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ROLE OF MYOSIN LIGHT CHAIN PHOSPHATASE IN CARDIAC PHYSIOLOGY AND PATHOPHYSIOLOGY

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Abstract

Maintenance of contractile performance of the heart is achieved in part by the constitutive 40% phosphorylation of myosin regulatory light chain (RLC) in sarcomeres. The importance of this extent of RLC phosphorylation for optimal cardiac performance becomes apparent when various mouse models and resultant phenotypes are compared. The absence or attenuation of RLC phosphorylation results in poor performance leading to heart failure, whereas increased RLC phosphorylation is associated with cardiac protection from stresses. Although information is limited, RLC phosphorylation appears compromised in human heart failure which is consistent with data from mouse studies. The extent of cardiac RLC phosphorylation is determined by the balanced activities of cardiac myosin light chain kinases and phosphatases, the regulatory mechanisms of which are now emerging. This review thusly focuses on kinases that may participate in phosphorylating RLC to make the substrate for cardiac myosin light chain phosphatases, in addition to providing perspectives on the family of myosin light chain phosphatases and involved signaling mechanisms. Because biochemical and physiological information about cardiac myosin light chain phosphatase is sparse, such studies represent an emerging area of investigation in health and disease.

Keywords

Sarcomere; myosin; regulatory light chain; myosin light chain kinase; myosin light chain phosphatase; cardiac contraction

1. Cardiac contractile protein system overview

The heart is an involuntary, striated muscle made up of cardiomyocytes that coordinate the continuous cycles of contraction and relaxation to pump blood out of atria and ventricles to the blood vessels of the pulmonary and systemic circulatory systems. Cardiomyocytes have

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a specialized membrane system to deliver and remove Ca^{2+} from the highly organized contractile protein system that initiates contraction and relaxation as part of the rhythmic pumping action of the heart [1].

1.1 Sarcomeres, myosin cross-bridge cycling, and Ca^{2+} regulation

Cardiomyocytes contain bundles of myofibrils with sarcomeres that represent the basic contractile units of the myocyte [2]. The sarcomere is composed primarily of thick filaments containing the molecular motor myosin, and thin filaments containing polymerized actin with its associated regulatory proteins tropomyosin and troponin, respectively (Fig. 1). Ca^{2+} binds to a specific troponin subunit resulting in a series of protein conformational changes in troponin and tropomyosin to remove them from an inhibitory state. This allows myosin cross bridges to bind cyclically to actin with the hydrolysis of ATP to generate contractile force [2-4].

1.2 Cardiac myosin

1.2.1 Function relative to structure—Dimerized heavy chains of myosin contain multiple subdomains including (1) a coiled coil of the two heavy chain subunits embedded in the thick filament, (2) an extended helix lever arm containing sites for the binding of two kinds of light chain subunits (essential and regulatory light chains (RLC), respectively), and (3) the cross-bridge head with its actin-activated ATPase motor domain, (Fig. 1) [5]. The coordinated movement of myosin cross bridges from rest (diastole) to contraction (systole) involves complex interactions with the backbone of the thick filament and myosin-binding protein C as well as with thin filaments [2, 6-9]. Cited recent reviews deal with these important topics in detail.

Fine-tuning of contractile performance is partly achieved by post-translational modifications of sarcomeric proteins occurring through signaling modules to regulate the pumping action of the heart to meet circulatory demands of the body [10-14]. RLCs in different kinds of muscles are phosphorylated by myosin light chain kinases (MLCKs) to activate or modulate the myosin, and dephosphorylated by myosin light chain phosphatases (MLCPs) [13, 15-20]. A serine in the N-terminus of different RLCs is phosphorylated by tissue-specific, dedicated MLCKs (Fig. 1) [13, 21]. In normal beating hearts, Serine 15 is 40% phosphorylated (0.40 mol phosphate/mol RLC) which is greater than the 10% or lower phosphorylation normally observed in resting skeletal and smooth muscles (Fig. 1) [13, 15-17, 21-23].

1.2.2 Biophysical effects of regulatory light chain phosphorylation on myosin cross bridges—In the absence of RLC phosphorylation in striated muscles a portion of the myosin heads are folded back on the myosin tail in a compact *off* conformation in which the long axes of the myosin heads are parallel to the thick filament axis (Fig. 1) [24-27]. However, some of the heads are not bound to the thick filament and are extended to the thin filament in an *on* state in striated muscles. Thus, there is an equilibrium between these two states. RLC phosphorylation in cardiac muscle controls myosin cross-bridge cycling properties on actin (increased myosin cross-bridge repulsion from the thick filament towards actin and the transition of cross-bridge attachment to the strongly bound, force generating

state) [28]. RLC phosphorylation also enhances the Ca^{2+}-dependent activation of thin filaments [26, 28]. RLC phosphorylation thus potentiates contractility in cardiac muscle by multiple effects on myosin cross bridges, including cooperative effects on adjacent unphosphorylated myosin cross bridges (Fig. 1). This leads to enhanced physiological reserve with contractile forces maintained at a lower Ca^{2+} concentration, and less energy spent pumping Ca^{2+} out of the myoplasm during the contraction cycle. Thus, RLC phosphorylation amplifies the effectiveness of Ca^{2+} release.

Although it is widely understood that the Ca^{2+}-dependence of cardiac contraction is regulated by the troponin complex, and that the phosphorylation of cardiac RLC plays a modulatory role in cardiac function, recent discoveries have advanced the physiological importance of this post-translational modification. Unlike the modulatory phosphorylations of other myofilament proteins, such as troponin I and myosin binding protein C, which are sensitive to acute changes in heart rate and inotropic agents, various investigators have reported baseline RLC phosphorylation in ventricular muscle does not change acutely [21, 29-32]. Lack of dynamic fluctuations in cardiac RLC phosphorylation *in vivo* is supported by the slow turnover rate of the phosphate bound to RLC in the intact rat heart [33]. When procedures are used that preserve phosphorylation, cardiac RLC shows an average 40% phosphorylation in a variety of animals [21, 28, 31, 33-42]. Nearly half-maximal phosphorylation of cardiac RLC from fish to humans suggests this functional reserve for cardiac performance is evolutionarily conserved. The present review will focus on ventricular cardiac RLC although it is appreciated that the distinct atrial RLC is also phosphorylated [30, 43].

