageQuant software to determine extents of RLC phosphorylation (2, 20). Briefly, left ventricles quickly removed from anesthetized animals were sliced transversely into apex, middle, and base portions and frozen in liquid nitrogen within 2 min or less. In a different set of hearts, the left ventricular free-wall was sliced into epicardial and endocardial sections with a razor blade. Changes in cRLC phosphorylation occur on the order of 30–45 min in heart, so immediate fixation in situ is not essential to measure the extent of phosphorylation that reflects in vivo values (23). Muscle samples were subjected to urea/glycerol-PAGE to separate phosphorylated and nonphosphorylated RLCs as described previously (20). Because the urea/glycerol-PAGE system separates nonphosphorylated from monophosphorylated RLC, we have a direct quantitative measure of RLC phosphorylation in terms of percent or mol of phosphate/mol of RLC. Antibodies to cRLC (rabbit polyclonal) or smooth/nonmuscle muscle RLC (mouse monoclonal) were used for the appropriate analyses with Western blotting (20), and quantitative measurements were processed on a Storm PhosphorImager and analyzed by ImageQuant software.

Additional Western blotting was performed on other proteins. Microsomes from heart were prepared with minor modifications (24). Samples were submitted to SDS-PAGE and blotted with antibodies to phospholamban as described previously (25). The endogenous Ca\(^{2+}\)/calmodulin-dependent calcineurin phosphatase activity was measured by assessing the expression of MCIPI.4 (modulatory calcineurin-interacting protein \(\lambda\), exon 4 isoform), which is a direct target of the calcineurin/NFAT (nuclear factor of activated \(\lambda\)-cells) pathway (26).

**Structural Analyses**—Before histological evaluation, hearts were arrested in diastole by injection of 10% KCl into the jugular vein of anesthetized mice and then processed into paraffin according to routine procedures (27, 28). Four-chamber, longitudinal views were sectioned at the level of the aortic and pulmonary valves, and transverse views were sectioned at the level of the papillary muscles. Slides of four-chamber, longitudinal sections were randomized, and a blinded observer attempted to sort the slides into groups by distinctions in microscopic morphology. Cardiomyocyte cross-sectional areas were measured using OpenLab software to calculate areas from outlined contours of cells in photomicrographs of transverse sections (29). Cells with a symmetry ratio of >0.6 (where 1 equals round) were used in the analysis to avoid measuring areas of cardiomyocytes sectioned tangentially.

For transmission electron microscopy, dissected ventricles were rinsed in 0.1 M cacodylate buffer, cut into blocks of 3 × 1.5 × 0.5 mm, and immersion-fixed in 2% glutaraldehyde in cacodylate buffer at 4 °C. Sections were cut by routine procedures, and images were made by AMT Advantage Software (version 4.1.0, for IC-PCI Frame Grabber with ORCA camera).

**Animal Protocols**—Exercise conditioning by voluntary wheel running was used to promote myocardial hypertrophy as described previously (30). Litter-matched male mice at 9–10 weeks of age were placed in individual cages where the number of revolutions/day was recorded; mice running 3 or more km/day were selected for analyses. Hearts were collected after 4...
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weeks of voluntary exercise. Other groups of matched mice were treated with isoproterenol for 7 days to induce cardiac hypertrophy. Isoproterenol at 40 mg/ml/g of mouse in saline or saline itself was injected into an Alzet® mini-osmotic pump (Model 2001, Durect Corp.), which releases at 1.0 µl/hr. Pumps were surgically implanted on the back during anesthesia.

At the end of treatment protocols, mice were anesthetized (50 mg/kg sodium pentobarbital) and weighed. Whole hearts were removed, dissected free of great vessels, lightly blotted to remove blood, and weighed. Tibial length was measured.

Statistical Analysis—Data are expressed as mean ± S.E. Statistical evaluation was carried out by using an unpaired Student’s t test for two comparisons or analysis of variance (plus the Newman-Keuls method) for multiple comparisons of data with variance homoscedasticity assessed by the Bartlett method. Kruskal-Wallis rank-sum and Nemenyi tests were used in multiple comparisons for data not meeting the homoscedastic variance test. Significance was accepted at a value of p < 0.05.

RESULTS

Transgenic Mice Express skMLCK in Heart—We generated transgenic mice producing the skMLCK transgene (TG-skMLCK) specifically in cardiomyocytes with the full-length rabbit skMLCK cDNA coding region driven by the mouse α-MHC promoter. PCR analysis showed a 492-bp fragment from rabbit skMLCK, a 256-bp mouse α-MHC promoter fragment in transgenic skMLCK mice, and only the 256-bp fragment in WT mice (Fig. 1). Southern blots showed that DNA from transgenic mice has a primary BamHI fragment at 2.5 kb that is absent in DNA from WT mice. Five founder lines were established, two of which (the AM and AN lines) displayed significant skMLCK expression in the heart and were used for additional characterization.

