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Myosin binding protein-C phosphorylation is the principal mediator of protein kinase A effects on thick filament structure in myocardium

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Abstract

Phosphorylation of cardiac myosin binding protein-C (cMyBP-C) is a regulator of pump function in healthy hearts. However, the mechanisms of regulation by cAMP-dependent protein kinase (PKA)-mediated cMyBP-C phosphorylation have not been completely dissociated from other myofilament substrates for PKA, especially cardiac troponin I (cTnI). We have used synchrotron X-ray diffraction in skinned trabeculae to elucidate the roles of cMyBP-C and cTnI phosphorylation in myocardial inotropy and lusitropy. Myocardium in this study was isolated from four transgenic mouse lines in which the phosphorylation state of either cMyBP-C or cTnI was constitutively altered by site-specific mutagenesis. Analysis of peak intensities in X-ray diffraction patterns from trabeculae showed that cross-bridges are displaced similarly from the thick filament and toward actin (1) when both cMyBP-C and cTnI are phosphorylated, (2) when only cMyBP-C is phosphorylated, and (3) when cMyBP-C phosphorylation is mimicked by replacement with negative charge in its PKA sites. These findings suggest that phosphorylation of cMyBP-C relieves a constraint on cross-bridges, thereby increasing the proximity of myosin to binding sites on actin. Measurements of Ca²⁺-activated force in myocardium defined distinct molecular effects due to phosphorylation of cMyBP-C or co-phosphorylation with cTnI. Echocardiography revealed that mimicking the charge of cMyBP-C phosphorylation protects hearts from hypertrophy and systolic dysfunction that develops with constitutive dephosphorylation or genetic ablation, underscoring the importance of cMyBP-C phosphorylation for proper pump function.

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Keywords

myosin binding protein C; troponin I; Ca^{2+} -sensitivity; cross-bridge cycling kinetics; protein kinase A; phosphorylation; low-angle x-ray diffraction; myocardium; trabeculae; myofilament structure

1. INTRODUCTION

On a beat-to-beat basis, the force of contraction and the dynamics of relaxation in the heart are finely tuned to match cardiac output to varying circulatory demands. Sympathetic activity contributes to the regulation of contractility via β_1 -adrenoreceptor stimulation, which results in generation of cAMP and protein kinase A (PKA)-mediated phosphorylations of membrane and myofilament proteins. Targets of PKA include Ca^{2+} handling proteins such as L-type Ca^{2+} channels, intracellular Ca^{2+} release channels, and the sarcoplasmic reticulum (SR) Ca^{2+} pump modulator phospholamban, and the myofilament regulatory proteins troponin I (cTnI) and myosin binding protein-C (cMyBP-C) (reviewed in [1-4]). The ensemble of PKA phosphorylations increases the strength and speed of myocardial contraction thereby increasing stroke volume and accelerates the rate of relaxation facilitating filling of the ventricles during diastole. During a twitch, cTnI phosphorylation by PKA reduces the Ca^{2+} sensitivity of force in the sarcomere, which in turn causes an *earlier onset of relaxation*. This is because cTnI phosphorylation leads to enhanced dissociation of Ca^{2+} from cardiac troponin C (cTnC) at higher $[\text{Ca}^{2+}]_i$ during the decay of the Ca^{2+} transient [5-8]. On the thick filament, PKA phosphorylation of cMyBP-C accelerates the rate of force development and also appears to *accelerate relaxation* as a consequence of accelerated cross-bridge cycling kinetics [8-12]. In addition to cTnI, cMyBP-C also contributes to a decreased Ca^{2+} -sensitivity of force when phosphorylated as previously shown by Cazorla *et al.*, (2006) [13]. One possibility is that the decreased Ca^{2+} -sensitivity of force results from a reduced dwell time for the cross-bridges once bound to thin filament sites [12-14].