1.3 Cardiac regulatory light chain phosphorylation and disease

The importance of constitutive RLC phosphorylation for normal cardiac performance is underscored by the reduction of RLC phosphorylation in human heart failure [44-47], and in animal models of myocardial infarction and pressure overload-induced heart failure [42, 48-54]. Pressure overload induced by transaortic constriction in wild-type mice reduced the extent of RLC phosphorylation by 40% and cardiac MLCK expression by 85% [54]. The importance of this extent of RLC phosphorylation on cardiac performance becomes apparent when various mouse models and resultant phenotypes are compared (Fig. 2).

Mice that express a non-phosphorylatable cardiac RLC showed RLC phosphorylation was required for optimal cardiac performance and a normal lifespan [28, 57]. Unlike human cardiac RLC, mouse cardiac RLC has two serines in tandem, Serines 15/16. Thus, when Serine 15 was mutated to Alanine, Serine 16 was phosphorylated *in vivo*, and a double mutation was necessary to remove the phosphorylation of cardiac RLC *in vivo* [28]. The double phosphorylation site knockin mutant has a shorter lifespan and a more severe phenotype than knockout of cMLCK (Fig 2). Despite the difference in severity of cardiac phenotype, both mouse models have attenuated hypertrophic response to transaortic constriction, attributed to the lack of RLC phosphorylation [28, 54].

There is a discrepancy in the amount of RLC phosphorylation that remains in two models of cMLCK knockout mice described by Warren *et al* [54] and Chang *et al* [21]. Both cMLCK null lines were generated by crossing cMLCK floxed mice with Cre recombinase transgenic

mice with a beta-actin promoter. The cMLCK knockout line described by Chang *et al* [21, 35] was produced by crossing cMLCK floxed mice with Cre transgenic with cytomegalovirus immediate early enhancer-chicken beta-actin hybrid (CAG) promoter, which deletes floxed genes at two-cell blastomere stage [58]. In both lines, cMLCK protein was not expressed, and mice had heart failure with a normal lifespan. However, Warren *et al* reported no RLC phosphorylation was detected by 2D-PAGE, whereas Chang *et al* [21, 35] reported that 10% of RLC phosphorylation remained in cMLCK gene-ablated mouse hearts, similar to the hypomorphic cMLCK-null line described by Ding [36]. The site phosphorylated in the 10% was confirmed by mass spectrometry to be the physiologically relevant Serine 15 [21]. Ten percent RLC phosphorylation in the cMLCK knockout mouse hearts is consistent with RLC phosphorylation detected in non-beating mouse hearts [21, 59]. It is unknown why there is a discrepancy in RLC phosphorylation results. It may be due to differences in sample preparation and RLC phosphorylation measurement techniques, which are discussed in the next section. Despite this difference, there was no compensatory phosphorylation of Serine 16 when Serine 15 phosphorylation was reduced [references]. Mice with lower than the basal 40% RLC phosphorylation all have compromised cardiac performance (Fig. 2).

RLC phosphorylation greater than the basal 40% was associated with improved cardiac performance and attenuation of hypertrophic responses to stress [38, 54]. In support of RLC phosphorylation as a direct determinant of cardiac performance, acute heart failure induced by the conditional knockdown of cardiac MLCK protein *in vivo* showed cardiac performance was reduced when RLC phosphorylation was reduced. Decreased cardiac performance in these mice was not secondary to fibrosis and myocyte disarray, which are characteristic of cardiomyopathies [21, 55].

2. Myosin regulatory light chain phosphorylation: Understanding conditions for making myosin light chain phosphatase substrate

The MLCK family is part of a large group of Ca^{2+}/calmodulin (CaM)-dependent protein kinases [60], and is comprised of four distinct kinases, MLCK1, 2, 3, and 4, which are each encoded by distinct genes (*MYLK1*, *MYLK2*, *MYLK3*, and *MYLK4*) [13, 17, 23]. Based on their muscle-type specific expression and activities, MLCK1 is known as smooth muscle MLCK (smMLCK), MLCK2 as skeletal muscle MLCK (skMLCK), and MLCK3 as cardiac muscle MLCK (cMLCK). MLCK4 has only recently been characterized, and is potentially another cardiac muscle MLCK based on its selective protein expression in cardiac myocytes [23].

2.1 Canonical regulatory light chain phosphorylation scheme from skeletal and smooth muscle studies

MLCK1 and 2 are Ca^{2+}/CaM-dependent kinases with autoregulatory segments C-terminal of the catalytic core, comprised of autoinhibitory and CaM binding sequences [61, 62]. In the absence of Ca^{2+}/CaM, the autoinhibitory sequence N-terminal to the CaM binding sequence binds to the surface of the kinase C-domain towards the catalytic cleft, blocking RLC binding and phosphorylation. Ca^{2+}/CaM binding to the CaM-binding sequence displaces the

autoregulatory segment, exposing the catalytic cleft for RLC-binding [63, 64]. Thus, the catalytic activity of MLCK1 and 2 are completely dependent on Ca^{2+}/CaM [13].

Phosphorylated RLC is dephosphorylated by myosin light chain phosphatase (MLCP), previously identified as a holoenzyme composed of three distinct subunits: a 38 kDa catalytic subunit (PP1cδ), a larger 110–130 kDa regulatory subunit (MYPT), and a small 20 kDa subunit of unknown function (see below) [65, 66]. RLC phosphorylation in smooth and skeletal muscles can thus be represented by a simple scheme showing an increase in cytosolic Ca^{2+} concentration leads to RLC phosphorylation (Fig. 3). This scheme does not present additional complex regulatory mechanisms involving MLCP in smooth muscle [16, 18, 65].

The extent of RLC phosphorylation by Ca^{2+}/CaM-dependent smMLCK determines force development in smooth muscle tissues [13, 67, 68]. Smooth muscle RLC is rapidly phosphorylated with smMLCK activation and rapidly dephosphorylated upon its inactivation. The rate of RLC dephosphorylation by MLCP is substantially greater in smooth vs. skeletal and cardiac muscles, and necessary for smooth muscle relaxation [13].

As noted above for cardiac muscle, Ca^{2+} binding to troponin in the thin filament in skeletal muscle initiates contraction. However, the properties for skeletal muscle RLC phosphorylation show (1) skMLCK is rapidly activated by Ca^{2+}/calmodulin and the interpulse interval between contractions determines the fraction of kinase activated, (2) the activity of activated skMLCK is limiting so that RLC is phosphorylated in seconds, not milliseconds, with contraction, (3) skMLCK activity is much greater than MLCP activity so a small fractional activation of kinase results in RLC phosphorylation that may be sustained, and (4) a slow rate of skMLCK inactivation combined with the slower MLCP activity provides a biochemical memory to enhance RLC phosphorylation after skeletal muscle fibers have relaxed [15, 69, 70].

