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The Significance of Regulatory Light Chain Phosphorylation in Cardiac Physiology

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Abstract

It has been over 35 years since the first identification of phosphorylation of myosin light chains in skeletal and cardiac muscle. Yet only in the past few years has the role of these phosphorylations in cardiac dynamics been more fully understood. Advances in this understanding have come about with further evidence on the control mechanisms regulating the level of phosphorylation by kinases and phosphatases. Moreover, studies clarifying the role of light chain phosphorylation in short and long term control of cardiac contractility and as a factor in cardiac remodeling have improved our knowledge. Especially important in these advances has been the use of gain and loss of function approaches, which have not only tested the role of kinases and phosphatases, but also the effects of loss of RLC phosphorylation sites. Major conclusions from these studies indicate that (i.) two negatively-charged post-translational modifications occupy the ventricular RLC N-terminus, with mouse RLC being doubly phosphorylated (Ser 14/15), and human RLC being singly phosphorylated (Ser 15) and singly deamidated (Asn 14/16 to Asp); (ii.) a distinct cardiac myosin light kinase (cMLCK) and a unique myosin phosphatase targeting peptide (MYPT2) control phosphoryl group transfer; and (iii.) ablation of RLC phosphorylation decreases ventricular power, lengthens the duration of ventricular ejection, and may also modify other sarcomeric proteins (e.g., troponin I) as substrates for kinases and/or phosphatases. A long term effect of low levels of RLC phosphorylation in mouse models also involves remodeling of the heart with hypertrophy, depressed contractility, and sarcomeric disarray. Data demonstrating altered levels of RLC phosphorylation in comparisons of samples from normal and stressed human hearts indicate the significance of these findings in translational medicine.

Keywords

myosin light chain kinase; myosin light chain phosphatase; MYPT2; myofilament Ca²⁺ sensitivity; hypertrophy; adrenergic control

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Introduction

Employing one dimensional gel electrophoresis techniques, early studies in the 1970s in the laboratory of S. V. Perry demonstrated that myosin light chains exist as charge variants, which led to the first identification of phosphorylation as a substantial mechanism for these charge variants in both skeletal [1] and in cardiac muscle [2]. A few years after these discoveries, Walsh et al. [3] isolated and purified a Ca^{2+} -calmodulin dependent myosin light chain kinase with specificity for the cardiac regulatory light chain (RLC). As illustrated in Figure 1, positioned in series downstream of the ELC, RLC is a likely candidate for having a central role in modulating cardiac contractility based upon its position at the fulcrum of the lever arm (S1-S2 junction of MHC) [4], and its ability to be modulated by charge [5]. It is highly likely that the RLC affects the stiffness and flexibility of the myosin head through its interactions with the C-terminal α -helix forming the myosin lever arm [6]. Figure 1 illustrates how the major thick filament associated proteins, RLC and MyBP-C act to modify radial movements of cross-bridges with respect to the thick filament backbone. There is strong evidence that this radial disposition of cross-bridges is a determinant of local concentrations of myosin heads in the vicinity of the thin filaments. Thus rates of entry of cross-bridges into force generating states have been demonstrated to be enhanced by radial movements of cross-bridges induced by RLC phosphorylation, which moves cross-bridges closer to the thin filament [7]. A second potential mode of control of RLC is through an N-terminal EF-hand domain, which binds $\text{Ca}^{2+}/\text{Mg}^{2+}$ [8] at activating concentrations (reviewed in Solaro [9]). The binding of Ca^{2+} to the closed state of RLC induces a conformational change to an open state in which the adjacent helices become perpendicular, and the overall helical content of the molecule increases [10]. Disrupting the loop binding of Ca^{2+} by replacing the critical Asp residue at position 12 with Ala (D47A) caused a decline in maximum tension and a decrease in the proportion of force-generating, cycling crossbridges in skeletal fibers [11], indicating that Ca^{2+} binding to RLC modulates crossbridge kinetics. However, the dissociation rate of Ca^{2+} from RLC is too slow to serve a beat-by-beat regulatory role in the heart [12]. Isoforms of RLC are expressed in smooth and striated muscle, albeit with relatively low homology (57%, Figure 2), suggesting unique regulatory roles. Indeed, smooth muscle RLC phosphorylation at serine-19 by smooth muscle MLCK is essential for activation of contraction [13]. However in striated muscle, contraction is activated by Ca^{2+} binding to TnC and thus the phosphorylation of RLC modulates rather than triggers contraction [14]. It is also apparent that metal binding by RLC and phosphorylation are independent inasmuch as phosphorylated and de-phosphorylated cardiac and fast skeletal RLCs have the same affinities for Ca^{2+} or Mg^{2+} [15].

