1. Introduction

Contraction of both heart and skeletal muscle is driven by cyclic interactions between myosin and actin [1,2]. In each cycle, the light-chain domain (LCD) of the myosin head amplifies small conformational changes induced by the release of ATP hydrolysis products from its catalytic domain to produce large rotational motions of the LCD. These motions change the orientation of the cRLC C-lobe in rigor conditions, showing that the orientation of this part of the myosin head is determined by its interaction with the thick filament even when the head is strongly bound to actin. These results suggest that cRLC phosphorylation controls the contractility of the heart by modulating the interaction of the cRLC region of the myosin heads with the thick filament backbone. A role for cRLC phosphorylation in the regulation of cardiac contractility [6-9]. Reported values of basal cRLC phosphorylation differ substantially however [10-12], and their interpretation is complicated by heterogeneity between different parts of the myocardium [7,13,14]. At a functional level, a significant role for cRLC phosphorylation is indicated by the effects of hypertrophic cardiomyopathy (HCM) mutations that abolish cRLC phosphorylation [15], and the severe cardiac dysfunction in transgenic mouse models expressing non-phosphorylatable cRLCs [12,16]. cRLC is phosphorylated by a cardiac-specific isoform of myosin light chain kinase (cMLCK) [17,18], and ablation of cMLCK expression is associated with a decrease in cRLC phosphorylation level in transgenic mice under basal conditions, leading to an impaired beta-adrenergic response and ventricular hypertrophy [19]. cMLCK activity has also been associated with control of sarcomere organisation in isolated mammalian cardiomyocytes [20] and with cardiac development in a zebrafish model [17]. However, the molecular mechanisms of these effects remain elusive.

The effects of cRLC phosphorylation on the mechanical properties of the myocardium have been extensively studied. Increased cRLC phosphorylation has been associated with an increase in calcium sensitivity [21], and in the rate of stretch activation [22] and cross-bridge cycling [23] in demembranated cardiac muscle preparations. The changes in
the equatorial X-ray reflections from cardiac trabeculae produced by in situ cRLC phosphorylation indicate a transfer of myosin heads towards the thin filaments [23], suggesting that these mechanical effects may be mediated by an increased probability of actin attachment (for review see [11]). Electron microscopy studies of isolated thick filaments from mammalian and invertebrate skeletal muscles [24,25] showed that the helically ordered organisation of myosin heads on the thick filament surface in the unphosphorylated state is lost on incubation with active MLCK. However, the molecular mechanism by which cRLC phosphorylation controls the conformation of the myosin heads is unknown.

We recently described a new method to determine the orientation of the cRLC region of the myosin heads with respect to the filament axis in demembranated ventricular trabeculae, based on measuring polarized fluorescence intensities from bifunctional sulforhodamine (BSR) probes on the cRLC [26]. We showed that, in the unphosphorylated state, cRLC orientation did not change significantly during calcium activation, and that its N-lobe did not change orientation even during strong attachment of myosin heads to actin in rigor. These results suggested that the cRLC interacts with the thick filament backbone via its N-lobe. Since the N-lobe contains the phosphorylation site, modulation of this interaction by cRLC phosphorylation might provide a mechanism for the control of myosin head conformation. Here we tested that hypothesis using a recombinant C-terminal fragment of the human cardiac isoform of MLCK to thio-phosphorylate BSR-labelled human cRLCs in vitro. The BSR-cRLCs were exchanged into demembranated right ventricular trabeculae with a very low endogenous cRLC phosphorylation background, and the orientation of the phosphorylated cRLC C-lobe was determined during relaxation, active isometric contraction and rigor. An important advantage of this in vitro labelling/cRLC exchange approach is that the orientation of the phosphorylated cRLCs can be determined directly in the presence of a mixed phosphorylated/unphosphorylated population, since only the phosphorylated cRLCs carry the probe. Comparison with our previous results for unphosphorylated BSR-cRLCs using the same cRLC exchange protocol [26] then allowed us to determine the change in the in situ orientation of the C-lobe produced by cRLC phosphorylation in each state.