2.2 Unique biochemical properties of cardiac myosin light chain kinase 3

Although it also has a catalytic core and autoregulatory segment highly homologous with skeletal and smooth muscle MLCKs, the biochemical properties of cardiac muscle-specific MLCK3 were not well defined initially in terms of Ca^{2+}/CaM activation [51, 71]. Recent studies showed the V_{max} value of cMLCK was orders of magnitude lower than those of the other three MLCK family members, whereas it's K_m (RLC and ATP) and K_{CaM} values were similar [23]. The K_{CaM} value shows high affinity (1 nM) similar to MLCK from skeletal and smooth muscles, predicting a diffusion-limited association of Ca^{2+}/CaM with cMLCK, and a slow rate of CaM dissociation and inactivation (3 sec^{-1}) of cMLCK upon reduction of Ca^{2+} concentrations [15, 68, 70]. In contrast to smMLCK and skMLCK that lack activity in the absence of Ca^{2+}/CaM, cMLCK has constitutive activity that is stimulated two-fold by Ca^{2+}/CaM. The constitutive, low activity of cMLCK appears to be intrinsic to its catalytic core structure rather than an autoinhibitory segment [23].

2.3 Myosin light chain kinase 4 in cardiac muscle: the unknown

MLCK4 was shown by RNA-Seq to be decreased by more than two-fold in heart samples from ischemic cardiomyopathy patients [72]. MLCK4 protein expression is greatest in

cardiac myocytes and is a kinase for cardiac RLC *in vitro* [23]. Although the regulatory mechanisms for both cMLCK and MLCK4 activities *in vivo* need further investigation, the measured enzymatic properties of cMLCK and MLCK4 are consistent with constitutive RLC phosphorylation *in vivo* in balance with constitutive phosphatase activities [23, 33].

MLCK4 gene ablation in mice has not yet been performed to test the hypothesis that it acts on RLC phosphorylation *in vivo*, but the high-resolution structure and biochemical assays show it lacks an autoinhibitory segment and is constitutively active [23]. MLCK4 and cMLCK double knockout is needed to confirm whether MLCK4 is the kinase responsible for the residual RLC phosphorylation at Serine 15 in cMLCK knockout mice [21]. Underscoring the importance of genetic models to confirm *in vitro* observations, conditional knockout of ZIPK in mice, another constitutively active kinase that phosphorylates cardiac RLC *in vitro* and in neonatal cardiac myocytes [73], did not reduce RLC phosphorylation *in vivo* (unpublished data), suggesting it is not a kinase for cardiac RLC in adult mouse hearts. Additionally, a double knockout of cMLCK and ZIPK did not further reduce RLC phosphorylation below 10% (unpublished data).

While constitutive phosphorylation of cardiac myosin RLC by the low constitutive activity of cMLCK has been investigated, whether dynamic transient phosphorylations by other kinases with higher activities, such as MLCK4 or ZIPK, contribute to myosin activity or other cellular functions is unknown.

2.4 Accurate measurements of regulatory light chain phosphorylation in ventricular cardiac tissue

Studies on regulation of cardiac MLCP activity *in vivo* require accurate quantitative measurements of the substrate, phosphorylated RLC. There are important considerations for measuring phosphorylation of proteins, including adequate tissue fixation, extraction of phosphoproteins, and measurements of the extent of phosphorylation of specific phosphorylation sites. The objectives of preparing tissue for analysis include: freezing the tissue rapidly enough to prevent changes associated with excision or other disturbances; preventing alterations during storage and during manipulation before extraction; and avoiding alteration of phosphoproteins by enzymes in the extraction medium [74]. We briefly discuss these issues in addition to different methods to quantify the extent of cardiac RLC phosphorylation. Common problems in assessing RLC phosphorylation are dephosphorylation during sample preparation and the lack of sufficient sensitivity in the measurements.

2.4.1 Tissue sample preparation—Freezing tissue rapidly enough to prevent artificial changes, due to the uncontrolled activities of protein kinases and phosphatases, needs careful consideration. In smooth and skeletal muscles RLC phosphorylation occurs in a second or less due to the high MLCK activity, thus rapid freezing techniques are essential [68, 70]. Although cardiac RLC phosphorylation is substantially slower, requiring several minutes for significant changes [31, 59], rapid freezing of cardiac tissue with pre-chilled clamps is recommended to facilitate analysis of other cardiac modulatory proteins which may have a higher phosphate turnover rates.

Tissue sample storage, handling and processing as well as buffer formulations are significant variables in the measurement of RLC phosphorylation. Although RLC is constitutively phosphorylated *in vivo*, it is rapidly dephosphorylated in heart homogenates [21]. Thus, care must be used to prevent RLC dephosphorylation when extracting RLC from quick-frozen heart muscle. A standard method of minimizing protein dephosphorylation [21, 29, 30, 34, 41, 46, 75-77], involves thawing frozen powdered heart in 10% trichloroacetic acid to denature and precipitate all proteins in the tissue sample, including kinases, phosphatases, and proteases [78].

In tissues homogenized directly in SDS sample buffer without phosphatase inhibitors and sufficient tissue dilution, RLC is dephosphorylated, resulting in low baseline tissue RLC phosphorylation values [79]. Consistent with high RLC phosphatase activity in tissue homogenates, cardiac RLC is also dephosphorylated when homogenized and denatured directly in 8 M urea without phosphatase inhibitors [31]. Thus, utilization of trichloroacetic acid to fix proteins in a phosphorylated state is a powerful tool for quantification of protein phosphorylation.

For procedures where fixation with trichloroacetic acid must be avoided, such as in subcellular fractionation studies, heart samples may be homogenized in aqueous buffers without protein denaturation [80, 81]. However, it is important that the homogenization buffer as well as the wash buffers contain protein phosphatase and kinase inhibitors, particularly chemical inhibitors of protein phosphatase 1 which is the catalytic subunit of MLCP. Calyculin A, microcystin, and okadaic acid are sufficient inhibitors [21, 82], but the effective concentration must be empirically determined. Calyculin A inhibits RLC dephosphorylation at 1 μM, but some commercially available phosphatase inhibitor cocktails contain less than 1 μM.