Regulatory Light Chain Phosphorylation

It is well-established that RLC is normally phosphorylated *in vivo* at a stoichiometry of approximately 0.4-0.5 mol P/mol protein (Figure 3). The proposed structural effect of RLC phosphorylation is that the negative charge induces a “swing-out” of the myosin head region (Figure 1), which decreases interfilament spacing and promotes the transition from weak to strong force-generating reactions at a given level of Ca^{2+} [16]. This mechanism is most likely responsible for the well-known effect of RLC phosphorylation to enhance cardiac myofilament response to Ca^{2+} [17]. Histological studies have identified a spatial gradient of RLC phosphorylation increasing from base to apex and from endocardium to epicardium, suggesting that RLC phosphorylation may support cardiac torsion and normalize wall stress [18, 19].

Precise functional roles for RLC phosphorylation in cardiac muscle were recently demonstrated *in vivo* by Sanbe et al. [20], Scruggs et al. [21], and Ding et al. [22]. Transgenic mice expressing a non-phosphorylatable RLC in which Ser-14/15/19 were

replaced with non-phosphorylatable Ala (TG-RLC(P-)) demonstrated depression of contractile function as measured by load-dependent measures of contractility [20]. We [21] also identified a slowing of time to peak elastance during ejection, and an inability to eject to a normal end-systolic volume when hearts were equally preloaded as NTG hearts at baseline. Furthermore, we observed an enigmatic blunting of a positive inotropic response to β 1-adrenergic stimulation in TG-RLC(P-), marked by no change or a decrease in load-independent measures of contractility. Biochemical alterations in TG-RLC(P-) occurred in both the thick and thin filaments, with both MyBP-C and TnI de-phosphorylated at baseline. However, following a five minute dobutamine stress, MyBP-C and TnI phosphorylation normalized between NTG and TG-RLC(P-), whereas the difference in RLC phosphorylation between NTG and TG-RLC(P-) nearly doubled. This disparity in RLC phosphorylation constituted the sole difference in the sarcomeric phospho-proteome between NTG and TG-RLC(P-). Our data strongly indicated that RLC phosphorylation plays a critical role in ejection, both at baseline and during β -adrenergic stimulation. Studies in a cMLCK knock-out model (cMLCK^{neo/neo}) by Ding et al. [22] generally agree with these findings. Basal RLC phosphorylation in this model was depressed as expected, and there was a depression in fractional shortening and induction of left and right ventricular hypertrophy in 4-5 month mice. A knock-down of the ortholog of cMLCK in zebra fish indicated a role in cardiogenesis, however there was no evidence of this effect in the cMLCK^{neo/neo} mouse hearts [22]. The non-phosphorylatable transgenic model, TG-RLC(P-), also developed cardiac hypertrophy, which occurred after 4 months of age [20, 21], yet the manifestation of cardiac dysfunction and blunted response to β 1-adrenergic stimulation, which was determined at exactly 13 weeks of age [21], occurred before this remodeling. Taken together, these data suggest that RLC phosphorylation is critical for normal cardiac dynamics at baseline and in the midst of sympathetic activation, however it appears not to be critical for cardiogenesis in mammalian systems.