cMyBP-C is localized to the C-zone [15, 16] of the A-band, where its interactions with myosin and possibly with actin [17-19] are likely to perturb the structural conformation of myosin heads [20-22] and also binds to titin [23, 24]. Similar to the myofibrillar protein titin, cMyBP-C is comprised of several immunoglobulin-I (Ig-I) and fibronectin-III (Fn-3)-like domains [25, 26]. At least two points of binding of unphosphorylated cMyBP-C, near its N- and C- termini, are needed to physically constrain the mobility of myosin cross-bridges [27] and conceivably limit any binding of cMyBP-C to actin. The C-terminal cMyBP-C contact is likely anchored to the myosin backbone [28], with 3 or 4 domains between C7 and C10 lying longitudinally along the filament surface, parallel to titin [20]. N-terminal cMyBP-C interactions with regions of myosin subfragment-2 (S2), closest to the light chain domain of the myosin head (S1), may also occur [29]. It therefore seems plausible that cMyBP-C slows cross-bridge cycling kinetics by constraining myosin access to the thin filament through two such thick filament contacts.

In the present study, we examined phosphorylation-induced structural and contractile changes in myosin cross-bridges in an attempt to determine the possible roles of the cMyBP-C PKA sites in mediating these changes. We used two complementary biophysical approaches, namely synchrotron X-ray diffraction and mechanical measurements of Ca^{2+} -activated force in skinned myocardium from transgenic mouse models. The mouse models include cardiac-specific transgenic expression of (I) mutant cTnI in which the two PKA phosphorylation sites were mutated to alanines, rendering cTnI constitutively non-phosphorylatable expressed on the cTnI null background (the cTnI_{Ala2} with two alanine

substitutions in cTnI: S22A and S23A [30]), (2) mutant cMyBP-C in which the three PKA phosphorylation sites were mutated to alanines, rendering cMyBP-C constitutively non-phosphorylatable (the cMyBP-C(t3SA) mouse with three alanine substitutions in cMyBP-C: S273A, S282A, and S302A, [11]), and (3) mutant cMyBP-C in which the three PKA phosphorylatable serines were mutated to aspartic acid to mimic the negative charge of phosphorylation (the cMyBP-C(t3SD) mouse with three aspartic acid substitutions in cMyBP-C: S273D, S282D, and S302D, which is introduced in this study) and then bred into the cMyBP-C^{-/-} background [31]. We also performed echocardiography on anesthetized cMyBP-C(t3SD) mice to assess *in vivo* left ventricular structure and systolic function. These mouse models allow us to examine the structural and functional effects of cMyBP-C phosphorylation in the presence or absence of cTnI phosphorylation. cTnI_{Ala2} mice express native cMyBP-C, while cMyBP-C(t3SA), cMyBP-C(tWT) control and cMyBP-C(t3SD) mice express native cTnI.

2. MATERIAL AND METHODS

2.1 X-ray diffraction and mechanical measurements

Figure 1A shows a typical X-ray diffraction pattern from skinned trabeculae and identifies the locations of the equatorial reflections that were the focus of this study. Lattice spacing and the ratio of intensities of the 1,0 and 1,1 equatorial reflections OR isometric force and the rate constant of force development (k_{tr}) were then measured as described [32]. Briefly, trabeculae were mounted in sealed plastic chambers and the sarcomere length set to 2.15 μm in relaxing solution pCa 9.0 at 22 °C. Low-angle X-ray diffraction patterns were collected using the small-angle instrument on the BioCAT undulator-based beamline 18-D at the Advanced Photon Source, Argonne National Laboratory [33, 34] and a CCD-based X-ray detector (PCCD 168080, Aviex L.L.C., Naperville, IL, USA). The spacings of the 1,0 and 1,1 equatorial reflections were converted to $d_{1,0}$ lattice spacings using Bragg's Law, as described [33, 34]. $d_{1,0}$ lattice spacing was then multiplied by 2/ 3 to yield the center-to-center distance between thick filaments, i.e. the inter-thick filament spacing (*IFS*). Intensities of the 1,0 and 1,1 equatorial reflections were determined from one-dimensional projections along the equator and analyzed independently by three people and the results averaged [35]. The ratio of the 1,0 and 1,1 equatorial intensities ($I_{1,1}/I_{1,0}$) can be used to estimate shifts of molecular mass (presumably cross-bridges) from the region of the thick filament to region of the thin filament. For example, the $I_{1,1}/I_{1,0}$ intensity ratio increases with increased proximity of myosin cross-bridges to actin [36, 37], although other factors can influence equatorial intensities [38-40].