Transgenic mice express TG-skMLCK in heart tissue. Immunoblotting shows that TG-skMLCK migrated at the expected molecular mass of 145 kDa (Fig. 1) and was present in ventricles from AM and AN lines in amounts greater than skMLCK expressed in fast-twitch, extensor digitorum longus skeletal muscle and with expression in AM greater than in the AN line. There was no detectable skMLCK in heart tissue analyzed by immunoblotting, consistent with previous reports (2, 31). To obtain a quantitative measure of the amount of skMLCK expressed in heart, we did Western blots with heart samples containing the transgene relative to the rabbit fast-twitch gracilis skeletal muscle. The cardiac tissue contained 6.3-fold and 0.63-fold amounts of kinase in the AM and AN lines, respectively (Fig. 1).

Measurement of Sarcomeric and Cytoplasmic Myosin RLC Phosphorylation—The specificities of our antibodies to sarcomeric cRLC and to smooth and nonmuscle RLC were first determined in immunoblots. Immunoblotting showed that the polyclonal antibody against cardiac sarcomeric myosin RLC recognized only purified cRLC, but not the smooth muscle or cytoplasmic nonmuscle RLC (Fig. 2A). The monoclonal antibody raised against smooth RLC identified both purified smooth muscle RLC and nonmuscle RLC, but not sarcomeric cRLC. Additional characterizations were performed with urea/
glycerol-PAGE that separates monophosphorylated from the nonphosphorylated RLC because of the two additional negative charges introduced by phosphate (32, 33). The monoclonal antibody raised against smooth RLC identified nonphosphorylated and monophosphorylated forms of RLC in ventricular tissue samples (Fig. 2B) that contain a mixture of cytoplasmic and smooth muscle RLCs that comigrate in urea/glycerol-PAGE (data not shown). The cytoplasmic nRLC in the tissue samples is derived from nonmuscle cells as well as from cardiomyocytes (12, 14), but an increase in nRLC phosphorylation associated with cardiomyocyte-specific expression of skMLCK will result from phosphorylation in cardiomyocytes. The polyclonal antibody against the sarcomeric cRLC recognized nonphosphorylated and monophosphorylated cRLC (Fig. 2B). Thus, the two antibodies may be used for quantitative phosphorylation analyses of sarcomeric cRLC and cytoplasmic nRLC in heart. This phosphorylation analysis is sufficiently sensitive to measure cRLC phosphorylation quantitatively in different segments of mouse heart (Fig. 2C).

skMLCK Expression in Cardiomyocytes Increases cRLC Phosphorylation—RLC phosphorylation was examined first in ventricular tissues from WT mice. The extent of cRLC phosphorylation in ventricles from 9-week-old WT animals decreased slightly from 0.46 ± 0.01 mol of phosphate/mol of cRLC at the apex to 0.36 ± 0.02 mol of phosphate/mol of cRLC at the base (Fig. 3A). The extent of cRLC phosphorylation was not statistically different in 6-month-old mice in comparing apex (0.50 ± 0.02 mol of phosphate/mol of cRLC) to base values (0.43 ± 0.03 mol of phosphate/mol of cRLC) (Fig. 4A). We also measured cRLC phosphorylation in ventricular free-wall sections from WT mice (n = 3). The extents of cRLC phosphorylation for left ventricular epicardium, endocardium, and septum were 0.35 ± 0.01, 0.40 ± 0.04, and 0.32 ± 0.03, respectively. These values were not significantly different. These quantitative measurements do not support the previous conclusion regarding a gradient of cRLC phosphorylation in ventricular heart muscle (7).

We analyzed changes in cRLC phosphorylation in hearts from transgenic animals overexpressing skMLCK. The extent of phosphorylation of cRLC in all regions of ventricular tissues from 9-week-old transgenic AM line mice was significantly greater than observed in WT mice (Fig. 3A). However, cRLC phosphorylation did not change in the transgenic AN line mice expressing lower amounts of the TG-skMLCK. These results for AM and AN mice are similar to results obtained in skeletal muscle with other transgenic mice (11). Overexpressing skMLCK at low amounts did not enhance RLC phosphorylation. The skMLCK phosphorylates skeletal, cardiac, and smooth muscle RLCs with similar kinetic properties in contrast to the smooth muscle MLCK, which is more selective to the