Previous studies *in vitro* have correlated an increase in skeletal RLC phosphorylation with the potentiation of twitch tension following a brief tetanus or during low-frequency repetitive stimulation [16]. Isolated skinned skeletal fibers bathed in exogenous MLCK demonstrate enhanced myofibrillar Ca^{2+} sensitivity and the rate of force production at sub-maximal levels of Ca^{2+} [14, 16, 17, 23-27]. This effect is also observed in cardiac muscle fibers [17, 24, 28-30], and is synergistically affected by addition of exogenous PKC [31-33]. Interestingly, we observed a lack of change in Ca^{2+} -sensitivity in fibers with a non-phosphorylatable RLC (TG-RLC(P-)), presumably due to the concomitant decrease in TnI phosphorylation [21]. This important finding highlights the necessity of profiling the global sarcomeric phospho-proteome when examining the effect of phosphorylation on cardiac mechanics. In addition, both our group and Olsson et al. [30] reported a positive correlation between RLC phosphorylation and maximal Ca^{2+} -activated tension. An additional novel finding of our study was that tension-cost (isometric force/ATPase rate at a given pCa), an index of cross-bridge detachment rate [34], was significantly decreased in TG-RLC(P-) fibers, suggesting that RLC phosphorylation enhances myosin cycling kinetics. Collectively, these data clearly demonstrate that RLC phosphorylation potentiates force production per unit Ca^{2+} , and enhances crossbridge cycling kinetics.

A limitation in previous studies was that the relevant *in vivo* sites of cardiac RLC phosphorylation were unknown. Site-specific RLC phospho-antibodies were generated [19] and transgenic mice harboring altered putative sites were generated [20], but the pattern in which these sites are phosphorylated *in vivo* was unknown. In accordance with our own observations, multiple laboratories had consistently seen multiple spots of phosphorylation separated with 2D-electrophoresis in numerous species (Figure 3), and a study by Venema, and Kuo [32] illustrated using phospho-peptide mapping that multiple sites may be phosphorylated by PKC and MLCK *in vitro*. White et al. [35] were the first to identify a

single phosphorylation in the N-terminal peptide 9-29 using MALDI-TOF peptide mass fingerprinting, however this peptide contained three potential sites. Recently, we [36] expanded on findings from these two studies by unequivocally identifying the *in vivo* sites of RLC phosphorylation in human and murine cardiac RLC. Murine RLC exists as three unique charged species—unphosphorylated, singly phosphorylated, and doubly phosphorylated at Ser-14/15. This was the first identification of a doubly-charged species of RLC in the murine heart [36]. Interestingly, human RLC, which contains a unique N-terminal sequence relative to mouse in which an Asn replaces Ser at position 14, exhibited a deamidation and phosphorylation, rather than the double phosphorylation as in the mouse [36]. Deamidation of Asn to Asp produces a negatively charged amino acid side chain, thus creating a charge similar to that of phosphorylation on the N-terminal region of RLC, albeit through different chemistry. In conclusion, at baseline, RLC exists as three distinct charged species in mouse and human *in vivo*—one unphosphorylated, one singly-phosphorylated, and one doubly phosphorylated (mouse) or phosphorylated/deamidated (human).