cTnI_{Ala2} transgenic myocardium allowed us to examine effects of PKA phosphorylation of cMyBP-C in the absence of phosphorylated cTnI. Separate preparations were isolated from mouse hearts for structural and functional measurements and were studied under closely matched conditions: skinned trabeculae were used for X-ray diffraction measurements but skinned multi-cellular myocardial preparations [9] were used for mechanical measurements of force and the kinetics of force development. Note that the X-ray diffraction results from WT trabeculae were reported earlier [32] but were collected during the same experimental sessions as the transgenic trabeculae reported here. Equatorial X-ray diffraction measurements were similarly assessed in trabeculae from transgenic mice in which non-phosphorylatable cMyBP-C was expressed on a cMyBP-C null background (cMyBP-C(t3SA)), wild-type cMyBP-C was expressed on the null background (cMyBP-C(tWT)) [11], or phosphomimetic cMyBP-C was expressed on the null background (cMyBP-C(t3SD)). Details regarding characterization of protein expression, heart morphology, and systolic function of the cMyBP-C(t3SD) mouse are included in the on-line supplemental information.

2.2 Protein phosphorylations

For PKA-mediated phosphorylations, skinned muscle preparations were first incubated for 1 h (~22°C) in solution of pCa 9.0 containing 1 U PKA/μL, and were then washed in the same solution without PKA (30 min, 3 solution changes). The extent of protein phosphorylation in skinned muscle preparations was determined densitometrically by SYPRO Ruby and Pro-Q Diamond stained SDS-PAGE. Representative gels are included in the on-line supplemental information and described earlier [27, 41].

3. RESULTS

3.1 Changes in distribution of cross-bridge mass due to PKA-mediated phosphorylation of native cMyBP-C in cTnI_{Ala2} trabeculae or expression of mutant cMyBP-C mimicking the charge of phosphorylation

Charge mutants at the PKA sites of cTnI and cMyBP-C were used to examine the specific effects of PKA treatment, constitutive phosphorylation, or constitutive dephosphorylation of either cTnI or cMyBP-C on average cross-bridge disposition between myofilaments in trabeculae. PKA-phosphorylated WT and cTnI_{Ala2} trabeculae and phosphomimetic cMyBP-C(t3SD) trabeculae exhibit equatorial $I_{1,1}/I_{1,0}$ ratios that were greater than their non-phosphorylated controls, indicating a movement of cross-bridge mass away from the thick filament backbone and towards actin, presumably as a consequence of charge modification in the cMyBP-C phosphorylation motif (Fig. 1, SI Table 2). First, prior to PKA treatment, WT and cTnI_{Ala2} trabeculae exhibited similar $I_{1,1}/I_{1,0}$ ratios (0.23 ± 0.02 versus 0.25 ± 0.01 , NS; Fig. 1B, SI Table 2), indicating that the mutations in cTnI_{Ala2} did not significantly alter cross-bridge disposition under baseline conditions. Next, PKA treatment increased $I_{1,1}/I_{1,0}$ ratios similarly in WT and cTnI_{Ala2} trabeculae (0.33 ± 0.03 vs. 0.34 ± 0.02 , NS; $p < 0.05$ compared to their non-PKA-treated controls; Fig. 1B, SI Table 2), suggesting that phosphorylation of cMyBP-C alone is sufficient to displace cross-bridges and that this structural effect is not due to PKA phosphorylation of cTnI. Third, cross-bridge displacement was dependent on both the presence and phosphorylation status of cMyBP-C in trabeculae, as $I_{1,1}/I_{1,0}$ ratios decrease in cMyBP-C(tWT) compared to null trabeculae (0.28 ± 0.02 vs 0.40 ± 0.06 [27]; $p < 0.03$, SI Table 2), and increase when constitutive phosphorylation is mimicked. We found that $I_{1,1}/I_{1,0}$ ratios from cMyBP-C(t3SD) trabeculae (0.33 ± 0.02) were greater than in cMyBP-C(t3SA) trabeculae (0.25 ± 0.02 ; $p < 0.0001$; Fig. 1C, SI Table 2), a difference that is similar to that observed in WT trabeculae before and after PKA. Thus, mimicking the charge of constitutive phosphorylation of cMyBP-C is sufficient to elicit the movement of cross-bridge mass observed upon PKA treatment of WT trabeculae. Supporting this conclusion, $I_{1,1}/I_{1,0}$ ratios from cMyBP-C(t3SD) were similar to those from PKA-treated WT and cTnI_{Ala2} trabeculae (0.33 ± 0.02 vs. 0.33 ± 0.03 vs. 0.34 ± 0.02 , NS; Fig. 1B-C, SI Table 2). Thus, cross-bridges were, on average, extended away from the surface of the thick filament and toward the thin filament with constitutive cMyBP-C phosphorylation as compared to constitutive dephosphorylation, and when cMyBP-C was phosphorylated irrespective of the phosphorylation state of cTnI. Thus, in addition to the presence of cMyBP-C, the charge introduced by phosphorylation of cMyBP-C is sufficient to move cross-bridges either via kinase activity or by site-directed mutagenesis.