2.4.2 Quantitative measurements—Denatured proteins may be solubilized in urea sample buffer, and nonphosphorylated and phosphorylated RLC separated in a native gel containing glycerol [83], where monophosphorylated RLC migrates faster than nonphosphorylated RLC due to the additional two negative charges from the phosphate group at pH 8.6. Since the description of this procedure in 1970, modifications include pre-electrophoresis to minimize pseudophosphorylation artifacts [84], and use of antibodies specific toward different isoforms of RLC to avoid nonspecific staining contributions from other proteins that co-migrate with RLC and to increase the sensitivity of the measurements [31, 38] . An alternative method includes Phos-tag SDS-PAGE where denatured proteins are solubilized in SDS sample buffer [85, 86]. The migration of monophosphorylated RLC is slower than nonphosphorylated RLC. Two-dimensional electrophoresis with isoelectric focusing can also be used to separate nonphosphorylated and monophosphorylated RLC, but it is a more laborious procedure [81]. Additionally, more sophisticated and detailed analyses of heart extracts utilizing top-down approaches of mass spectrometry have been described which provide measurements of various post-translational modifications of RLC and other sarcomeric proteins [22, 87].

The separation of nonphosphorylated from phosphorylated RLC by urea-glycerol PAGE, Phos-tag SDS-PAGE or 2D-PAGE provides valuable information on the extent of RLC

phosphorylation relevant to physiological and pathophysiological studies. In all of these procedures it is essential to establish that the amount of protein loaded is within a range for quantitative measurements, particularly if the amount of RLC phosphorylation is 10% or less.

There are also other useful, but less quantitative procedures, demonstrating relative changes in RLC phosphorylation including Pro-Q Diamond staining [80] and phospho-antibody to cardiac RLC phosphorylated Serine 15 [81]. Although relative comparisons in RLC phosphorylation are easier to perform, it is recommended that they be supported with quantitative measurements, as a relative change of 100% increase in RLC phosphorylation could potentially be an increase from 1% to 2%, or 10% to 20% etc., leading to over interpretation of the significance of a change in phosphorylation.

3. Protein phosphatases

3.1 Protein phosphatase classification with focus on protein phosphatase 1 catalytic subunit and its isoforms

A simplified nomenclature system originally separated serine/threonine protein phosphatases (PP) into two types, PP1 and PP2, based on observed specificity toward PKA-phosphorylated α- and β-subunits of phosphorylase kinase [88]. PP1 is inhibited by Inhibitor-1 and Inhibitor-2, whereas PP2 is insensitive to Inhibitor-1 and Inhibitor-2. Subtypes PP2A, PP2B, PP2C are differentially dependent on distinct divalent cations. Subsequent to classification of the serine/threonine phosphatases to two types, additional protein phosphatases were identified (types 3-6, PPM1, DxDxT, and PGAM5), raising the possibility of more unidentified distinct phosphatases [89, 90].

Countering the hundreds of serine/threonine kinases in cells, combinatorial binding of nearly 200 PP1 interacting proteins (PIPs) to distinct docking sites on PP1 directs the specificity of this ubiquitously expressed catalytic subunit, which has only five distinct isoforms in mammalian cells (α_1, α_2, β/δ, γ_1, γ_2) [91, 92]. The majority of PIPs dock with PP1 via an RVxF motif and recruit the catalytic subunit to distinct substrates. The PP1 subtype that has been shown to dephosphorylate RLC was originally identified as PP1cδ by peptide mapping of proteins exhaustively purified from animal muscle tissues and subsequently defined by antibody cross-reactivity [82, 93]. The PIPs that recruit PP1cδ to myosins are members of the myosin phosphatase target subunit (MYPT) family [90].

3.2 Protein phosphatase 1 regulator: myosin phosphatase target subunit (MYPT) family

The myosin phosphatase target subunit family is comprised of five members: MYPT1 *(PPP1R12A)* [94], MYPT2 *(PPP1R12B)* [95], MYPT3 *(PPP1R16A)* [96], MBS85 *(PPP1R12C)* [97], and TIMAP *(PPP1R16B)* [98]. Although catalytic subunit binding specificity has not been confirmed for all members, similar motifs for PP1c binding (Myosin Phosphatase N-terminal Extension (MyPhoNE): RxxQ[VIL][KR]x[YW], RVxF and ankyrin repeats) are found in the N-terminus (Fig. 4) [18, 99].

3.2.1 MYPT1—MYPT1 from smooth and nonmuscle cells was biochemically characterized as a scaffolding protein that targets PP1cδ to myosin and potentiates the phosphatase activity

toward phosphorylated RLC [65, 82]. Binding of PP1cδ, but not PP1cα or PP1cγ isoforms to MYPT1 was shown in co-expression studies [101] and detailed in a high-resolution structure [100]. The interaction was confirmed *in vivo*, which showed concomitant decrease in PP1cδ with MYPT1 protein knockout [102-104]. However, additional studies are needed to ascertain specific MYPT1-binding sequences in PP1cδ, as biochemical and structural studies are not in agreement [100, 101].

The specificity and regulatory activity of smooth muscle MLCP comprised of MYPT1 and PP1cδ is based on numerous biochemical studies using purified recombinant proteins and overexpression systems. An important property of smooth muscle MLCP is that although the catalytic subunit is PP1, the MLCP holoenzyme is insensitive to both Inhibitor-1 and Inhibitor-2 [105]. The activity of MYPT1-targeted PP1cδ toward phosphorylated RLC is regulated by a phosphorylation-dependent intramolecular autoinhibition mechanism [106, 107]. While there are numerous phosphorylation sites on MYPT1, Thr696 (human sequence) has emerged from studies *in vitro* and *in vivo* as a primary inhibitory site for MLCP activity [104, 106, 108]. This site is constitutively phosphorylated in many kinds of smooth muscles but the responsible protein kinase is not yet identified.

There are reports of MYPT1 localization to areas where myosin is not present, such as the nucleus [109, 110], or membranes [111], but interpretations are limited due to overexpression in non-muscle cells or a lack of quantitative measurements. Dynamic redistribution of MYPT1 in response to PGF2α was reported, but control images with a soluble non-phosphatase protein, z-stack images, or live cell microscopy are needed to confirm conclusions drawn from confocal microscopy of fixed detergent-treated cells [111]. Quantitative survey of MYPT1 localization in various resting and contracting smooth muscle tissues is needed to determine whether MYPT1 targets PP1cδ to substrates other than RLC in myosin.