2. Materials and methods

Details of protein production, preparation of cardiac trabeculae, protein exchange protocols, fluorescence polarization experiments and tissue sampling procedures are provided in the supplemental materials.

3. Results

3.1. Preparative thiophosphorylation of BSR-cRLCs by cMLCK

A fragment of the human cardiac isoform of myosin light chain kinase (cMLCK, UniProtKB entry: Q32MK0) spanning the catalytic and regulatory domains was expressed in and purified from S9 cells to over 95% homogeneity as described in the Materials and methods section. The purified cMLCK fragment was partially phosphorylated (34% unphosphorylated, 55% mono- and 11% bis-phosphorylated as determined by Phostag™-SDS-PAGE; data not shown) and the phosphate groups were removed by Lambda Protein phosphatase treatment. There was no significant difference between the catalytic activities of the untreated and dephosphorylated cMLCK fragments.

Previous studies have led to contradictory conclusions about the calcium- and calmodulin-dependence of cMLCK activity [17,18]. In the present work, in vitro kinase assays at different enzyme-to-substrate ratios in the presence and absence of Ca2+, calmodulin and EGTA revealed a strong dependence of cMLCK activity on Ca2+ and calmodulin (Fig. 2A), as expected from the canonical C-terminal calmodulin binding site of cMLCK. However, some calcium-independent catalytic activity was observed at high enzyme-to-substrate ratios.

Multiple phospho-species of cRLC have been identified in rodent cardiac muscle [27], and human cRLC contains several residues that could serve as potential substrates for protein kinases. Two series in the N-terminal extension (S15 and S19) of cRLC and a tyrosine (Y118) in its C-lobe have been identified by mass spectrometry and site-specific methods as potentially phosphorylated in vivo (PhosphoSitePlus®, www.phosphosite.org) [28]. To unambiguously identify which residue is phosphorylated by the purified cMLCK (and to exclude the possible contribution of any co-purified kinases) we mutated the two serine residues (S15 and S19) in the N-terminal extension separately or together to alanines. The wildtype and mutant cRLCs were tested in in vitro kinase assays (Fig. 2). Only the wildtype and S19A mutant could be phosphorylated, indicating that the purified cMLCK specifically phosphorylates serine 15 in the human cRLC N-terminal extension (Fig. 2A). Specific mono-phosphorylation of the recombinant wildtype cRLC was confirmed by ESI mass spectrometry. The measured (calculated) masses (in Da) for the cRLC before and after phosphorylation by cMLCK were 19020.6 (19020.5) and 19101.6 (19100.5), respectively. Additionally, although rat cRLC has an additional phosphorylatable serine at position 14 that is replaced by asparagine in the human cRLC sequence, we found that the recombinant cMLCK also mono-phosphorylates rat cRLC in vitro (Fig. S1), indicating that cMLCK has a high specificity for serine 15.

ATP·γS was used to preparatively thiophosphorylate BSR-labelled cRLCs, on the basis that thiophosphorylated proteins are expected to be relatively resistant to dephosphorylation by any protein phosphatases that might be present in freshly skinned trabeculae. In this study,
only thiophosphorylated cRLCs labelled on the C-terminal lobe were used (Fig. 1), because labelling of helices in the N-terminal lobe (particularly the A- and D-helices) interfered with phosphorylation of the cRLCs by cMLCK (Fig. S2). Surprisingly, BSR-crosslinking of cysteine residues introduced at positions 6 and 10, relatively close to the phosphorylatable serine residue 15, did not significantly interfere with the phosphorylation reaction (Fig. 2C). All cRLCs with probes attached in the C-terminal lobe (BSR-cRLCs E, F, G and FG) were preparatively mono-thiophosphorylated within 2 h to 4 h to over 95% homogeneity (Fig. 2C).