3.2 Effects of PKA phosphorylations on interfilament spacing

Interfilament spacing (*IFS*) is reduced in by ~3 nm in both TnI_{Ala2} and cMyBP-C(t3SD) myocardium in the absence of phosphorylation (Fig. 2; SI Table 2), suggesting that changes in *IFS* are mediated by alterations in charge distribution as a result of the mutations. This reduction is similar to that observed when cMyBP-C is phosphorylated by PKA, whether or not cTnI is phosphorylated and there is thus a greater *net* negative charge on the thick filament. It is unclear whether the observed changes in filament spacing is due to

electrostatic interactions or changes in inter- or intra-molecular structural properties of cMyBP-C and/or cTnI, such as a change in motif length.

Konhilas *et al.* [42] also found that PKA phosphorylation of cMyBP-C in the presence of non-phosphorylatable slow skeletal TnI reduces *IFS* by ~3 nm, which is consistent with the idea that cMyBP-C phosphorylation changes *IFS*. However, analysis of these results is complicated by the fact that while the cTnI_{Ala2} mutation leads to a reduction in *IFS* by ~3 nm, mutation of cTnI to the ssTnI isoform (by deletion of the 32 amino acid N-terminal extension in cTnI; [43]) increases *IFS* by ~3 nm. This suggests that that removal of the phosphorylatable PKA sites in cTnI and removal of the entire N-terminal extension differentially affect *IFS*. The phosphomimetic cMyBP-C mouse model studied by Sadayappan [44] had increased lattice spacing, in contrast with our results (Fig. 2). Differences in *IFS* could be influenced by RLC phosphorylation level (basal phosphorylation vs dephosphorylation) [32], as well as the different backgrounds of the mouse models (true complete protein genetic ablation vs truncation) [11, 31].

3.3 PKA phosphorylation of cTnI or cMyBP-C decreases Ca²⁺-sensitivity of force but only phosphorylation of cMyBP-C accelerates cross-bridge cycling kinetics

Resting force, maximum Ca²⁺-activated force, the steepness of the force-pCa relationship, and the Ca²⁺-sensitivity of force did not differ between untreated WT and cTnI_{Ala2} myocardium (Fig. 3, SI Table 3). Also, the pCa₅₀ for isometric force did not differ in untreated WT and cTnI_{Ala2} myocardium (5.81 ± 0.01 vs. 5.83 ± 0.01) (Fig. 3A). Following treatment with PKA, the Ca²⁺-sensitivity of force significantly decreased by ~0.12 pCa units in WT myocardium (pCa₅₀ = pCa 5.69 ± 0.01 vs. 5.81 ± 0.01 ; Fig. 3A), whereas in cTnI_{Ala2} myocardium, the Ca²⁺ sensitivity significantly decreased by ~0.07 pCa units (pCa 5.76 ± 0.01 vs. 5.83 ± 0.01) and the steepness of the force-pCa relationship was also significantly reduced (n_H : 3.99 ± 0.11 vs. 4.27 ± 0.09 ; Fig. 3B). Thus, PKA phosphorylation reduces the Ca²⁺-sensitivity of force in cTnI_{Ala2} myocardium expressing non-PKA-phosphorylatable cTnI and native cMyBP-C, although the effect was half that observed in WT myocardium (Δ pCa₅₀: ~0.07 vs. 0.12; Fig. 3, SI Table 3). These results suggest that both cTnI and cMyBP-C contribute to the reduced Ca²⁺-sensitivity of force following PKA treatment.