Lethality of a conventional MYPT1 knockout confirmed its importance in embryogenesis [112], but when conditionally knocked out in smooth muscle cells in adult mice, myosin dephosphorylation and smooth muscle relaxation were not dramatically affected [102-104, 108, 113], indicating it is not necessary for smooth muscle contractile function. It was recently suggested that another MLCP in smooth muscle tissues is not dependent on MYPT1 [102].

3.2.2 MYPT2—Similar to MYPT1 in the bladder smooth muscle cells [95], striated muscle specific MYPT2 in the heart is not detectable in soluble fractions, presumably due to binding to cardiac myosin, which is insoluble in low ionic strength homogenates [21]. Additionally, comparison of MYPT2 to MYPT1 showed its function was biochemically similar to MYPT1 [114]: 1) both regulatory subunits co-immunoprecipitated overexpressed PP1cδ but not PP1cα or PP1cγ, 2) both MYPTs biochemically activated the phosphatase activity of PP1cδ toward phosphorylated RLC, and 3) phosphorylation of both MYPTs biochemically by purified Rho-kinase inhibited the phosphatase activity of PP1cδ.

MYPT1 Thr696 corresponds to MYPT2 Thr646 in human sequence alignments. Contrary to biochemical studies where MLCP activity was inhibited by incubation with Rho-kinase

[114], MYPT1 Thr696 and MYPT2 Thr646 were constitutively phosphorylated *in vivo* [21, 102]; based on MYPT1 Thr696Ala mutant knock-in mouse studies and the fact that MYPT1 T696 phosphorylation is not inhibited by Rho-kinase inhibitor [108], the protein kinase that phosphorylates MYTP1 T696 constitutively in smooth muscle is not yet identified. Similarly, the protein kinase that constitutively phosphorylates MYPT2 T646 in cardiac muscle is not identified. Overexpression of MYPT2 in the heart caused accumulation of the catalytic subunit PP1cδ bound to excess MYPT2, and a decrease in RLC phosphorylation [56].

3.2.3 MBS85—Although MBS85 was discovered from a substrate screen with the myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK) in rat brain homogenates, comparison of MBS85 mRNA by Northern blot showed highest amount in the heart [97]. Similar to MYPT1 T696 and MYPT2 T646 phosphorylation, MBS85 has an inhibitory phosphorylation site Thr560, which was biochemically shown to increase binding to PP1cδ [97]. Muscle-type distribution of MBS85 protein, and potential contributions to dephosphorylation of RLC remains a point of investigation. MBS85 has received little attention after its initial discovery, but was recently identified from a screen for AMPK substrates and is thought to be involved in mitosis [115]. Additionally, MBS85 expression increased in ileal smooth muscle from MYPT1-deficient mice where it may have contributed to dephosphorylation of RLC [104]. It is unknown what role MBS85 may have in the developing and adult heart, and it remains to be seen whether it contributes to regulation of RLC dephosphorylation.

3.2.4 MYPT3 and TIMAP—Like other MYPT family members, MYPT3 and TIMAP have consensus PP1c-binding motifs, but do not have the conserved inhibitory phosphorylation site as MYPT1, 2, and MBS85. A unique property of MYPT3 and TIMAP is the presence of a prenylation motif CAAX, which targets the proteins to cell membranes. MYPT3 mRNA and protein is expressed in the heart [96], but TIMAP mRNA is very low in striated muscles and protein expression in other organs appears to be localized to endothelial cells [98]. Additionally, there is no published record of TIMAP-PP1 contributing to the regulation of MLCP activity.

Purified recombinant MYPT3 inhibits smooth muscle myosin phosphatase activity of PP1cδ and PP1cγ1 *in vitro,* but appears to be localized to membranes in cells rather than contractile proteins [96, 116]. Comparison of endogenous interactions with specific isoforms of PP1 and quantification of contributions to total phosphatase activity toward bonafide substrates at the membrane is needed to determine if MYPT3 and TIMAP potentially regulate cardiac function.

3.3 Myosin-bound and soluble cardiac MLCP

Conditional knockout of the catalytic subunit PP1cδ in cardiomyocytes increased the extent of RLC and myosin binding protein C phosphorylation compared to control myofilaments [80]. There are potentially numerous substrates for PP1cδ within the cardiac myocyte that could account for the resulting severe cardiac phenotype in these knockout animals. Given that cardiac RLC phosphorylation is insensitive to acute changes in heart rate, unlike myosin

binding protein C [80], PP1cδ may be controlled by distinct regulatory subunits. Failure to abolish RLC dephosphorylation by knockout of PP1cδ, suggests another catalytic subunit could be targeted to myosin by MYPT2. This is in conflict with the specificity of MYPT2-PP1cδ binding established by the high-resolution structure of MYPT1-bound PP1cδ [100], increased PP1cδ in MYPT2 transgenic animals [56], and co-immunoprecipitation studies that show selective binding of overexpressed MYPT2 and PP1cδ [114]. The putative regulatory subunit bound to the soluble PP1cδ fraction remains unidentified, and whether phosphatase activity targeted by MYPT2 is sufficient and necessary for RLC dephosphorylation *in vivo* is unknown. Additionally other isoforms of PP1c or another protein phosphatase may participate in dephosphorylating RLC.

As smooth muscle contractions are regulated by the phosphorylation status of RLC, MYPT1 was widely accepted as a regulatory protein that is necessary for RLC dephosphorylation and thusly, smooth muscle relaxation. However, recent characterization of a conditional MYPT1 knockout in smooth muscles of adult mice showed it was not necessary for smooth muscle relaxation, suggesting the presence of another MLCP [102-104, 108, 113]. A large proportion of soluble PP1cδ not targeted to myosin by MYPT1 dephosphorylates biochemically the physiological substrate, RLC in myosin [102]. As PP1cδ alone has limited activity towards myosin RLC, soluble PP1cδ may be bound to a soluble regulatory subunit.

