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Cardiac myosin regulatory light chain kinase modulates cardiac contractility by phosphorylating both myosin regulatory light chain and troponin I

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Running Title: cMLCK phosphorylates human cardiac troponin I

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ABSTRACT
Heart muscle contractility and performance are controlled by posttranslational modifications of sarcomeric proteins. Although myosin regulatory light chain (RLC) phosphorylation has been extensively studied both in vitro and in vivo, the precise role of cardiac myosin light chain kinase (cMLCK), the primary kinase acting upon RLC, in the regulation of cardiomyocyte contractility remains poorly understood. In the current study, using recombinantly expressed and purified proteins, various analytical methods, in vitro and in situ kinase assays, and mechanical measurements in isolated ventricular trabeculae, we demonstrate that human cMLCK is not a dedicated kinase for RLC, but can phosphorylate other sarcomeric proteins with well-characterized regulatory functions. We show that cMLCK specifically mono-phosphorylates Ser-23 of human cardiac troponin I (cTnI) both in isolation and in the trimeric troponin complex in vitro and in situ in the native environment of the muscle myofilament lattice. Moreover, we observed that human cMLCK phosphorylates rodent cTnI to a much smaller extent both in vitro and in situ, suggesting a species-specific adaptation of cMLCK. Although cMLCK treatment of ventricular trabeculae exchanged with either rat or human troponin increased their cross-bridge kinetics, the increase in sensitivity of myofilaments to calcium was significantly blunted by human TnI, suggesting that human cTnI phosphorylation by cMLCK modifies the functional consequences of RLC phosphorylation. We propose that cMLCK-mediated phosphorylation of TnI is functionally significant and represents a critical signaling pathway that coordinates the regulatory states of thick and thin filaments in both physiological and potentially pathophysiological conditions of the heart.

Cardiac muscle contraction is driven by the cyclic interactions of myosin and actin, coupled to ATP hydrolysis. Calcium binding to troponin triggers the activation of the actin-containing thin filament mediated by the azimuthal movement of tropomyosin on its surface, which allows myosin head domains from the neighboring myosin-containing thick filaments to strongly attach to available actin-binding sites. Subsequently, small conformational changes in the catalytic part of the myosin head domain, associated with the release of inorganic phosphate, are amplified by the
myosin light chain-containing ‘lever arm’ or light chain domain (LCD), which results in nm-scale displacement of the thin filaments towards the center of the sarcomere or generation of pN-scale forces (1). Heart muscle contractility and performance are also controlled by post-translational modifications of sarcomeric proteins, including phosphorylation of the myosin regulatory light chain (RLC) by the cardiac isoform of myosin light chain kinase (cMLCK). RLC phosphorylation has been shown to be an important regulator of cardiac muscle function, with ablation of either cMLCK or phosphorylation of RLC per se leading to cardiac muscle dysfunction and pathological hypertrophy in animal models (2-4). In contrast, increasing RLC phosphorylation in transgenic animal models has a cardio-protective function (5). RLC phosphorylation in transgenic animal models is controlled by post-translational mechanisms (11,14).

The clinical significance of cMLCK is highlighted by the fact that mutations in the gene encoding for cMLCK (MLYK3) have been associated with the development of dilated cardiomyopathy in human (15,16), and increased protein turn-over of cMLCK has been suggested to underlie the transition from compensatory hypertrophy to decompensated heart failure in animal models (17). In contrast, increasing RLC phosphorylation prevents the development of hypertrophic cardiomyopathy-associated heart failure (18), and increases cardiac output, suggesting that cMLCK may prevent the clinical onset of cardiomyopathies and increase the performance of the failing heart. However, the exact role of cMLCK in cardiomyocyte contractile regulation and sarcomere organization remains to be established.

In the current study, we show that cMLCK is not a dedicated kinase and in fact phosphorylates human cardiac troponin I, an important regulator of myofilament calcium sensitivity and relaxation kinetics. We show that cMLCK specifically mono-phosphorylates human cTnI on serine 23 in vitro in isolated protein preparations and in situ in the native environment of the muscle lattice. Moreover, cTnI phosphorylation by cMLCK is species-specific, and cMLCK does not phosphorylate cTnI in rodent muscle due to sequence variations around the phosphorylatable serine residue. We also show that the functional consequences of cMLCK activation differ significantly in the presence of either human or rodent troponin, which in the case of the former modifies the functional effects of RLC phosphorylation previously observed in isolated cardiac fibers from rodent heart (7). The current results provide new mechanistic insights into the regulation of cardiac muscle contractility by cMLCK and its species-specific differences.

Results

cMLCK phosphorylates serine 23 of human cTnI in vitro

We compared the primary sequence of known myofilament phospho-proteins from different species with the cMLCK consensus sequence in RLC and identified serines 22/23 in the cardiac specific N-terminal extension (NTE) of human cardiac troponin I (hTnI) as potential substrates (Fig. 1A, red). In contrast, rodent cardiac troponin I differs considerably in its primary sequence surrounding the phosphorylatable serines 22/23 in its NTE, suggesting that rodent (including rat and mouse) cTnI might not be a substrate for cMLCK. The phosphorylatable serines 22/23 are followed by an asparagine residue (P+1 position) in human cTnI (Fig. 1A), which is similar to the consensus sequence found in both rodent and human cardiac RLC. In contrast, in rodent cTnI
the phosphorylatable serines are followed by an alanine in the P+1 and an asparagine in the P+2 position (Fig. 1A), which might interfere with substrate recognition by cMLCK. To test this hypothesis, we used either recombinant human or rat cardiac troponin complex as substrates in cMLCK in vitro kinase assays and analyzed their phosphorylation profiles by Phostag™-SDS-PAGE. As shown in Fig. 1B, cMLCK specifically phosphorylated hcTnI to >0.6 mol P/mol cTnI within 30 min of incubation. No phosphorylation of either cardiac troponin T or C was observed under the same conditions, suggesting that cMLCK has a high specificity towards hcTnI in vitro. In contrast, rat cardiac troponin I (rcTnI) was phosphorylated to less than 0.02 mol P/mol cTnI under the same conditions, suggesting that it is a poor substrate for cMLCK in vitro. Note that unphosphorylated rat cTnT and cTnI migrate faster and slower in SDS-PAGE, respectively, than the corresponding human proteins. Prolonged incubation of both troponin complexes for >60 min resulted in almost complete mono-phosphorylation of hcTnI (>0.9 mol P/mol cTnI), but less than 0.15 mol P/mol cTnI phosphate incorporation for the rat troponin complex (Fig. 1C). In contrast to cTnI, cMLCK mono-phosphorylates both isolated human and rat cRLC under the same conditions with a similar time-course to ≈1 mol P/mol cRLC within 60 min (10).