FIGURE 2. Specificity of antibodies against sarcomeric cRLC and nRLC. A, purified RLC (cardiac, smooth; 10 ng each) or brain myosin (2 μg) was subjected to SDS-PAGE and blotted with antibodies raised against cardiac RLC (cRLC) or smooth muscle RLC (sRLC). B, ventricles from 9-week-old adult mice were homogenized in 1ysis buffer, and proteins were immediately precipitated with trichloroacetic acid (control; C) or incubated for dephosphorylation and then precipitated (D). Proteins were subjected to urea/glycerol-PAGE to separate nonphosphorylated RLC (Non-P) from its monophosphorylated form (Mono-P). C, representative blots for cRLC phosphorylation in different heart regions are shown.

FIGURE 3. Effect of skMLCK transgene expression on phosphorylation of cRLC and nRLC in heart regions from 9-week-old mice. Samples were obtained from the apex, middle, and base portions of left ventricular muscle. A, cRLC phosphorylation in hearts from wild-type and transgenic skMLCK AM and AN mouse lines. B, nRLC phosphorylation results for WT, TG (AM), and TG (AN). Values are means ± S.E. of 4 –10 samples. *, p < 0.05; **, p < 0.01 versus WT for matched apex, mid-ventricle, and base portions; ##, p < 0.01 versus apex within type.
smooth and cytoplasmic nonmuscle RLCs (15). Thus, we expected some overexpression of the kinase would lead to smaller but significant increases in RLC phosphorylation in heart and skeletal muscle.

At 6 months of age, cRLC phosphorylation remained elevated in transgenic AM mice compared with WT animals in all three regions of the heart. No significant differences were found among the three heart regions within WT or transgenic animals (Fig. 4A). Thus, enhanced cRLC phosphorylation produced by TG-skMLCK was maintained. Moreover, exercise training that produces cardiac hypertrophy (see below) did not affect the extent of cRLC phosphorylation in TG or WT mice (Fig. 4B).

skMLCK Expression Increases nRLC Phosphorylation—Similar to results obtained with sarcomeric cRLC, there was no consistent significant difference in the extent of nRLC phosphorylation in the apex compared with the base of ventricles from WT mice (Fig. 3B). Overexpression of TG-skMLCK in hearts of the AM, but not AN, mouse line resulted in an increase in nRLC phosphorylation in 9-week-old animals (Fig. 3B) that was still significantly increased at 6 months (Fig. 4C).

skMLCK Expression Does Not Cause Cardiac Hypertrophy—Hearts from transgenic mice expressing TG-skMLCK showed no hypertrophy in 9-week-old and 6-month-old animals (Table 1). In 9-week-old transgenic mice, the heart weight to tibial length ratio was significantly less than the ratio for WT animals, but there were no differences at 6 months. Histological examinations of hearts from TG-skMLCK (AM) mice also showed no differences from WT at either age. Hearts had no cardiomyocyte hypertrophy, necrosis, or interstitial fibrosis (supplemental Fig. S1). Transmission electron micrographs of both WT and transgenic hearts from either sedentary or exercise-conditioned mice showed normal sarcomeric structure and no filament disarray (supplemental Fig. S2).

Stress-induced Cardiac Hypertrophy Is Attenuated in Transgenic skMLCK Mice—Exercise conditioning induced significant cardiac hypertrophy. Heart weight to tibial length ratios increased for both WT and TG mice; however, there was less hypertrophy in exercised TG mice compared with WT (Fig. 5A). Exercised WT mice had an increase in cardiomyocyte size, whereas exercised TG mice exhibited smaller increases (Fig. 5B). Quantification of cardiomyocyte areas revealed that the cellular hypertrophic response to exercise was greatly attenuated in the hearts from TG mice as compared with WT (Fig. 5C). WT and TG mice ran similar distances per day (8.2 ± 0.7 versus 7.9 ± 0.6 km, respectively) as well as total distances (247.2 ± 22.6 versus 231.8 ± 18.1 km, respectively). Thus, the differences in the heart sizes were not due to differences in exercise intensities.

Isoproterenol induced cardiac hypertrophy in both WT and TG (AM) mice. The ratio of heart weight to tibial length in isoproterenol-treated WT and TG mice significantly increased compared with vehicle-treated mice (Fig. 6A). However, the ratio of heart weight to tibial length in TG mice increased less than that in WT mice (p < 0.05). Isoproterenol-treated WT mice had a recognizable increase in cardiomyocyte size, whereas isoproterenol-treated TG mice exhibited smaller cardiomyocytes (Fig. 6B). Quantification of cardiomyocyte areas revealed that the hypertrophic response to isoproterenol was significantly attenuated in the hearts from TG mice as compared with WT (Fig. 6C).