4. Summary and future directions: physiology and disease

In early studies on RLC phosphorylation in different tissues, many investigators focused biochemical, biophysical, and physiological investigations on smooth and skeletal muscles where RLC phosphorylation appeared to play a dynamic role in initiating or modulating contractions, respectively [15, 16, 27, 67, 117]. Early biophysical studies on skinned cardiac fibers illustrated the functional importance of RLC phosphorylation for enhancing contraction similar to skeletal muscle fibers [118, 119], but RLC phosphorylation appeared not to change dynamically with different pharmacological or physiological interventions due to the slow rate of phosphate turnover in cardiac RLC [21, 31, 33, 59]. As genetic approaches were introduced for modifying cardiac sarcomeric proteins, the physiological importance of RLC phosphorylation was further revealed [57]. These studies occurred about the same time it was appreciated that mutations in sarcomeric proteins, including RLC, resulted in familial hypertrophic cardiomyopathy [120, 121]. Genetic approaches clarified muscle-specific MLCKs with cMLCK identified initially in human heart failure [51, 71] and its role in maintaining constitutive RLC phosphorylation for normal cardiac performance [36].

Herein, we have described recent contributions from many investigators that have refined the function of RLC phosphorylation on myosin cross bridges, but we primarily focus on how RLC is phosphorylated and dephosphorylated. Fig. 5 summarizes the key ideas of this signaling module where it is proposed in the beating heart that cMLCK is saturated with Ca^{2+}/CaM due to fast association and slow dissociation rates. The bound Ca^{2+}/CaM enhances cMLCK activity to maintain 40% RLC phosphorylation necessary for normal cardiac performance. This phosphorylation reaction is in equilibrium with dephosphorylation by MLCP represented by both myosin bound and soluble forms. Because

MYPT2 T646 is maximally phosphorylated in beating hearts, this form of MLCP is predicted to be inhibited, presumably by an intramolecular autoinhibition mechanism [107]. Thus, the slow dephosphorylation of cardiac RLC in beating hearts may occur by other forms of PP1cδ or other protein phosphatases. In the non-beating heart the lack of cyclic increases in cytosolic Ca^{2+} prevents the formation of an effective Ca^{2+}/CaM complex so cMLCK is reduced to its Ca^{2+}/CaM-independent activity. Additionally, MYPT2 is dephosphorylated by an unknown mechanism, relieving the inhibition of PP1cδ activity bound to MYPT2. These combined effects result in the reduction of RLC phosphorylation to 10% [21, 31, 59]. RLC phosphorylation in non-beating hearts was shown to slowly decrease to 6 ± 2% after 2 hours [21].

The scheme presented in Fig. 5 provides an overview of current perspectives on the cardiac RLC phosphorylation signaling module but there are still many unknowns, particularly in relation to human disease. Gene ablation studies are needed with MLCK4 to determine if it contributes to RLC phosphorylation and whether its activity is regulated, perhaps by phosphorylation or other post-translational modifications. Conceivably, cMLCK may also be regulated by phosphorylation. Insights are provided on MLCP but the amount of published information is limited. Would MYPT2 knockout in cardiomyocytes be similar to the MYPT1 knockout in smooth muscles where there are minimal effects? If MYPT2 phosphorylation inhibits the bound PP1cδ, its knockout may have no effect on RLC phosphorylation. What additional phosphorylation sites in MYPT2 may be functionally important? What are the other protein phosphatases that dephosphorylate RLC? Future investigations should include identification of the protein phosphatases that maintain RLC phosphorylation at 40%. In addition, as the kinases and phosphatases that regulate RLC phosphorylation are themselves substrates for other regulatory kinases and phosphatases, upstream effectors of RLC phosphorylation need to be defined in order to ascertain how the MLCPs are regulated. It will be particularly important to assess the potential contributions of MLCPs in affecting RLC phosphorylation in heart diseases leading to cardiac failure.

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Highlights

- Myosin regulatory light chain (RLC) is constitutively phosphorylated 40% *in vivo*.
- Constitutive RLC phosphorylation is necessary for normal cardiac performance.
- RLC phosphorylation is maintained by cardiac specific myosin light chain kinase.
- Myosin light chain phosphatase (MLCP) also maintains RLC phosphorylation.
- MLCP may consist of two distinct forms, soluble and bound to myosin filaments.

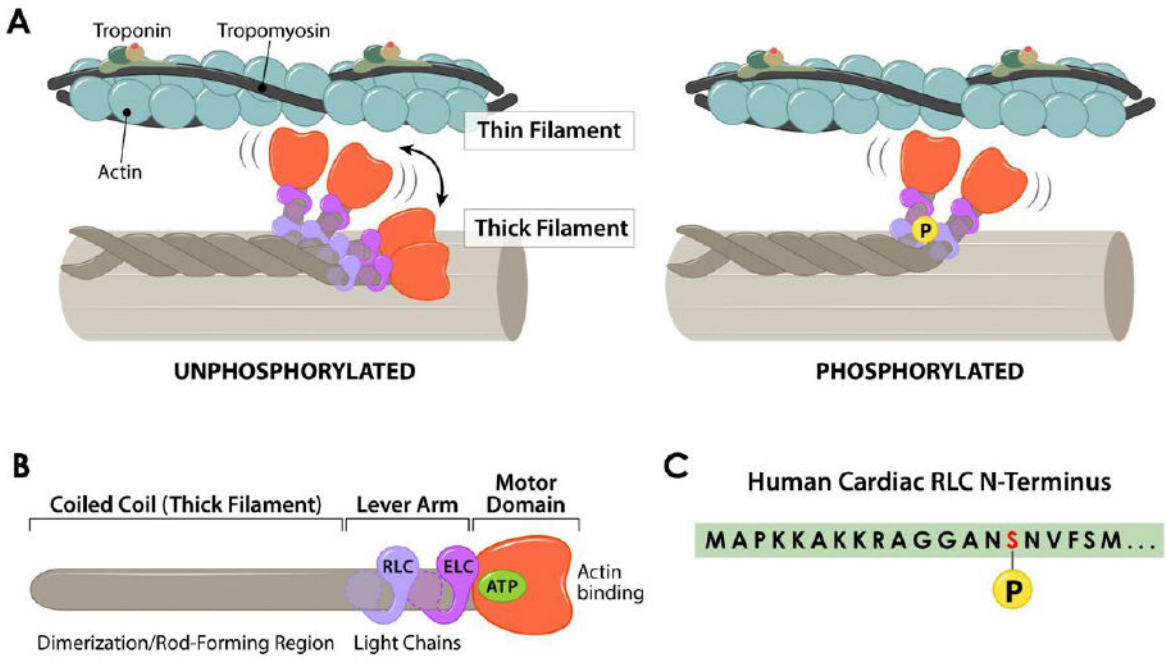
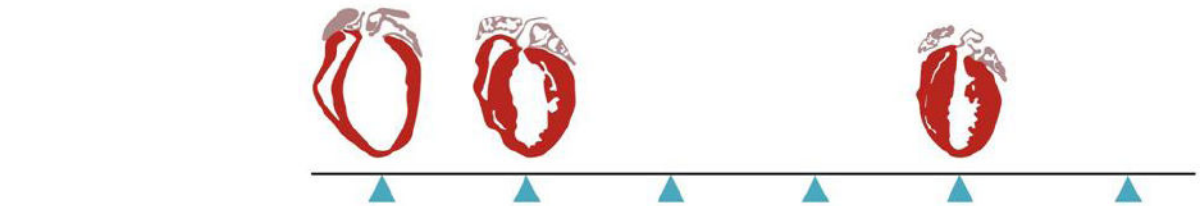