Next, we tested the specificity of cMLCK for hcTnI by incubating human cardiac troponin complex with increasing concentrations of cMLCK (i.e. from 1:100 to 1:5 enzyme to substrate ratio) for 1h, and analyzed the phosphorylation profiles of cardiac troponin T, C and I by Phos-tag™-SDS-PAGE and electron spray ionization (ESI) mass spectrometry (Figs. 1D, 1E and Tab. S1). Only hcTnI was specifically mono-phosphorylated to ≈1 mol P/mol cTnI under all conditions tested, supporting the hypothesis that cMLCK specifically phosphorylates a single residue in cTnI, i.e. either serine 22 or 23. We confirmed this result by phosphorylating isolated hcTnI with cMLCK (Fig. 1F) followed by Phos-tag™-SDS-PAGE and Western-blot using a cTnI pS22/pS23-specific antibody (Fig. 1G). The phospo-specific antibody clearly recognized the mono-phosphorylated species, supporting the above conclusion that either S22 or S23, or both are substrates for cMLCK in vitro. To identify the phosphorylated serine residue, we incubated isolated wildtype hcTnI, or hcTnI with either S22- or S23-substituted to alanine (S-A) with Ca²⁺/calmodulin-activated cMLCK (Fig. 1H). Only wildtype and S22A-substituted cTnI were phosphorylated by cMLCK, localizing the phosphorylation site to S23, in good agreement with the sequence similarity to RLC (Fig. 1A). A minor bis-phosphorylated human cardiac troponin I species was observed when the protein was used in isolation in kinase assays (Figs. 1F and 1H), suggesting that cMLCK phosphorylated a secondary unspecific site that is, however, not accessible in the trimeric troponin complex.

A sequence alignment of the N-terminal extension of cTnI from different species revealed that the P+1 position is an asparagine in all old- and new-world primate species, and human (Fig. S2), and an alanine in other vertebrates, suggesting that cTnI phosphorylation by cMLCK might be a specific evolutionary adaptation.

**In situ phosphorylation of cTnI by cMLCK**

Next, we confirmed the results for isolated proteins described above in experiments on isolated cardiac myofibrils (CMF), in which intact thin and thick filaments are organized into the native myofilament lattice. CMFs isolated from rat ventricular tissue were pre-treated with lambda protein phosphatase (λPP) to homogenously reduce protein phosphorylation levels. λPP-treatment resulted in efficient dephosphorylation of cRLC, cTnI, cardiac troponin T, desmin and cMyBP-C as assessed by SDS-PAGE and Pro-Q Diamond phospho-protein staining (Fig. S3). Myofibrillar proteins after either λPP treatment, or sequential treatment with λPP followed by incubation with Ca²⁺/CaM/cMLCK for 1h, were separated by SDS-PAGE, and phosphorylated proteins identified by phospho-protein staining with Pro-Q Diamond and total protein staining with Coomassie or SYPRO Ruby. As described previously (7), cMLCK specifically phosphorylated an abundant rat myofilament protein with an apparent molecular weight of ~18 kDa (Fig. 2A, grey arrowhead), in good agreement with the calculated mass of cRLC. An additional band in the Pro-Q staining with an apparent molecular weight of ~37 kDa (Fig. 2A, black arrow head) corresponds to cMLCK, which is endogenously phosphorylated (10). We confirmed cRLC phosphorylation in treated rat CMFs by Phos-tag™-SDS-PAGE and Western-blot against cRLC (Fig. 2A, right). As expected, cRLC was highly phosphorylated after cMLCK
treatment (~0.7 mol Pi/mol cRLC). In contrast, Phos-tag™-Western-blot against cTnI with the same samples showed that although λPP-treatment resulted in efficient dephosphorylation of cTnI, the rat variant was not phosphorylated by cMLCK in situ.

We repeated the in situ phosphorylation experiments in CMFs prepared from both marmoset (Callithrix jacchus) and human left ventricle, - which exhibit a significantly higher cTnI primary sequence identity (~92% rat vs human cTnI; and ~96% marmoset vs human cTnI; Fig. S2), including an asparagine in the P+1 position following serine 23 in the NTE. Pro-Q phospho-protein staining of λPP/cMLCK-treated marmoset and human CMF showed three bands with apparent molecular weights of ~18 kDa, ~24 kDa and ~37 kDa (Figs. 2B and 2C; grey, white and black arrowheads, respectively), consistent with the expected molecular weights of cRLC, cTnI and cMLCK, respectively. As before, we used Phos-tag™-SDS-PAGE and Western-blot to confirm that cRLCs in both CMFs were phosphorylated after cMLCK treatment (~0.8 mol P/mol cRLC). However, in contrast to rat CMF, cTnI was phosphorylated in the same samples to about 0.3-0.6 mol P/mol cTnI (Fig. 2B, right), confirming that cMLCK phosphorylates native marmoset and human cTnI in the myofilament lattice in situ. Specific phosphorylation of marmoset and human cTnI at serine 23 in situ was further confirmed by Western-blot using a pS22/pS23-specific antibody (Fig. S4A). Incubation of human CMF with smooth muscle MLCK did not result in any phosphorylation of cTnI, suggesting that phosphorylation of cTnI is specific to the cardiac isoform of MLCK (Fig. 2D).