3.2. Native cRLC phosphorylation level

The distribution of phosphorylated cRLC species in rat trabeculae was measured by Phostag™-SDS-PAGE [29] followed by Western Blot against cRLC (Fig. 3). The endogenous cRLCs in demembranated trabeculae were almost entirely dephosphorylated. Neither the skinning procedure nor treatment of the tissue with 2,3-butanedione monoxime (BDM) had a significant effect on the cRLC phosphorylation level (Figs. 3A and 3B), suggesting that the low cRLC phosphorylation level might be endogenous to rat trabeculae under basal conditions. Tissue samples removed and snap-frozen immediately (within two minutes) after sacrificing the animal also showed a homogenously low cRLC phosphorylation level in the right and left ventricles (Fig. 3C), further supporting this conclusion. Incubation of skinned trabeculae with 1 μM cMLCK led to almost full phosphorylation of the endogenous cRLC within 2 h (Fig. 3D). The recombinant cMLCK only mono-phosphorylated the endogenous cRLCs in situ, consistent with the in vitro phosphorylation data described above (Fig. S1).

A similarly low endogenous cRLC phosphorylation level was observed for mouse ventricular tissue (Fig. S3). Freshly prepared whole ventricular samples and the enriched myofibrillar fraction showed a homogenously low cRLC phosphorylation level, indicating...
that sample preparation did not affect the cRLC phosphorylation level measurements.

3.3. Exchange of thio phosphorylated BSR-cRLCs into skinned trabeculae

Thiophosphorylated BSR-cRLCs (subsequently referred to as ‘phosphorylated’) were exchanged into skinned trabeculae from rat right ventricle by bathing the trabeculae in EDTA rigor solution containing 0.5 mg/ml of the phosphorylated BSR-cRLC for 30 min at 22 °C. Subsequently, the trabeculae were bathed in relaxing solution containing 0.5 mg/ml CtnC and Ctn for 20 min and 1 h, respectively to replace any of these probes lost during cRLC exchange. The extent of exchange was estimated by SDS-PAGE followed by either Coomassie staining or by Western Blot against cRLC (Fig. S4), which allowed separation of the endogenous and phosphorylated BSR-labelled cRLCs. Densitometric analysis of the Coomassie-stained gels showed that about 30% of the endogenous cRLCs were replaced by phosphorylated BSR-cRLC-G. In contrast, SDS-PAGE followed by Western Blot against cRLC indicated that 18% ± 2% of the endogenous cRLCs were replaced. Similar exchange ratios were obtained for the other phosphorylated BSR-cRLCs (12% ± 6%, 20% ± 5% and 14% ± 8% for BSR-cRLC-E, -F and -FG, respectively, mean ± SD; n = 3), in approximate agreement with the exchange fraction of 12% for the unphosphorylated BSR-cRLCs estimated by fluorescence intensity [26]. Incorporation of phosphorylated BSR-cRLCs had no effect on the protein stoichiometry of skinned ventricular trabeculae (Fig. S5), but the phosphorylation levels of Ctn and cMyBP-C decreased. In the case of Ctn this effect may be due to replacement of endogenous phosphorylated tropinin lost during the cRLC exchange protocol with recombinant unphosphorylated tropinin complex (see Materials and Methods). The origin of the lower cMyBP-C phosphorylation observed following the cRLC exchange protocol is unknown.

Replacement of 10%–30% of the endogenous unphosphorylated cRLC with phosphorylated BSR-cRLC had no significant effect on maximal force at pCa 4.5 and 2.1 μm sarcomere length; force recovery after cRLC exchange of un- and phosphorylated BSR-cRLCs was 88% ± 11% (mean ± SD; n = 25) and 88% ± 9% (mean ± SD; n = 23), respectively.

3.4. Polarized fluorescence from phosphorylated BSR-cRLCs

The polarized fluorescence intensities obtained from the BSR probes on the N-terminus, on the E, F, G helices and cMyBP-C decreased. In the case of Ctn this effect may be due to replacement of endogenous phosphorylated tropinin lost during the cRLC exchange protocol with recombinant unphosphorylated tropinin complex (see Materials and Methods). The origin of the lower cMyBP-C phosphorylation observed following the cRLC exchange protocol is unknown.