**Table 1.**

|                  | 9 weeks                  | 6 months                 |
|------------------|--------------------------|--------------------------|
| **Measurements** | **WT**                   | **skMLCK (AM)**          |
|                  | **WT**                   | **skMLCK (AM)**          |
| Body weight (g)  | 34.7 ± 0.8               | 35.8 ± 1.5               |
| Heart weight (mg)| 148.6 ± 3.6              | 134.7 ± 5.3              |
| Tibial length (mm)| 18.2 ± 0.2              | 18.4 ± 0.2              |
| Heart/body weight ratio | 4.28 ± 0.06 | 3.77 ± 0.07 |
| Heart/tibial length ratio | 8.12 ± 0.16 | 7.30 ± 0.24 |

*p < 0.05

**p < 0.01 versus WT mice at the same age.**

**REFERENCES**

1. Mol Phosphate / Mol cRLCMol Phosphate / Mol nRLC

**FIGURE 4.**

**Phosphorylation of cRLC and nRLC in heart regions from 6-month-old mice and exercised mice.** Transgenic skMLCK mice (AM) at 6 months of age display a significant increase in phosphorylation of cRLC and nRLC (C) in apex, middle, and base portions of left ventricular muscle compared with age-matched WT mice. Hearts from exercised, 14-week-old, transgenic skMLCK mice (AM) show increased phosphorylation of cRLC (B) and nRLC (D) in the apex compared with age-matched, exercised WT mice. Values are means ± S.E. of 5–8 samples. *, p < 0.05; **, p < 0.01 versus WT.
mice (Fig. 7). There were also no differences in the expression of total calmodulin in hearts from WT and TG mice as determined by Western blotting (data not shown). The calcineurin/NFAT pathway is a key signaling pathway that promotes cardiac hypertrophy (30). A decrease in calcineurin phosphatase activity would diminish expression of downstream targets,
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**DISCUSSION**

The kinase in heart that phosphorylates cRLC physiologically has been difficult to identify. There are two well known MLCKs representing products of two genes, the smooth and skeletal muscle MLCKs (15). One problem with cardiac muscle is the exceptionally low MLCK activity in heart compared with MLCK activities in skeletal and smooth muscles (39–41). skMLCK was recently cloned from cardiac muscle (7), but the skMLCK knock-out had no effect on cRLC phosphorylation (2). The skMLCK mRNA appears sufficiently rare so that significant amounts of skMLCK are undetectable by Western blotting of ventricular samples. These results are consistent with the proposal that skMLCK is not expressed in physiologically significant amounts in cardiomyocytes (2, 31). This conclusion is supported by two recent reports that identified another MLCK expressed only in heart (42, 43). The cardiac MLCK is structurally related to skeletal and smooth muscle MLCKs and appears to play a role in sarcomere formation. Evidence was presented that this kinase phosphorylates cRLC, but it is not known if the kinase phosphorylates cytoplasmic nRLC.

The extent of cRLC phosphorylation in beating hearts of various animal species typically varies from 0.35 to 0.55 mol of phosphate/mol of cRLC (44). However, these values represent tissue averages, because both left and right ventricles are usually quick-frozen before analysis. Davis et al. (7) proposed that there was a spatial gradient of cRLC phosphorylation decreasing from apex to base based on immunofluorescent staining of tissue sections with an antibody raised to a peptide containing the cRLC-phosphorylated serine. It is difficult to assess these results because they did not present control immunofluorescent images for a generic gene product that would demonstrate homogenous distribution by their procedures. They also did not describe the procedures for obtaining and processing the tissues that would retain RLC phosphorylation. We used a more quantitative approach to measure nonphosphorylated and monophosphorylated RLC forms in cardiac tissues by urea/glycerol-PAGE, where care was taken to obtain tissues in a timely manner to retain RLC phosphorylation (32, 33). A modest gradient of cRLC phosphorylation was shown from apex to base of the left ventricle in a few conditions. When there was an apparent change, the magnitude was consistently small, <0.10 mol of phosphate/mol of cRLC. Measurements of cRLC phosphorylation in left ventricular endocardium and epicardium demonstrated no significant differences. These small changes, when they occur, probably have no significant effects on contractile performance (8).