To assess the functional consequences of hcTnI phosphorylation by cMLCK, we exchanged unphosphorylated rat or human cardiac troponin complex into demembranated rat right ventricular trabeculae with a roughly 70% exchange efficiency (Fig. 3A). Endogenous cTnI in demembranated ventricular trabeculae is highly phosphorylated due to residual kinase activity present in the quiescent preparations (Fig. 3B) and overnight exchange with the unphosphorylated recombinant troponins reduced cTnI phosphorylation from ~1.8 mol P/mol cTnI to ~0.9 mol P/mol cTnI in both groups (Figs. 3C and 3D). As previously described, the level of RLC phosphorylation was low in demembranated trabeculae (< 0.05 mol P/mol RLC) (10) and recombinant troponin exchange had no further effect (Figs. 3C and 3D, bottom). Incubation in activating solution (pCa 4.5) containing 25 μmol/L blebbistatin and 1 μmol/L cMLCK/CaM had no effect on the cTnI phosphorylation level of rat troponin exchanged trabeculae, but significantly increased cTnI phosphorylation in the presence of human cardiac troponin to ~1.3 mol P/mol cTnI (Figs. 3D and 3E, top). In contrast, RLC was highly phosphorylated in both groups of trabeculae after cMLCK treatment (Figs. 3D and 3E, bottom).

In summary, these results show that cMLCK phosphorylates hcTnI in situ in the native environment of the myofilament lattice.

**Functional consequences of cTnI phosphorylation by cMLCK in cardiac muscle**

The functional consequences of hcTnI phosphorylation by cMLCK were assessed by measuring the calcium sensitivity and cross-bridge kinetics of troponin-exchanged ventricular trabeculae before and after incubation with cMLCK/Ca²⁺/CaM. However, exchange of ~70% endogenous with recombinant troponin had only a moderate effect on the cTnI phosphorylation level observed after exchange, with about 30% still present as mono-phosphorylated and 30% as bis-phosphorylated form (Fig. 3C and 3D), suggesting that recombinant unphosphorylated cTnI was partially phosphorylated during the protein exchange by kinases still present in the freshly prepared ventricular trabeculae (Fig. 3B). Because of the high basal level of phosphorylation, the effects of kinase treatment on cTnI phosphorylation and myofilament function could therefore not be unambiguously interpreted. We therefore employed an additional dephosphorylation step using λPP as described previously for mouse ventricular trabeculae (19). First, we tested the effects of λPP treatment on cardiac muscle mechanics. Incubation of rat ventricular trabeculae with λPP increased the calcium sensitivity of force as indexed by an increase in pCa₅₀ from 5.81 ± 0.01 to 5.94 ± 0.01 (Means ± SEM, n=4), but decreased the steepness of the force-calcium relation (nₜₐₜ of 7.47 ± 0.56 vs. 4.56 ± 0.37, mean ± SEM, n=4) (Fig. 4A). Moreover, λPP-treatment decreased the rate of force re-development at intermediate and high levels of activation (Figs. 4B and 4C), but had little or no effect on either passive or maximal calcium-activated isometric force. After the experiments trabeculae were dismounted and dissolved in SDS-PAGE loading buffer, and cTnI...
phosphorylation levels determined by Phos-tag™-SDS-PAGE followed by Western-blot against cTnI (Fig. 4D). Trabeculae from the same hearts that did not undergo λPP-treatment were used as negative controls. As before, endogenous cTnI phosphorylation levels in untreated control trabeculae were high (~1.8 mol P/mol cTnI) and incubation with λPP efficiently reduced cTnI phosphorylation to less than 0.1 mol P/mol cTnI, in good agreement with the CMF experiments described above. λPP-treatment also resulted in efficient dephosphorylation of cMyBP-C, which was confirmed by Western-blot using a phospho-specific antibody (Fig. S4B). The comparison of the protein phosphorylation levels with the functional effects suggests that λPP-treatment increases calcium sensitivity and decreases crossbridge kinetics of ventricular trabeculae via dephosphorylation of cTnI and cMyBP-C, respectively. Therefore in order to achieve a homogenously low cTnI phosphorylation level, we first exchanged trabeculae overnight with either unphosphorylated human or rat cardiac troponin followed by λPP-treatment directly before the mechanical experiments and subsequent incubation with Ca^{2+}/CaM/cMLCK. Incubation of rat cTn-exchanged and λPP-dephosphorylated trabeculae with Ca^{2+}/CaM-activated cMLCK increased the calcium sensitivity of force by 0.07 ± 0.01 pCa (mean ± SEM, n=5) (Fig. 5A; Tab. S1), but had no effect on the steepness of the force calcium relation (n_{s1} of 4.76 ± 0.46 vs. 4.44 ± 0.42, mean ± SEM), in good agreement with previous results obtained from native rat ventricular trabeculae (7). This result suggests that cTn-exchange and dephosphorylation with λPP did not affect the physiological response of trabeculae to RLC phosphorylation by cMLCK. In contrast, the increase in calcium sensitivity after cMLCK treatment was strongly reduced in trabeculae exchanged with human cardiac troponin to 0.02 ± 0.01 pCa (mean ± SEM, n=5) (Figs. 5B and 5C; Tab. S1), suggesting that hcTnI phosphorylation by cMLCK in the same preparations mitigated the increase in calcium sensitivity associated with phosphorylation of cRLC. Moreover, cMLCK treatment reduced the steepness of the force calcium relation from n_{s1} 5.51 ± 0.19 to 4.11 ± 0.25 (means ± SEM, n=5) in the presence of human cTn. cMLCK treatment slightly increased passive and active force of both human and rat cTn-exchanged trabeculae.

We determined the effect of cMLCK phosphorylation on cross-bridge kinetics by measuring the rate of force-redevelopment (k_{fr}) at different levels of activation by a rapid shortening-re-stretch maneuver. Both rat and human cTn-exchanged trabeculae exhibited activation-dependent changes in k_{fr} (Figs. 5D and E), ranging from ~5 s^{-1} at low levels of activation to ~15 s^{-1} at maximal activation. Data points were fitted to a quadratic equation to extract k_{fr} at half-maximal levels of force (k_{fr} 50\%_{max}). In both human and rat cTn-exchanged trabeculae cMLCK treatment increased k_{fr} 50\%_{max} by ~2 s^{-1} (Fig. 4F; Tab. S1), suggesting increased crossbridge kinetics at intermediate and high levels of activation.