Replacement of 10%–30% of the endogenous unphosphorylated cRLC with phosphorylated BSR-cRLC had no significant effect on maximal force at pCa 4.5 and 2.1 μm sarcomere length; force recovery after cRLC exchange of un- and phosphorylated BSR-cRLCs was 88% ± 11% (mean ± SD; n = 25) and 88% ± 9% (mean ± SD; n = 23), respectively.

The orientation of the cRLC-C-lobe was described in terms of its local EG-helix frame (see Materials and Methods section), with β describing the angle between the E-helix and the filament axis, and γ describing rotation of the C-lobe around the E-helix. The orientation distribution of the unphosphorylated cRLC-C-lobe in relaxation and active contraction (Fig. 4A) can be described in terms of four populations, referred to as C1 to C4 in order of increasing β [26]. There is very little change in the phosphorylated cRLC-C-lobe orientation distribution on calcium activation, but the C1 and C3 peaks become much weaker in rigor. An analogous study of the phosphorylated cRLC-N-lobe showed similar orientation distributions in relaxation, active isometric contraction and rigor [26], suggesting that the cRLC region of myosin might stays docked onto the thick filament backbone through interactions with the cRLC-N-lobe in all these states when the cRLC is unphosphorylated.

When the cRLC is phosphorylated (Fig. 4B), the orientation distributions of the C-lobe of the cRLC in relaxation and isometric contraction show two main peaks, corresponding to the C2 and C3 peaks seen for the unphosphorylated cRLC, with (θEG, γEC) = (60°, −60°) and (85°, 35°), respectively. Phosphorylated cRLC-C-lobes are less likely to be in the more parallel orientations C1 and C4, with (θEG, γEC) = (30°, 15°) and (125°, −30°), respectively. Qualitatively similar effects of phosphorylation on cRLC-C-lobe orientation were observed when different cRLC reference structures were used for the ME calculations (Fig. S7). Thus the conclusions about the effects of cRLC phosphorylation on its orientation are unlikely to be influenced by a change in the overall fold of the light chain domain, which is almost unaffected by phosphorylation in vitro [33]. The orientation distributions in Fig. 4 were also not significantly affected by the experimental variability in the order parameters <P2> and <P4> (Tab. S1), as shown by repeating the ME calculations with values chosen at random from normal distributions of the measured order parameters (Fig. S8).

As noted previously [26], peak C4 of the cRLC-C-lobe orientation distribution, which is more prominent in the unphosphorylated state (Fig. 4A), is close to the orientations of the thick filament in the myosin heads in isolated thick filaments from vertebrate skeletal muscle (Fig. 1) [34]. In this asymmetric structure, sometimes called the ‘J-motif’ or ‘interacting heads motif’, which seems to be conserved among muscle types and species [35,36], the myosin heads are folded...
HMM from smooth muscle [37] (PDB entry 3J04). These are shown as similar to those observed in 2D crystals of isolated phosphorylated cRLC. The more perpendicular C2 and C3 orientations for phosphorylated cRLC are more likely to peak C4. Squid S1 with bound ADP (cyan) is close to the nucleotide-free chicken skeletal S1 in the actin-S1 rigor complex, are shown as orange, yellow, cyan, green and pink diamonds respectively. The C-lobe orientations of the light-chain and catalytic domains in published S1 crystal structures, a diversity that is matched by that of the cRLC C-lobe orientations in cardiac muscle cells.

4. Discussion

4.1. Phosphorylation level of endogenous ventricular myosin regulatory light chain

As noted in the Introduction, there is no clear consensus about the basal level of cRLC phosphorylation in mammalian cardiac muscle. Some studies have reported a constant level of 0.4–0.5 mol P_i/mol cRLC [39] (for review see [11]), whereas others have reported much lower ventricular RLC phosphorylation levels [6,12,40]. These differences may be associated with heterogeneity between different parts of the heart [7,13,41] and potential effects of species, gender and tissue sampling procedures [42]. The cardiac muscle preparations from rat ventricle used in the present experiments had a consistently low cRLC phosphorylation level of less than 0.05 mol P_i/mol cRLC, and this was independent of the tissue treatment. Moreover the same low level was measured in the intact ventricle immediately snap-frozen after excision (Fig. 3). Freshly prepared mouse heart myofibrils and whole ventricular preparations that were immediately snap-frozen after excision of the heart showed that the mouse cRLCs are also almost entirely unphosphorylated (Fig. S3) suggesting that the low ventricular CRLC phosphorylation level is conserved among rodent species and independent of the sample preparation. Similar low cRLC phosphorylation levels were recently measured in swine myocardium by mass spectrometry [10], suggesting that the basal phosphorylation level of mammalian CRLCs might be lower or more dynamic than previously thought.