Figure 1. Cardiac contractile proteins and myosin regulatory light chain phosphorylation

A) Left panel: The cardiac sarcomere is composed primarily of thick filaments containing the molecular motor myosin, and thin filaments containing polymerized actin with its associated regulatory proteins tropomyosin and troponin. The binding of Ca^{2+} to the troponin complex results in movement of troponin/tropomyosin from an inhibitory state exposing myosin binding sites on actin, thus permitting actin-activation of myosin ATPase activity and ensuing contractions to occur. Myosin heads fluctuate between positions against the thick filament or in the inter-filament space, as indicated by the double-headed arrow. Right panel: Phosphorylation (**P**) of RLC favors the inter-filament position and thus modulates the activity of cardiac myosin by promoting its attachment to actin. B) Subdomains of the myosin protomer. The myosin heavy chain folded into a motor domain catalyzes the actin-activated ATPase activity. The lever arm binds an essential light chain (ELC) and a regulatory light chain (RLC). The rod-forming region dimerizes with a second protomer through coiled coil assembly to form the myosin molecule. Thus, myosin II is a hexamer comprised of two each of the heavy chain, ELC, and RLC. Bundles of myosins form the thick filament. C) Amino acid sequence near the human cardiac RLC phosphorylation site, Serine 15 (red).



Percent RLC Phosphorylation:	0	10	20	30	40	65

MOUSE MODEL	Non-P Knockin	cMLCK KO	Overexpressed MYPT2	Wildtype	Overexpressed MLCK
PHENOTYPE	Severe Failure	Failure	Failure	Normal	Normal
LIFE-SPAN	Short	Normal	Normal	Normal	Normal
STRESSED PHENOTYPE	Severe Failure			Hypertrophy	Attenuated Hypertrophy
STRESSED LIFE-SPAN	Short			Normal	Normal
HUMAN HEART	End-stage failure			Normal	

Figure 2. Cardiac phenotype trends related to the extent of RLC phosphorylation

Phenotype and lifespan of sedentary and stressed mouse models are shown as a function of RLC phosphorylation. Tracings of heart sections from representative mice are shown above the table. In hearts from normal mice and humans, 40% of RLC (0.4 mol phosphate/ mol RLC) is constitutively phosphorylated. In mice, decreased phosphorylation by phosphorylation site knockin mutant [28], cMLCK gene ablation (KO) [21, 35, 36, 54, 55], or overexpression of myosin phosphatase target subunit (MYPT2) [56], is associated with heart failure, which is exacerbated by cardiovascular stresses such as hypertension [54]. Increased phosphorylation by overexpression of MLCK in the heart attenuates hypertrophic responses to stress [38, 54]. Human hearts in failure have reduced RLC phosphorylation [44-47].

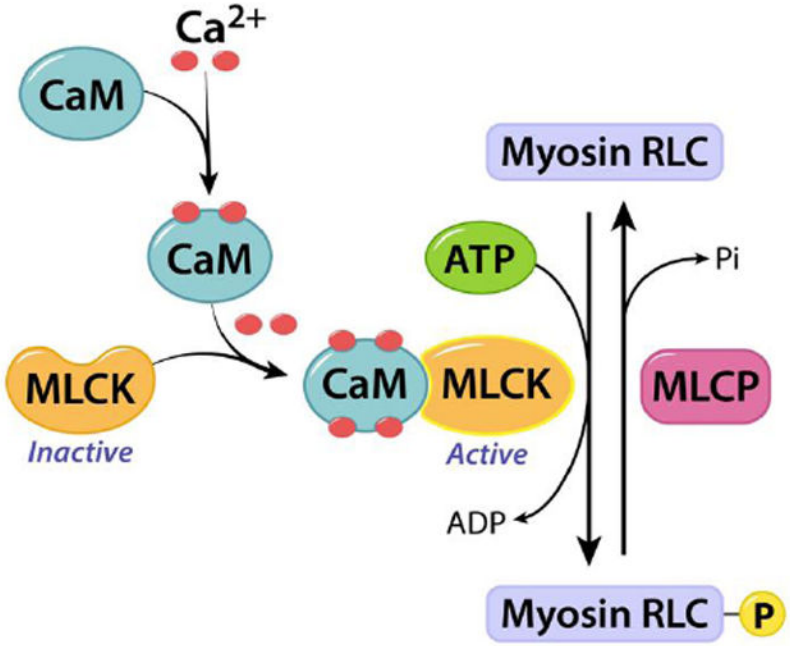


Figure 3. Canonical scheme for Ca^{2+}/CaM-dependent activation of myosin light chain kinase (MLCK) and phosphorylation of myosin RLC

Ca^{2+}/CaM-dependent MLCKs are inactive until bound to Ca^{2+}/CaM, leading to phosphorylation of myosin RLC. Phosphorylated myosin RLC is dephosphorylated by myosin light chain phosphatases (MLCP).