After the experiments, trabeculae were dismounted and phosphorylation levels determined by Phos-tag™-SDS-PAGE and Western-blot (Fig. S4C), RLC was phosphorylated to 0.28 ± 0.08 mol P/mol RLC (mean ± SEM, n=5) and 0.36 ± 0.05 mol P/mol cRLC in rat and human cTn-exchanged trabeculae, respectively. cTnI was largely dephosphorylated in rat cTn exchanged trabeculae. Because mono-phosphorylated hcTnI and unphosphorylated rcTnI co-migrate in Phos-tag™-SDS-PAGE (see also Fig. 1B), we could not directly determine the level of troponin I phosphorylation, but it was estimated to be ~0.3 mol P/mol assuming a troponin-exchange efficiency of 70%.

**Discussion**

**Cardiac myosin light chain kinase is not a dedicated kinase**

Although myosin light chain kinases have long been considered ‘dedicated kinases’ that specifically phosphorylate the myosin heavy chain-associated RLCs (13), recent research has suggested that MLCKs can phosphorylate other targets, e.g. the skeletal isoform of MLCK has been shown to regulate skeletal muscle myogenesis via phosphorylation of the transcription factor MEF2c (20) and smooth muscle MLCK has been recently suggested to phosphorylate a multitude of proteins that, interestingly, overlap with PKA signaling targets in non-muscle cells (21). In the current study, we show that the human cardiac isoform of MLCK specifically mono-phosphorylates human cardiac troponin I in a Ca^{2+}-calmodulin-dependent manner, in agreement with its Ca^{2+}-dependent activity reported previously (10) (Fig. 1F). cMLCK
phosphorylated cTnI on serine 23 not only on the isolated cTnI subunit, but also when it was incorporated within the trimeric troponin complex or the myofilament lattice (Figs. 1, 2 and 3), suggesting that cMLCK has a high specificity towards hCTnI serine 23. The N-terminal extension (NTE) of hCTnI (amino acids 1-30) displays a sequence motif (Fig. 1A) similar to that found in cMLCK’s primary substrate RLC. Serine 23 in hCTnI is followed by an asparagine and an amphipathic amino acid residue (pSNY), which is similar to the conserved amino acid sequence found in the NTE of all RLC isoforms (pSNVF). Step-wise deletion of this conserved sequence in smooth muscle RLC decreased the maximal rate of phosphorylation, and it was suggested that these residues contribute to substrate recognition by binding to a pocket found next to the catalytic cleft of smooth muscle MLCK (22,23). Moreover, hCTnI’s NTE contains a series of positively charged amino acid residues upstream of the phosphorylatable serines 22/23, which are responsible for specific interactions with the catalytic core of protein kinases (24). Specifically, cTnI’s NTE has an arginine in the P-3 position, and the analogous residue in RLC has been proposed to be crucial for binding to two conserved glutamate residues found in the catalytic core of both smooth and skeletal cMLCK (25). Mutation of the P-3 arginine to alanine strongly reduced smooth and skeletal MLCK activity towards RLC (22). The sequence conservation with the catalytic core of cardiac MLCK therefore suggests a similar mechanism of substrate recognition of hCTnI’s NTE by cMLCK (11).

Similar to cMLCK, another member of the same family of calmodulin-dependent kinases (CAMK), protein kinase D (PKD), has been shown to mono-phosphorylate cTnI on either serine 22 or 23 in response to ß-adrenergic stimulation of cardiomyocytes (19). Moreover, PKD phosphorylates other sarcomere-associated proteins such as cMyBP-C and telethonin, suggesting that cMLCK might also have broader substrate specificity. In fact, cMLCK efficiently phosphorylates the isolated tail domain of human telethonin [AA104-161] on two sites in vitro (Fig. S5A). However, cMLCK did not phosphorylate N-terminal domains of cMyBP-C (C0C2) even at very high enzyme to substrate ratios (Fig. S5B), consistent with published results indicating that cMyBP-C is not a substrate for cMLCK (7).

Activity of kinases can also be controlled by tethering them to specific subcellular compartments, e.g. PKA activity is locally regulated within cells via interactions with so-called A-kinase anchoring proteins which bind PKA with high specificity and affinity (26). In contrast, cMLCK has mainly been found diffusely localized in the cytosol of cardiomyocytes with some striated patterns (14,27), suggesting that cMLCK can access both myofibril and non-myofibril compartments of cardiomyocytes. Interestingly, the striated patterns do not co-localize with myosin, its primary substrate, but with actin.

It remains unclear what the upstream regulators are for cMLCK’s localization and substrate-specificity. Perhaps cMLCK’s N-terminal extension, which includes putative phosphorylation sites for several physiologically and pathophysiologically relevant kinases (12), can regulate its localization, substrate specificity and function. Consistent with this idea, both adrenergic and prostanoid F receptor stimulation have been shown to increase RLC phosphorylation, probably by stimulation of cMLCK activity (11,28).

Functional implications of cTnI phosphorylation by cMLCK for cardiac myofilament function