An increase in cRLC phosphorylation level in intact cardiac muscle has been associated with an increase in the stimulation frequency,
muscle pre-load, and isoprenaline treatment [6–9], suggesting that these stimuli work upstream of cRLC phosphorylation and might be involved in the activation of cardiac myosin light chain kinase (cMLCK). Prolonged incubation of skinned cardiac muscle preparations with recombinant cMLCK led to almost full phosphorylation of the endogenous cRLCs in our preparations, suggesting that both cRLCs of the double-headed myosin molecule are available for phosphorylation by cMLCK in situ. However, the cMLCK phosphorylated the cRLCs to a maximum level of ca 1 mol/mol both in situ and in vitro, suggesting that different signalling pathways are involved in the multiple phosphorylated cRLC species identified in rodent cardiac muscle in vivo [43–45].

4.2. Effects of phosphorylation on cRLC dynamics

Phosphorylation of the cRLC produced significant changes in the order parameter \( \langle P_{2}\rangle \) for the BSR probes (Table S1), which describes the amplitude of fast probe motion (“wobble”) on the surface of the cRLC. The observed changes were much larger than those associated with the transition from relaxation to active isometric contraction or rigor, suggesting that cRLC phosphorylation leads to a much larger change in the local environment of the probes. These effects were not confined to the probe close to the phosphorylatable serine 15 in the cRLC N-terminal extension (BSR-cRLC-N), suggesting that they are due to global changes in cRLC environment associated with changes in its tertiary structure or with intermolecular interactions. The observation that the crystal structure of the scallop smooth muscle light chain domain does not change significantly on RLC phosphorylation [33] argues against the former explanation.

A more likely explanation, suggested previously as a mechanism for activation of smooth muscle contraction by RLC phosphorylation [46], is that interactions between the cRLC N-terminal extension and other regions on the surface of the cRLC are altered by phosphorylation of serine 15. A similar mechanism was recently proposed for the effect of phosphorylation of serines 22/23 of cardiac troponin I, involving contacts between positively charged residues in its N-terminal extension, close to serines 22/23, and negatively charged residues in the N-lobe of tropo- nin C [47].

4.3. cRLC phosphorylation controls myosin head orientation in diastole and systole

The distribution of the orientations of phosphorylated cRLC with respect to the thick filament axis in the relaxed (low calcium or diastolic) state showed two distinct peaks (Fig. 4B), in contrast with the four states, suggesting that strong attachment of myosin to actin during active contraction (in systole) does not alter the orientation of the C-lobe of cRLC. This is consistent with our previous conclusion [26] that the primary function of the cRLC region of myosin is to regulate the availability of myosin heads for attachment to actin. The present results show that this transition between the ‘OFF’ and ‘ON’ conformations of the myosin heads is controlled by the phosphorylation state of the cRLC (Fig. 5).

In contrast with the large effects of cRLC phosphorylation described above, the orientation of the cRLC region did not change significantly on calcium activation in either the phosphorylated or unphosphorylated states, suggesting that strong attachment of myosin to actin during active contraction (in systole) does not alter the orientation of the C-lobe of cRLC.