Kinase and Phosphatase Control of RLC Phosphorylation

Based upon findings in smooth muscle, it is assumed that cardiac RLC is phosphorylated by myosin light chain kinase (MLCK) and dephosphorylated by myosin light chain phosphatase (MLCP). The relevant MLCK isoform, cMLCK, in the heart had been a subject of debate. The original purification of MLCK from heart carried by Walsh et al. [3] showed that MLCK is Ca^{2+} calmodulin dependent. However, Chan et al. [37] cloned the first cardiac-specific MLCK (cMLCK). cMLCK is highly and equally expressed in atria and ventricles, exerts a positive inotropic effect on isolated cardiac myocytes in culture as measured by rate of cell shortening, and surprisingly, is Ca^{2+} -calmodulin-independent. [37]. A more recent study [22] in which cMLCK was ablated in a mouse knockout model suggests that its activity is dependent on Ca^{2+} -calmodulin, but no definitive experiments were carried out. This is a critical question inasmuch as spatial and temporal changes in intracellular Ca^{2+} have been proposed to control levels of cMLCK and therefore RLC phosphorylation [18, 19]. Despite these important findings, the activation of cMLCK and the signaling involved in modulating cardiac RLC phosphorylation have not been extensively studied. The involvement of adrenergic signaling is apparent from both a study by Westwood and Perry [38] and our observations demonstrating that RLC phosphorylation increases at a time post-peak functional response following β_1 stimulation in mice [21]. Previous studies analyzing RLC phosphorylation at the peak functional response found no change [38-41]. Furthermore, a change in RLC phosphorylation has been observed following alpha-adrenergic receptor activation [42-45]. Two kinases activated by the alpha-adrenergic pathway, Rho-associated kinase (ROCK) and protein kinase C (PKC) have been associated with an increase in RLC phosphorylation [32, 46, 47], and phospho-peptide mapping demonstrated PKC phosphorylation of RLC at sites distinct from MLCK [32]. Of particular interest is the presence of a unique, cardiac-specific N-terminal sequence in cMLCK [37], that displays numerous Ser and Thr residues with significantly high phosphorylation potential (>0.9 , NetPhos www.cbs.dtu.dk/services/NetPhos) and favorable consensus sites (>0.8 , NetPhosK, www.cbs.dtu.dk/services/NetPhosK) for PKA and PKC (Figure 4). Collectively, the results of these studies suggest that in addition to RLC, cMLCK may be a relevant endogenous downstream target of phosphorylation following Gs- and Gq-coupled pathway activation. ZIPK, a kinase known to phosphorylate RLC in non-muscle cells [48] and in smooth muscle [49] in a Ca^{2+} -independent manner, has recently been demonstrated to be abundantly expressed in heart and to phosphorylate cardiac RLC [50]. Control of ZIPK by processes independent of Ca^{2+} -calmodulin appears likely. There is evidence [51] in non-cardiac systems that ZIPK is a substrate for Rho kinase, which phosphorylates and activates its activity. Chang et al. [50] also make the interesting speculation that ZIPK, which appears to play a role in suppression of stresses involving inflammation, apoptosis, and autophagy

[52], may serve a role in compensation for these threats by promoting contractility through RLC phosphorylation. In summary, these data suggest that RLC may be phosphorylated by kinases other than cMLCK. While it is clear from Ding et al. [22] that baseline phosphorylation of RLC is predominantly controlled by cMLCK, when cMLCK^{neo/neo} animals were stressed with a 7-day isoproterenol infusion, it was reported that RLC phosphorylation did in fact increase. This finding, in conjunction with above data, suggests that alternate kinases play a role in levels of RLC phosphorylation upon perturbation of basal cellular signaling. Furthermore, additional studies are warranted to determine whether alterations in RLC phosphorylation can occur through modulation of cMLCK activity via phosphorylation, presumably in the N-terminal, cardiac-specific domain.

Nishio et al. [53] initially clarified the mechanism by which RLC is dephosphorylated in their studies identifying that the major phosphatase is PP1c- δ . PP1c- δ is composed of a 38-kDa catalytic unit, which acts in concert with a larger ~100-130 kDa myosin binding unit that is similar to that discovered in smooth muscle, and now known as MYPT. Subsequent studies demonstrated that the major isoform in the heart is MYPT2 [54]. There is also a relatively small 20-21-kDa unit of the holoenzyme [55], discovered in smooth muscle, which also exists as a cardiac specific isoform, hHS-M(21). When present in permeabilized cardiac muscle fibers, the small unit increased Ca²⁺ sensitivity of force development by a mechanism presumably involving an interaction with MYPT1/2. In support of this, Schichi et al. reported that two isoforms of hHS-M(21) exist, hHS-M(21)A and hHS-M(21)B, which bind the C-terminal regions of MYPT1 and MYPT2 *in vitro* [56]. Studies reported by Mizutani et al. [57] employing transgenic over-expression of MYPT2 provide *in vivo* evidence strongly supporting a critical role for MYPT2 in cardiac physiology. Hearts of these mice demonstrated a specific increase in the activity of PP1c- δ , and reduced levels of RLC phosphorylation associated with a depression in myofilament response to Ca²⁺. The over-expression of MYPT2 also induced an increase in left ventricular mass and sarcomeric disarray with cardiac dysfunction, as indicated by a significant depression in fractional shortening. These data support evidence cited above that chronic depression of RLC phosphorylation through the mutation of phosphorylation sites [20], and by knockout of cMLCK [22] induce increases in cardiac mass with depressed contractility. There is also evidence that MYPT's actions on RLC may be modulated through an α -adrenergic receptor-dependent activation of Rho kinase [58]. Data reported by Rajashree et al. [58] demonstrated that stimulation of α -adrenergic receptors in cardiac ventricular myocytes increased levels of both RLC and MYPT2 phosphorylation with an associated increase in Ca²⁺-sensitivity of force generation. Schichi et al. recently demonstrated that hHS-M(21)A, but not hHS-M(21)B, enhanced the inhibitory phosphorylation of MYPT1 at Thr-696 (human sequence), which was diminished upon Rho kinase inhibition [56]. Furthermore, they determined that Rho kinase, MYPT1, and hHS-M(21)A form a trimeric complex *in vitro*, and that hHS-M(21)A potentiates the enzymatic activity of Rho kinase. Of note, the modulatory actions hHS-M(21)A and hHS-M(21)B on MYPT2 phosphorylation were not examined in this study. Taken together, these data suggest a prominent role for α -adrenergic signaling in modulation of RLC phosphorylation via inhibition of the myosin phosphatase.