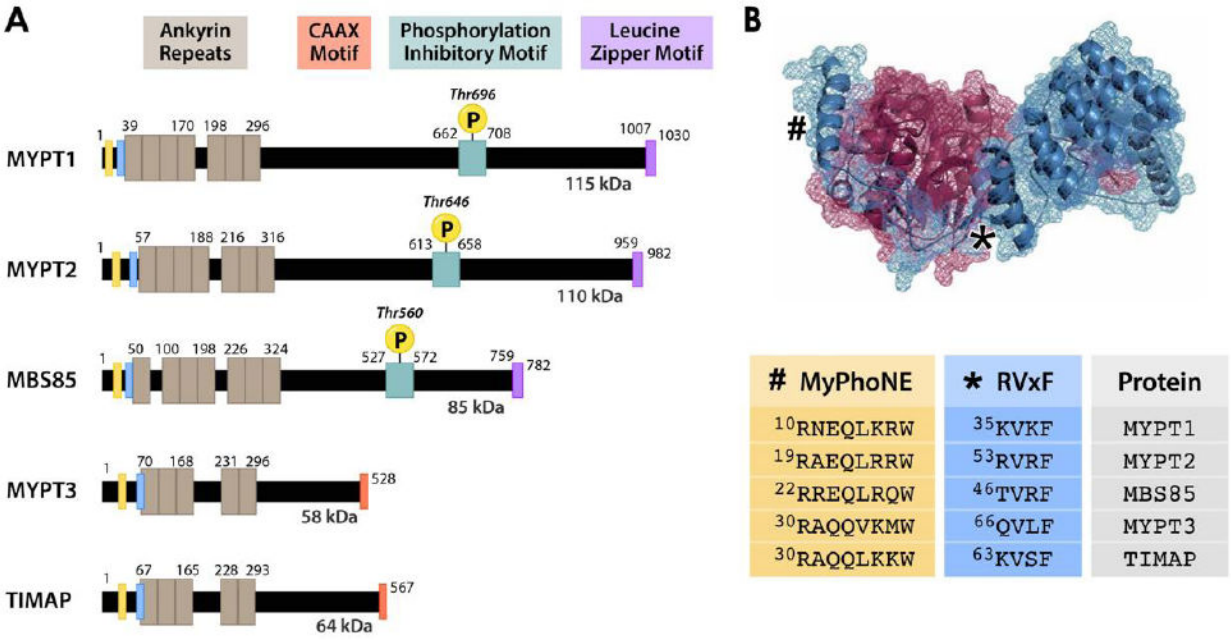


Figure 4. The myosin phosphatase target subunit family

A) Domain alignment of MYPT family members. All members have conserved PP1c binding sequences: myosin phosphatase N-terminal extension (MyPhoNE; yellow), RVxF (blue), and ankyrin repeats (taupe). MYPT1, MYPT2, and MBS85 have a common phosphorylation site that inhibits the activity of bound catalytic subunit PP1cδ, and a C-terminal leucine zipper motif. MYPT3 and TIMAP are smaller family members with a prenylation motif (CAAX). B) Top: Ribbon and mesh diagram for the refined structure of the N-terminus of MYPT1 (residues 1-299; blue) bound to PP1cδ (burgundy) positioned with the catalytic hydrophobic groove at the top [100]. Locations of the MyPhoNE (hashtag) and RVxF (asterisk) sequences are indicated. Bottom: Table shows sequences of MyPhoNE and RVxF domains in MYPT family members.

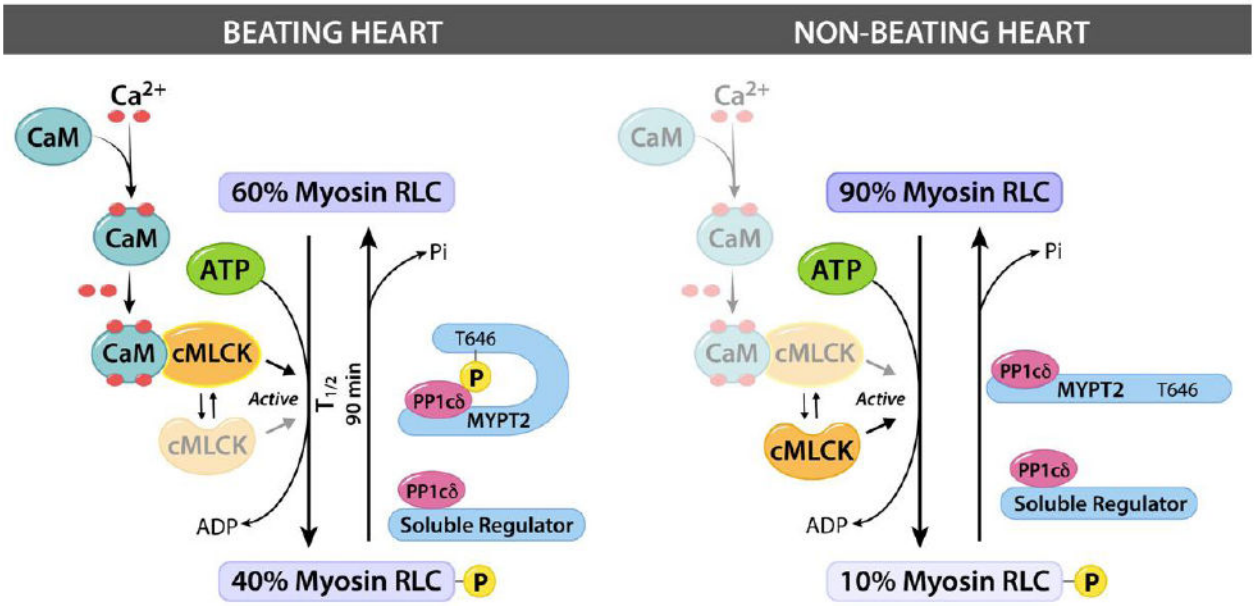


Figure 5. Schematic for regulation of RLC phosphorylation in the beating and non-beating heart

Left: In the beating heart, high-affinity binding of Ca^{2+}/CaM saturates cMLCK to maintain maximal kinase activity with little free cMLCK (ghosted). Balanced activities of Ca^{2+}/CaM-stimulated cMLCK and cMLCPs maintain 40% constitutive RLC phosphorylation. Long half-life of RLC phosphate ($T_{1/2}$) in the beating heart is attributed to the low constitutive activities of both cMLCK and cMLCPs. Right: In the non-beating heart, intracellular Ca^{2+} is reduced, resulting in no Ca^{2+}/CaM bound to cMLCK (ghosted). The lower Ca^{2+}/CaM-independent cMLCK activity is unable to maintain RLC phosphorylation at 40%. In the non-beating heart, MYPT2 is dephosphorylated, leading to enhanced MLCP activity. Details for regulation of cardiac MLCPs have yet to be defined.