The phosphorylatable N-terminal extensions of both cRLC and cTnI are intrinsically disordered regions, which facilitates substrate recognition by protein kinases and allows control of downstream regulatory mechanisms via dynamic protein-protein interactions. Consistent with this idea, the NTE of both cTnI and cRLC have been shown to regulate myofilament function in a phosphorylation dependent manner (7,29). Although both serines 22 and 23 in cTnI-NTE are subject to phosphorylation by a series of protein kinases (e.g. PKA, PKD, PKC) and bis-phosphorylation has been proposed to be necessary for the downstream functional effects (30), a recent study has shown that mono-phosphorylation on either S22 or S23 is sufficient to reduce myofilament calcium sensitivity to the same extent as bis-phosphorylation (19). This suggests that mono-phosphorylation of cTnI on serine 23 by cMLCK is functionally significant for the myocardium, and that different phosphorylation sites and combinations thereof might have different functional effects (31). Moreover, cTnI phosphorylation by PKA is hierarchical with serine 23 being phosphorylated...
before serine 22 (32,33), further suggesting that cMLCK phosphorylation of serine 23 might prime serine 22 for phosphorylation by PKA and other kinases. Phosphorylation of hcTnI’s NTE by cMLCK therefore represents a novel signaling mechanism in the regulation of cardiac contractile function under both physiological and potentially pathophysiological conditions. In the presence of human cardiac troponin, cMLCK incubation of ventricular trabeculae increased cross-bridge kinetics via phosphorylation of RLC. However, the associated increase in calcium sensitivity observed in native rat cardiac fibres is largely abolished (7), suggesting that phosphorylation of human cTnI in the same preparations mitigated the increase in calcium sensitivity associated with phosphorylation of RLC (Fig. 5). Moreover, cMLCK incubation reduced the cooperativity of myofilament calcium activation in the presence of human cTnI as indexed by a decrease in the Hill coefficient nH, further supporting the hypothesis that cTnI phosphorylation by cMLCK modulates the functional effects of cRLC phosphorylation. The present results therefore suggest that the Ca2+-dependent activation of cMLCK in the human heart has two functional consequences: 1. activation of the thick filament structure via phosphorylation of cRLC associated with increased calcium sensitivity, cross-bridge kinetics and isometric force production (6,7), and 2.) decreased calcium sensitivity of the thin filaments associated with faster rate of relaxation via phosphorylation of cTnI (29). The net result is an increased force production, and increased rate of both myofilament activation and relaxation, without significantly altering the calcium sensitivity of the myocardium. This suggests that cMLCK can coordinate both thick and thin filament activation states in the human heart, and modulate both its inotropy and lusitropy in response to changes in the intracellular calcium transient. Simultaneous phosphorylation of both cRLC and cTnI by cMLCK might contribute to the positive force-frequency relation and the slow force response to stretch of the healthy myocardium (34-36), which increases maximal force, and the rates of both myofilament activation and relaxation. However, other signaling pathways likely will also feed into these regulatory mechanisms, which might act synergistically or compensatory to cMLCK signaling. The functional effects of cMLCK activation in the human heart likely depend on the phosphorylation background of other sarcomeric regulatory proteins. During low β-adrenergic stimulation, cMLCK can phosphorylate both cRLC and cTnI. However, at high levels of β-adrenergic stimulation, cTnI-NTE will be phosphorylated by PKA, and cMLCK activation will lead to phosphorylation of primarily cRLC, which will increase cross-bridge kinetics, calcium sensitivity and the rate of myofilament activation. Therefore depending on the phosphorylation background, β-adrenergic stimulation and cMLCK activation might have antagonistic effects on myofilament function. Phosphorylation of cRLC by cMLCK has been the focus of considerable attention in clinical research over the last few years, because decreased levels of RLC phosphorylation have been associated with heart disease and heart failure (37). In contrast, increased levels prevent the onset of heart diseases in animal models (18), suggesting that modulation of RLC phosphorylation via increasing cMLCK activity is a potential target for the development of novel heart failure therapeutics. The current results suggest that cMLCK’s physiological function is likely to be more complex, however, and this should be taken into account in that development. Cardiac troponin I dephosphorylation due to β-adrenergic receptor desensitization during heart failure development contributes to cardiac muscle dysfunction via impaired length-dependent activation, the cellular analogue of the Frank-Starling law of the heart (38). Increasing cTnI phosphorylation in the failing heart by modulating cMLCK activity is therefore a potential new target for the development of new heart failure interventions.

Species-specific modulation of heart muscle function by cMLCK

Our results suggest that there are likely fundamental differences between the physiological functions of cMLCK in rodent and human heart muscle. Although human and rodent cTnI have ~92% sequence identity, human cMLCK specifically phosphorylates only the human but not the rodent protein isoform in situ (Fig. 3), with only minor phosphorylation of rat cTnI after prolonged incubation in vitro (Fig. 1C). The catalytic domains of human and rat cMLCK exhibit very high sequence identity and similarity (>98%), but the current results cannot exclude the possibility that rat cMLCK can phosphorylate rat cTnI.
The P+1 position following serine 23 is an alanine in rodent, but an asparagine in human cTnI, and it was previously shown that deletion of the analogous asparagine in RLC greatly reduces MLCK’s activity towards RLC (22). The comparison suggests that the additional alanine interferes with substrate recognition of rat cTnI by cMLCK, likely by increasing the distance between serine 23 and the posterior residues that anchor cTnI-NTE to a specific pocket close to the catalytic core. These results demonstrate that small protein sequence variations between species can have significant effects on myofilament regulation and their associated functional consequences. In agreement with this hypothesis, pharmacological inhibition of PKA and PKC did not affect the contractile performance of isolated human donor and failing heart muscle, although similar interventions had a significant effect on isolated rabbit myocardium (39,40), suggesting species-specific effects of kinase signaling. Moreover, sequence variations in the N-terminus of human and mouse cMyBP-C, with the latter exhibiting eight additional amino acids, have been shown to significantly modulate its interaction with myosin (41).

The concept of species-specific myofilament regulation has more fundamental implications for the study of heart failure, its aetiology and the development of pharmacological interventions using animal models. Loss-of-function mutations in cMLCK have been associated with dilated cardiomyopathy in humans (15,16) whereas cMLCK ablation leads to a hypertrophic phenotype in mouse models (3), consistent with the view that the molecular functions of cMLCK differ between rodent and human.

Experimental procedures

Protein production

The catalytic fragment of human cardiac myosin light chain kinase was prepared as described previously (10) based on cDNA AJ247087. Human cardiac troponin complex was kindly provided by Mitla Garcia-Maya (King’s College London). Rat cardiac troponin C, I and T were cloned from a rat heart cDNA library (BioChain Inc.) into a pET3a vector, codon-optimized for bacterial expression, and separately expressed in BL21(DE3)-RIPL cells (Agilent Technologies) according to manufacturer’s instructions. Rat cardiac TnC (rcTnC) was purified as specified before (42). Rat cardiac troponin I (rcTnI) was expressed at 37°C in inclusion bodies and purified on a CM Sepharose ion-exchange column using 6 mol/L urea (buffer composition in mmol/L: 50 Tris-HCl pH 8.0, 1 EDTA, 5 DTT) and a linear gradient of 0-0.5 mol/L NaCl. Rat cardiac troponin T (rcTnT) was expressed in the soluble fraction and purified using a similar method. The troponin components were mixed in a 1:1:1 ratio in the presence of 6 mol/L urea, and the urea concentration was reduced by serial dilution against a buffer containing 50 mmol/L Tris-HCl pH 8, 300 mmol/L NaCl, 5 mmol/L MgCl₂, 1 mmol/L CaCl₂ and 1 mmol/L DTT. The salt concentration was reduced to 200 mmol/L followed by 100 mmol/L in the last two steps of dialysis. The complex was purified by ion-exchange chromatography on a Resource-Q column with a two-step linear gradient of 0-200 mmol/L NaCl over 5 column volumes and 200-450 mmol/L NaCl over 30 column volumes. The final concentrated product was characterized by HPLC, electron spray ionization mass spectrometry (ESI-MS) and size exclusion-multi angle light scattering (SEC-MALS) (Fig. S1). All measurements were consistent with a 1:1:1 complex of rat cardiac troponin T, I and C. Troponin was dialyzed into a K₂EGTA buffer (composition in mmol/L: 25 Imidazole, 78.4 potassium propionate (KPr), 6.8 MgCl₂, 10 K₂EGTA, 1 DTT, pH 7.1) and aliquots stored at -80°C. Na₂Creatine phosphate (Na₂CrP) and Na₂ATP were supplemented before troponin reconstitution in trabeculae (mmol/L: 15 and 5.65, respectively).