Techniques for measuring endogenous RLC phosphorylation

RLC separates nicely with 2DE due to its small M_r (18.9 kDa) and acidic, but not extreme, pI (4.86). The most effective way to separate the endogenous species of RLC, both atrial and ventricular isoforms, has been with tube gel electrophoresis using mobile carrier ampholytes over a narrow pH range (Figure 3), as first demonstrated with cardiac RLC by Westwood and Perry [38]. For analyzing large amounts of sample, tube gels are superior over IPG strips for RLC separation, as one can effectively separate as much as 1 mg of myofilament-enriched homogenate over a 4.5-5.4 pH range. This is important for providing sufficient

protein material per spot when the downstream objective is to obtain profitable mass spectrometric results when searching for low-abundant post-translational modifications. Gels are cast in glass tubes of 180 mm (length) $\times$ 1.5 mm (ID) with the following constituents: urea 9.2 M, acrylamide 4.5%, CHAPS 1.5%, Nonidet P-40 0.5%, ampholytes (pH 4.5–5.4) 5%, and are run for 200 V for 2h, 500 V for 2h, then 800 V for 16h. The acidic buffer (lower chamber) is 10 mM H_3PO_4 and the basic buffer (upper chamber) is 100 mM NaOH. Gels are ejected from tubes and equilibrated in Laemmli buffer [59] for 10 min prior to being laid atop 13% second dimension SDS-PAGE gels. We consistently observe three spots for murine cardiac RLC, the most prominent being the most alkaline spot, followed by two spots of increasing acidity and decreasing intensity. Staining these spots with ProQ Diamond (Molecular Probes), a phospho-specific stain, confirms that the two more acidic spots are phosphorylated. Treatment of the protein with protein phosphatase 1 (PP1) completely de-phosphorylates RLC (Figure 3). One-dimensional isoelectric focusing in-gel was the first technique applied for the separation of cardiac RLC phospho-species by charge [2]. This technique is very effective and is still widely-used [60]. However, if proteomic analysis is desired subsequent to the separation of RLC via one-dimensional methods, contaminating proteins within the same molecular weight range may complicate the analysis. In summary, the charged species of RLC are relatively simple to resolve using standard biochemical methods, however the method of choice should be selected based upon the desired downstream analysis.

Conclusions

Although we have known that the RLC of heart myofilaments undergoes phosphorylation, new discoveries continue to enhance our understanding of the relevance of this PTM in heart function, growth and remodeling. An important aspect of these discoveries is that they indicate the importance of determining how RLC phosphorylation integrates with complex signaling networks. It is also critical to continue to investigate the spatial distribution of RLC phosphorylation as a determinant of ejection in the heart beat.

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Abbreviations

ELC	essential light chain
FHC	familial hypertrophic cardiomyopathy
LMM	light meromyosin
MHC	myosin heavy chain
MLCK	myosin light chain kinase
MyBP-C	myosin binding protein C
PTM	post-translational modification
RLC	regulatory light chain
PP1	protein phosphatase 1

MYPT	myosin phosphatase target subunit
hHS-M(21)	heart specific small subunit of myosin light chain phosphatase
S-1	myosin sub-fragment 1
S-2	myosin sub-fragment 2

Figure 1.