One potential limitation of the present experiments is that less than 30% of endogenous unphosphorylated rat cRLCs were replaced by phosphorylated BSR-RLCs, so that the majority of myosin heads in the trabeculae were unphosphorylated. Although the in vitro phosphorylation and labelling protocol used here specifically measures the orientation of the phosphorylated cRLCs within a mixed population of phosphorylated and unphosphorylated myosin heads in situ, we cannot exclude the possibility that intra- or intermolecular interactions between the heads [35, 36] might attenuate the observed effects of phosphorylation when a low fraction of heads are phosphorylated. Such an effect would imply that an even larger effect of cRLC phosphorylation on its orientation distribution might be observed at higher phosphorylation levels, and might explain the residual C1 and C4 features seen for the phosphorylated cRLCs in relaxation and active contraction in the present experiments (Fig. 4B). However, given the very low level of basal cRLC phosphorylation in the preparation used here, the levels of cRLC phosphorylation achieved here are likely to be in the physiological range. The present results clearly establish that relatively low changes in phosphorylation level in this range are sufficient to produce significant

![Fig. 5. Schematic model for the effect of cRLC phosphorylation on myosin head orientation.](image-url)
changes in cRLC orientation, consistent with previous studies on isolated thick filaments from vertebrate skeletal muscle, in which phosphorylation of only 10% of the RLCs was sufficient to disorder the helical array of myosin heads [24].

4.4. Structural mechanism for the thick filament-based regulation of cardiac contractility by cRLC phosphorylation

Since the cRLC region of the myosin heads remains ordered after cRLC phosphorylation (Fig. 4B), even in the calcium-activated systolic state, the present results suggest that an interaction between the cRLC and the thick filament backbone is maintained after cRLC phosphorylation. Thus the present results imply a modification of the mechanism by which RLC phosphorylation controls the conformation of the myosin heads suggested previously by electron micrographs of isolated thick filaments from invertebrates [25,48] and vertebrates [24], and by X-ray diffraction studies on skinned mammalian cardiac muscle [23]. These studies suggested that the effect of RLC phosphorylation on myosin head conformation was mediated by a weakening or breaking of the interaction between cRLC and the filament backbone; the present results, in contrast, suggest that the cRLC region of myosin stays bound to the thick filament backbone in a preferred conformation. The effect of RLC phosphorylation is to shift the conformational equilibrium between distinct RLC:backbone interactions that correspond to distinct RLC orientations and by implication distinct conformations of the myosin motor domain (Fig. 5).

The effect of cRLC phosphorylation on actin-bound myosin heads in rigor provides further support for the above conclusion. In the unphosphorylated state, the cRLC C-lobe adopts a conformation (Fig. 4; peak C4) similar to that of nucleotide-free scallop myosin S1 in the rigor state of cRLC at its interaction point with the thick filament (Fig. 4; yellow diamond). After phosphorylation, however, the predominant orientations are those populated during relaxation and active contraction (peaks C2 and C3), and C4 becomes a minor population. Since cRLC phosphorylation is unlikely to change the actin-interaction of the catalytic domain in rigor, this change in cRLC orientation implies a phosphorylation-mediated change in the preferred conformation of the cRLC on the thick filament surface in a myosin head that is strongly bound to actin. It follows that all or part of the cRLC is not part of the functional lever arm of the myosin motor in cardiac muscle, which would therefore be shorter than previously assumed [26]. This effect may require an intact thick filament, since cRLC phosphorylation leads to an increase in mean step-size in isolated cardiac myosin [51].

Control of myosin head conformation by mediating conformational states of cRLC at its interaction point with the thick filament might represent a common mechanism for thick filament-based control of cardiac contractility. The interactions of cRLC with the N-terminus of cMyBP-C [52] and with the A-band region of titin [36] could also feed into this regulatory pathway to constrain the conformational and regulatory state of the myosin heads.

5. Conclusions

We found that the basal in vivo cRLC phosphorylation level in the rodent heart is lower than previously thought, at less than 0.05 mol P/mol cRLC. Serine 15 of cRLC is phosphorylated by cMLCK in a calcium/calmodulin dependent manner. cRLC phosphorylation does not disorder the cRLC region of the myosin heads, but alters the conformational equilibrium towards states in which the heads can interact more readily with the thin filament. cRLC phosphorylation controls myosin head orientation and the contractility of the heart by modulating interactions between the cRLC region of myosin and the thick filament backbone.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1616/j.jmcc.2015.06.002.

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Disclosures

None