High performance liquid chromatography, electron spray ionization-mass spectrometry and size exclusion-multi angle light scattering

Analytical scale HPLC was performed with an Agilent 1200 Series system on octadecyl carbon chain (C18)-bonded silica columns (5 µm pore size, 4.6 mm x 250 mm, #218TP54, Vydac). The solvent system was HPLC-grade H₂O (#W/0106/PB17, Fischer Scientific) and HPLC-grade acetonitrile (ACN) (#A/0627/PB17, Fischer Scientific); buffer A: HPLC-grade H₂O containing 0.1% (v/v) HPLC-grade trifluoroacetic acid (TFA) (#T/3258/04, Fischer Scientific); buffer B: 80% (v/v) ACN in HPLC-grade H₂O containing 0.1% (v/v) TFA.

Mass spectrometric analysis was performed on the Agilent Series 1100 LC-MS system in positive mode of electrospray ionization (ESI). The solvent system was: buffer A: LC-MS grade H₂O (#W/0112/17, Fischer Scientific) containing 0.1% (v/v) formic acid (FA) (#06440, Fluka); buffer B: 80% (v/v) ACN in MS-LC-grade H₂O.
containing 0.1% (v/v) FA. Data analysis was performed with LC/MSD ChemStation software. Size exclusion-multi angle light scattering (SEC-MALS) was performed on Superdex 200 5/150 GL column (GE Healthcare) in SEC-MALS buffer (50 mmol/L Tris-HCl pH 7.4, 5 mM MgCl₂, 1 mmol/L CaCl₂, 100 mM NaCl and 100 mmol/L TCEP). Recombinant troponins were extensively dialyzed against SEC-MALS buffer before experiments. Light scattering (LS) and refractive index (RI) were measured on a Mini DAWN and OPTILAB DSP (Wyatt Technology, UK), respectively. Data were analyzed with custom written software.

**Preparation of cardiac myofibrils and ventricular trabeculae**
All animals were treated in accordance with the guidelines approved by the UK Animal Scientific procedures Act (1986) and European Union Directive 2010/63/EU. Wistar rats (male, 200-250 g) were sacrificed by cervical dislocation without the use of anesthetics (Schedule 1 procedure in accordance with UK Animal Scientific Procedure Act, 1986) and demembranated right ventricular trabeculae were prepared as described previously (42). Some trabeculae were prepared in the presence of 10 µmol/L H-89 and 1 µmol/L Staurosporine, and dissolved in SDS-PAGE loading buffer for analysis of protein phosphorylation levels. Human samples were obtained after informed consent and with approval of the Institutional Review Board of the University of Kentucky (08-0338-F2L; approval date 18th January 2017) and King’s College London Research Ethics Panel (LRS-16/17-4698; approval date 15th June 2017). This investigation conformed with the principles in the Declaration of Helsinki (1997). Marmoset (Callithrix jacchus) ventricular tissue was kindly provided by the Biological Service Unit of King’s College London. Cardiac myofibrils were prepared as described previously (43). For dephosphorylation experiments, cardiac myofibrils were washed three times in λ-protein phosphatase (λ PP) assay buffer (composition in mmol/L: 50 HEPES, 100 NaCl, 2 DTT, 1 MnCl₂, 0.01% (v/v) Brij-35, pH 7.5), λPP was added to a final concentration of 4000 U/mL and the mixture incubated at 30°C for 1h. After incubation, cardiac myofibrils (CMFs) were washed three times with relaxing buffer (composition in mmol/L: 20 MOPS pH 7, 35 NaCl, 5 MgCl₂, 1 EGTA, 1 CaCl₂, 1 DTT, pCa 9) without ATP to remove Mn²⁺ and λPP.

**In vitro and in situ kinase assays**
Cardiac troponins were gel-fermented into cMLCK assay buffer (in mmol/L: 50 HEPES, 50 NaCl, 2 MgCl₂, 1 CaCl₂, 1 DTT) via NAP5 columns (GE Healthcare). Concentrations determined by UV absorbance at 280 nm using calculated extinction coefficients and reactions started by addition of calmodulin and cMLCK. Reactions were stopped by addition of SDS-PAGE loading buffer, samples denatured at 100°C for 2 min and phospho-species separated by Phos-tag™-SDS-PAGE (containing 50 µmol/L Phos-tag-reagent and 100 µmol/L MnCl₂). Gels were subsequently stained with Coomassie and band intensities quantified using ImageJ. CMFs (2-4 mg/ml) were in situ phosphorylated by incubation in cardiac activating solution (in mmol/L: 25 imidazole, 15 Na₂Cr₂, 58.7 KPr, 5.65 Na₂ATP, 6.3 MgCl₂, 10 CaCl₂, 10 K₂EGTA, 1 DTT, pH 7.1) containing 25 µmol/L Blebbistatin and 1.2 µmol/L cMLCK/CaM (0.5 nmol cMLCK/CaM per mg CMF protein) for 1 hour at 30°C. After incubation, CMFs were briefly washed in 10 mmol/L Tris-HCl pH 7 to remove EGTA, and CMFs dissolved in SDS-PAGE loading buffer and samples run on Phostag™-SDS-PAGE as described above. The assays were repeated at least two times to make sure that they are reproducible and at least three times for quantitation.