Illustration of the transition between diastole and systole within a region of overlap between thick and thin filaments of the cardiac sarcomere. *Left:* The relaxed state is shown with thin filament troponins (TnC, TnT, TnI) and tropomyosin (Tm) blocking the reaction of crossbridges with actin. *Right:* The binding of Ca^{2+} to TnC releases Tm and TnI from the blocking position, and crossbridges—intricately tethered to associating light chains (RLC and ELC), MyBP-C, and Titin—react with actin and mechanically work to pull the thin filaments towards the center of the sarcomere. It is conceivable that the net negative charges (illustrated by yellow stars) induced by phosphorylation of both the N-terminus of RLC and the cardiac-specific domain of MyBP-C, create a steric opposition between RLC and MyBP-C, which may contribute to the positioning of myosin heads relative to actin. This mechanism may in part explain how RLC and MyBP-C act to modify radial movements of crossbridges with respect to the thick filament backbone.

Figure 2. Isoform sequence alignment of murine atrial, ventricular, skeletal, and smooth muscle RLCs. Sequences were obtained from SwissProt database (www.expasy.org) with the following accession numbers: atrial (Q9QVP4), ventricular (P51667), skeletal (P97457), and smooth (Q3THE2).

Figure 3.

Two-dimensional analysis of RLC. (A) Myofibrillar-enriched mouse ventricular tissue separated using tube-gel electrophoresis over a narrow pH range (4.5-5.4); (B) Atrial and ventricular tissue combined illustrating the clear separation between the two isoforms and phosphorylated counterparts; (C) phosphatase assay of ventricular RLC demonstrating that P1 and P2 are phosphorylated (ProQ Diamond stain, top) counterparts of U; (D) ventricular RLC profiles in different species illustrating phospho-profiles on the top (ProQ Diamond stain) and the total protein profiles on the bottom (Blue Silver Colloidal Coomassie stain).

BNetPhosK 1.0 kinase prediction results for cMLCK unique N-terminus

1	MSGVSEEDPEGLAPQGLPAL	21	GGACLATMDKKLNVLTEKVD
41	RLHFQEDVTEKLQCVCQGM	61	DHLEQDLHRLEASRELSLAG
81	SGSTPPTTAQAAWPEVLELV	101	RAVRQEGAQHGARLEALFKM
121	VVAVDRAITLVGSTFQNSKV	141	ADFIMQGTVPGRKGS ^S LADGP
161	EENKEQAEVAGVKPNHVLTT	181	GGVQADASRTLWEESQKEDI
201	PVRTVEGLPLIINTSLKGAD	221	LTQAGASLRQGVEVLGPGQV
241	PLPTEAESRLPETASENTGA	261	TLELSVAIDRISEVLTSKLM
281	SQGGGQETSSSKPDCWLSEE	301	AMRL ^S SGPLPQPLGPTPDS
321	DIHSGDALPRIPINMQEMAT	341	PGELLETSQSGSPIGSAEAPG
361	LGTVLEDQIPKGARPFPPPLP	381	KRSSNNGGMSAEEEEIGSGAE
401	PMRGP ^S LATRDWRDET ^S VTGT	421	DLQQGIDPGAVSPEPGKDHA
441	AQGPGRTEAG		

High potential PKC site: **T-148**; 0.85

High potential PKA sites: **S-155**, 0.86; **S-305**, 0.81; **S-406**, 0.85

Figure 4.

Characteristic features of cardiac-specific myosin light chain kinase. (A) Comparison of MLCK isoforms; figure re-drawn based on Chan *et al.* [37]. NetPhos 2.0 [61] analysis demonstrates that the cardiac-specific N-terminus of cMLCK contains numerous Ser and Thr residues with high phosphorylation potential. Those that are nearly ideal substrates for PKC and PKA as determined by NetPhosK 1.0 [62] are shown with their score (1.0 being an ideal substrate) in (B) along with the full sequence of the unique N-terminus as published in [37].