The lack of physiologically meaningful amounts of skMLCK in cardiomyocytes provides an opportunity to test hypotheses related to physiological and pathophysiological cardiomyocyte growth and development related to cRLC and nRLC phosphorylation. The skMLCK is a dedicated protein kinase with substrate determinants in the primary sequences around the phosphorylatable serine in addition to subdomains I and II in the light chain (45). No other physiological substrates are known, although RLCs from different myosins are equally good substrates (44). Overexpression of skMLCK in cardiomyocytes of transgenic mice leads to a robust increase in both cRLC and nRLC phosphorylation for at least 6 months. There were no changes in cardiac mass or structure in hearts from TG animals compared with WT animals. Thus, a marked increase in cRLC and nRLC phosphorylation in mouse ventricular myocytes is not sufficient to induce a hypertrophic response.

Physiological and pathophysiological stresses induce cardiac hypertrophy through a variety of cardiomyocyte signaling pathways, some dependent on Ca2+/calmodulin via the phosphatase calcineurin and Ca2+/calmodulin-dependent protein kinases (35, 46). Therefore, it was of interest to determine whether overexpression of skMLCK in cardiomyocytes resulting in increased phosphorylation of both cRLC and nRLC...
would enhance hypertrophic responses to stressful stimuli. Exercise conditioning by voluntary wheel running caused increases in heart mass and the myocyte cross-sectional area that were significantly attenuated in transgenic mice. There was a similar pattern of responses with isoproterenol infusion. The reason that the skMLCK transgene and the resultant increase in RLC phosphorylation inhibit hypertrophic responses could arise from two mechanisms not mutually exclusive.

First, TG-skMLCK binds Ca\(^{2+}\)/calmodulin and thus may diminish the availability of a limiting amount of free Ca\(^{2+}\)/calmodulin (20, 47, 48) and thereby inhibit activation of Ca\(^{2+}\)/calmodulin-dependent enzymes such as CaM kinase II and calcineurin. The amount of TG-skMLCK expressed in hearts is calmodulin-dependent enzymes such as CaM kinase II and calcineurin. The amount of TG-skMLCK expressed in hearts is greater than the amount expressed in skeletal muscle, leading to a calculated cellular kinase concentration of 1.2 \(\mu\text{M}\). Considering the biosensor kinase contains an additional calmodulin binding site, the effective Ca\(^{2+}\)/calmodulin target is about 2.4 \(\mu\text{M}\). This concentration is significantly less than the 5–6 \(\mu\text{M}\) total calmodulin concentration recently measured in adult cardiac myocytes (48) but much greater than the 0.5–75 \text{nM} free Ca\(^{2+}\) concentration (21). Thus, cardiac myocytes appear to have a large reservoir of buffered calmodulin to maintain temporal and spatial dynamic competition among Ca\(^{2+}\)/calmodulin targets (21, 49). Additionally, a significant portion of the total calmodulin may be apocalmodulin close to target sites such as the ryanodine channel (50). A prebound apocalmodulin close to its target may not readily enter into the cytoplasmic pool containing free Ca\(^{2+}\)/calmodulin. It appears that both low affinity (CaM kinase II) and high affinity (calcineurin) targets have sufficient access to the buffered calmodulin to maintain their respective functional capacity in myocytes overexpressing the Ca\(^{2+}\)/calmodulin-binding TG-skMLCK. In some cells such as smooth muscle, the buffering capacity may be insufficiently low to limit activation of target enzymes in the calmodulin network despite a high (40 \(\mu\text{M}\)) total calmodulin content (20). In skeletal and cardiac muscles, the functional buffering capacity appears to be sufficiently high even at a total calmodulin content less than smooth muscle, so Ca\(^{2+}\)/calmodulin is not limiting (Ref. 11 and references herein).

An alternative explanation may account for the attenuation of the physiological and pathophysiological hypertrophy responses by enhanced RLC phosphorylation. Both types of hypertrophic stimuli initiate unique signaling pathways imposed on the heart with a normal extent of RLC phosphorylation (35, 37). Activation of \(\beta\)-adrenergic receptors by isoproterenol appears to influence Ca\(^{2+}\) release in cardiac myocytes to increase the activity of Ca\(^{2+}\)-dependent prohypertrophic calcineurin signaling (34). Because RLC phosphorylation improves contractile performance by acting on myosin crossbridge cycling kinetics (1–7, 10), a specific amount of force development may be obtained at a lower Ca\(^{2+}\) concentration released for contraction. Thus, less Ca\(^{2+}\) may be released with increased RLC phosphorylation, thereby contributing to enhanced contractile performance and efficiency (1, 10). The stresses imposed by exercise conditioning and chronic \(\beta\)-adrenergic stimulation may thus be blunted, leading to a diminished compensatory growth response. The attenuation of the hypertrophic responses may be related to the beneficial effects of RLC phosphorylation on cardiac hemodynamic properties.

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