**SDS-PAGE and Western-blotting**
After Phos-tag™-SDS-PAGE, gels were washed with transfer buffer (composition in mmol/L: 25 Tris-HCl pH 8.3, 192 glycine, 0.08% (v/v) SDS, 15% (v/v) methanol) containing 5 mmol/L EDTA for 10 min, followed by three washes in buffer without EDTA. Proteins were blotted onto nitrocellulose or PVDF membranes for 1-2h at 1 mA/cm² in transfer buffer using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BIORAD). Post-blotting, membranes were blocked with Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBS-T), and either 5% (w/v) semi skimmed milk powder or 5% (w/v) bovine serum albumin (BSA). Membranes were incubated with the following primary antibodies (source, working concentration) overnight in TBS-T containing either 5% (w/v) semi skimmed milk powder or 5% (w/v) BSA: rabbit monoclonal anti-myosin light chain 2 (ABCAM; 1:10000),
mouse monoclonal anti-cardiac troponin I (MF4, GeneTex, 1:10000), rabbit polyclonal anti-cardiac troponin I (ABCAM, 1:1000), rabbit polyclonal anti-cardiac troponin I (phospho S22 + S23) (Cell Signalling, 1:1000), rabbit polyclonal anti-cardiac troponin T (ABCAM, 1:1000), rabbit monoclonal anti-pS282 cMyBP-C (ENZO, 1:1000). Membranes were washed three times in TBS-T and incubated for 1 h at room temperature with secondary antibody (1:2000 dilution, HRP-conjugated donkey anti-rabbit IgG, GE Healthcare, NA934V; or HRP-conjugated goat anti-mouse IgG, Santa Cruz Biotechnology) in TBS-T containing either 1% (w/v) semi-skimmed milk powder or 1% (w/v) BSA. Blots were washed in TBS-T, immersed in ECL Plus reagent (GE HEALTHCARE) and bands visualized on a BIO-RAD Gel-Doc Imager. SDS-PAGE gels were stained with Pro-Q Diamond and SYPRO total protein stain according to manufacturer’s instructions (Life Technologies, Inc.) and imaged on a BIO-RAD Gel-Doc Imager using the appropriate excitation and emission filter settings.

Troponin exchange, lambda protein phosphatase treatment and experimental protocols for mechanical measurements in trabeculae

Endogenous troponin was exchanged by incubation of trabeculae in relaxing solution (composition in mmol/L: 25 imidazole, 15 Na$_2$CrP, 78.4 KPr, 5.65 Na$_2$ATP, 6.8 MgCl$_2$, 10 K$_2$EGTA, 1 DTT, pH 7.1) containing 1.5 mg/ml troponin complex and 30 mmol/L BDM overnight at 4°C. Trabeculae were mounted between a force transducer (KRONEX, model A-801, resonance frequency ~2 kHz) and length controller (Aurora Scientific, Model 312C). For dephosphorylation experiments, trabeculae were washed three times in MgCl$_2$-rigor buffer (in mmol/L: 25 Imidazole, 152.6 KPr, 1.5 MgCl$_2$, 10 EGTA, 1 DTT) followed by three washes in Mn$^{2+}$-rigor buffer (in mmol/L: 25 Imidazole, 152.6 KPr, 1.5 MgCl$_2$, 1 MnCl$_2$, 1 DTT) and incubated in Mn$^{2+}$-rigor buffer containing 3000 U/mL λPP for 1 h at room temperature. Subsequently, trabeculae were washed three times for 10 min in relaxing buffer and sarcomere length adjusted to ~2.1 µm by laser diffraction. Trabeculae were phosphorylated by incubation in activating solution (in mmol/L: 25 imidazole, 15 Na$_2$CrP, 58.7 KPr, 5.65 Na$_2$ATP, 6.3 MgCl$_2$, 10 CaCl$_2$, 10 K$_2$EGTA, 1 DTT, pH 7.1) containing 30 mmol/L BDM, and 1 µmol/L Calmodulin and 1 µmol/L cMLCK for 1h at room temperature. Subsequently, trabeculae were washed three times for 10 min in relaxing buffer and sarcomere length adjusted to ~2.1 µm by laser diffraction. Each activation was preceded by a 2-min incubation in pre-activating solution (composition in mmol/L: 25 imidazole, 15 Na$_2$CrP, 108.2 KPr, 5.65 Na$_2$ATP, 6.3 MgCl$_2$, 0.2 K$_2$EGTA, 1 DTT, pH 7.1). Solutions with varying concentrations of free [Ca$^{2+}$] were prepared by mixing relaxing and activating solutions using MAXCHELATOR software (maxchelator.stanford.edu). The dependence of force on free calcium concentration was fitted to data from individual trabeculae using non-linear least-squares regression to the modified Hill equation:

$$ F = \frac{1}{1 + 10^{n_H(pCa-pCa_{50})}} $$

where pCa = -log$_{10}$([Ca$^{2+}$]), pCa$_{50}$ is the negative logarithm of [Ca$^{2+}$] corresponding to half-maximal change in F and $n_H$ is the Hill coefficient. Trabeculae which showed a decline in maximal calcium activated force of more than 15% after the pCa titrations were discarded.

The rate of force re-development was measured by a fast release and re-stretch protocol (44). Briefly, isometrically contracting trabeculae were released by 20% of their initial length (~500 µs step response), held at the new length for ~30 ms, and re-stretched to the original length. The time-course of force redevelopment was fitted to a single exponential, yielding the rate constant k$_{tr}$. 

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions
TK and IS designed research; TK and IS performed research; IS, SP, MG, BB, KSC and YBS contributed new reagents/analytic tools; TK and IS analyzed data; and TK, IS and MI wrote the paper.
References


FOOTNOTES
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The abbreviations used are: cMLCK, cardiac myosin light chain kinase; CaM, calmodulin; CMF, cardiac myofibrils; cMyBP-C, cardiac isoform of myosin binding protein-C; cRLC, cardiac isoform of myosin regulatory light chain; cTnC cardiac isoform of troponin C; cTnI, cardiac isoform of troponin I; cTnT, cardiac isoform of troponin T; λPP, lambda-protein phosphatase; NTE, N-terminal extension, PKA, protein kinase A; PKC, protein kinase C; PKD, protein kinase D; S-A, serine to alanine mutation.
Figures and Figure Legends

**Figure 1.** *In vitro* phosphorylation of human cardiac troponin I on serine 23 by cMLCK. (A) Protein primary sequence alignment of the N-terminal extension of rat and human cTnI with the sequence surrounding the phosphorylated serines in rat and human cRLC. Phosphorylatable serines are highlighted in red. (B) *In vitro* kinase assays of human (left) and rat cardiac troponin (right) with cMLCK analyzed by Phos-tag™-SDS-PAGE. Troponin T, I and C are labelled accordingly. (C) Densitometric analysis of human and rat cTnI phosphorylation levels after >60 min incubation of troponin complexes with cMLCK. (D) Kinase assays at different human cTn to cMLCK stoichiometries analyzed by Phos-tag-SDS-PAGE. Please note that cMLCK phosphorylated cTnI to ~1 mol P/mol cTnI under all conditions tested. (E) ESI mass spectrometry analysis of cTnI before (top) and after incubation of cTn complex with cMLCK (bottom, 1:5 enzyme to substrate ratio) from (D). The increase in mass by ~80 Da indicates mono-phosphorylation. (F) In vitro kinase assay of isolated hcTnI with cMLCK followed by (G) Western-blot using a pS22/pS23 antibody. (H) Alanine substitution of either serine 22 or 23 demonstrates that cMLCK specifically phosphorylates serine 23 in the hcTnI N-terminal extension. Means ± SEM (n=3). Statistical significance of difference was assessed with an unpaired, two-tailed Student’s t-test: ***p<0.001.
Figure 2. *In situ* phosphorylation of cTnI and cRLC by cMLCK in native cardiac myofibrils. (A) Pro-Q diamond and total protein staining (left) of λ-protein phosphatase-treated rat cardiac myofibrils before (-) and after cMLCK incubation (+). The black and grey arrowheads indicate phosphorylated cMLCK and cRLC, respectively. Cardiac cTnI and cRLC phosphorylation levels were further confirmed by Phos-tag\textsuperscript{TM}-SDS-PAGE and Western-blot (right). Degradation products of cTnI are labelled accordingly (deg). (B) Pro-Q diamond and total protein staining (left) of λ-protein phosphatase-treated marmoset CMFs before (-) and after cMLCK incubation (+). The black, white and grey arrowheads indicate phosphorylated cMLCK, cTnI and cRLC, respectively. Cardiac cTnI and cRLC phosphorylation levels in the same samples were further confirmed by Phos-tag\textsuperscript{TM}-SDS-PAGE and Western-blot (right). (C) and (D) Same assays as in (B), but using cardiac myofibrils isolated from human ventricular tissue treated with either cardiac (C) or smooth muscle MLCK (D).
Figure 3. In situ phosphorylation of cTnI and cRLC by cMLCK in troponin-exchanged rat ventricular trabeculae. (A) Recombinant cardiac troponin-exchange efficiency in ventricular trabeculae determined by Western-blot against cTnT. Endogenous native rat cTnT (left) migrates faster in SDS-PAGE than recombinant human cTnT (right), allowing estimation of the cTn-exchange efficiency into rat ventricular trabeculae (middle). Mean ± SEM, n=3. (B) Low molecular weight portion of SDS-PAGE of ventricular trabeculae treated without (-) and with (+) protein kinase inhibitors (H-89 and Staurosporine). Gels were stained with phospho-specific Pro-Q Diamond or SYPRO total protein stain. Densitometric analysis for cTnI is shown on the right. Means ± SEM, n=6. (C) Rat cTn- or (D) human cTn-exchanged demembranated ventricular trabeculae were incubated without (-cMLCK, time-matched control) or with cMLCK (+cMLCK) and phosphorylation levels determined by Phos-tag\textsuperscript{TM} SDS-PAGE followed by Western-blot against cTnI (top) and cRLC (bottom). Please note that unphosphorylated human cTnI (indicated by 0P') migrates faster than unphosphorylated rat cTnI (0P). TnI degradation products are labelled accordingly (Deg). Ponceau stains for endogenous rat (rcTnT) and exogenous human cardiac troponin T (hcTnT) are shown in the middle panels. (E) Densitometric analysis of cTnI (top) and cRLC phosphorylation (bottom) levels before (-) and after cMLCK (+) incubation shown in (C) and (D). Means ± SEM, n=3-6. Statistical significance of differences was assessed with an unpaired, two-tailed student’s t-test: ns-not significant, *p<0.05, ***p<0.001.
Figure 4. Effect of $\lambda$-protein phosphatase ($\lambda$PP) treatment on cardiac muscle mechanics. (A) Force-pCa relation of demembranated rat ventricular trabeculae before (closed circles) and after $\lambda$PP-treatment (open circles). (B) Representative recordings of force re-development after release-restretch protocol before (grey) and after $\lambda$PP-treatment (black) at ~50% maximal activation. (C) Force-$k_r$ relation of demembranated rat ventricular trabeculae before (closed circles) and after $\lambda$PP-treatment (open circles). (D) Top: cTnI phosphorylation levels of control and experimental trabeculae that underwent $\lambda$PP-treatment analyzed by Phos-tag$^{\text{TM}}$-SDS-PAGE and Western-Blot against cTnI. Deg indicates cTnI degradation products. Bottom: Ponceau stain showing the actin loading control for individual trabeculae. Means ± SEM, n=3-4.
Figure 5.  Effect of cMLCK incubation on mechanical properties of rat and human cTn-exchanged rat ventricular trabeculae. Normalized force-pCa relations of λPP-treated (A) rat cTn- and (B) human cTn-exchanged trabeculae before (closed circles) and after cMLCK incubation (open circles). (C) Bar graph indicating the increase in calcium sensitivity (ΔpCa_{50}) upon cMLCK-treatment in the two groups. Force-k_{tr} relation of (D) rat cTn- and (E) human cTn-exchanged trabeculae before (closed circles) and after cMLCK incubation (open circles). (F) Bar graph indicating the increase in the rate of force re-development at 50% maximal activation (Δk_{tr} 50%_{max}) upon cMLCK-treatment in the two groups. Means ± SEM, n=4-5. Statistical significance of differences between the two groups was assessed with an unpaired, two-tailed Student’s t-test: ns-not significant, ***P<0.001.