Both before and after PKA treatment, the rates of force development at submaximal activation were noticeably faster in cTnI_{Ala2} than in WT myocardium (Fig. 4 A, B) There is likely to be a structural change in cTnI following the substitution of *Ala* for *Ser* at residues 23 and 24, such that molecular interactions within the troponin complex or the thin filament regulatory strand are altered. Since there is no change in Ca²⁺ sensitivity of force due to the cTnI_{Ala2} mutation, but the rate of force development is accelerated even prior to phosphorylation, it seems plausible that non-phosphorylatable cTnI on the thin filament would reduce the effects of the regulatory strand to retard activation, in a manner analogous to cross-bridges residing in the pre-activated state by pre-equilibration with nucleotide prior to Ca²⁺-activation. Thus, cross-bridge cycling kinetics would be accelerated without affecting pCa₅₀. Phosphogel analyses also showed that the absence of cTnI phosphorylation had no affect on the basal level of cMyBP-C phosphorylation (SI Fig. 3A; [30, 45]).

3.4 Expression of cMyBP-C(t3SD) prevents development of cardiac hypertrophy in the cMyBP-C^{-/-} mouse

The phosphomimetic cMyBP-C(t3SD) mice exhibited a generally normal physical appearance and no evidence of early mortality or increased morbidity. cMyBP-C(t3SD) exhibited similar heart weight to body weight ratios as cMyBP-C(t3WT). cMyBP-C(t3SD) reversed the severe hypertrophy exhibited by knockout (cMyBP-C^{-/-}) and non-PKA-phosphorylatable cMyBP-C (cMyBP-C(t3SA)) hearts [11, 31] (SI Table 1), similar to the

observation by Sadayappan *et al.*, using a transgenic mouse expressing a phosphorylation mimic of cMyBP-C [44, 46]. This suggests that the release of cross-bridges upon phosphorylation is critical to normal cardiac function and might even suggest that the release of a small number of cross-bridges due to basal phosphorylation of cMyBP-C contributes to baseline contractility. cMyBP-C null and cMyBP-C-t3SA mutations each reduce the dynamic range of myocardial function, which presumably reduces the responsiveness of the heart to altered loading conditions [11, 47]. Phosphomimetic mutations (cMyBP-C(t3SD)) also reduce dynamic range, but mutant mice exhibit normal heart/body weight ratios (SI Table 1). This is surprising, since the inability to modulate cMyBP-C phosphorylation status and cross-bridge disposition in cMyBP-C^{-/-} and cMyBP-C(t3SA) hearts leads to hypertrophy [11]. Thus, it seems likely that phosphorylated cMyBP-C, or the potential for it to be phosphorylated, is important for maintenance of normal contractile function. The cTnI_{Ala2} mutation did not result in detectable cardiac hypertrophy or cardiomyopathy [30].

3.4 Echocardiography

We performed echocardiography to assess *in vivo* left ventricular structure and systolic function on anesthetized 3-month old mice (Table 1). Similar heart rates were maintained to ensure similar study conditions. The constitutively phosphorylated mimetic cMyBP-C(t3SD) hearts exhibit similar chamber dimensions and ejection fraction (EF) as control cMyBP-C(tWT) hearts with normally phosphorylated WT cMyBP-C. cMyBP-C(t3SD) hearts demonstrate improved EF and slightly smaller left ventricular inner diameter at diastole in comparison to non-phosphorylatable cMyBP-C(t3SA) hearts. Consistent with prior studies [11], cMyBP-C(t3SA) hearts exhibit hypertrophy as evidenced by increased LV posterior wall thickness during diastole (PWd). Thus, constitutively phosphorylated mimetic cMyBP-C(t3SD) improves ventricular structure and systolic function compared to constitutively dephosphorylated cMyBP-C(t3SA).

4. DISCUSSION

4.1 PKA phosphorylation of cMyBP-C induces cross-bridge displacement toward actin in relaxed myocardium

Equatorial intensity ratios suggest that when cMyBP-C is either minimally or unphosphorylated, a population of myosin heads is localized near the thick filament backbone. The unphosphorylated state of cMyBP-C is critical to this cross-bridge arrangement, as molecular mass moves away from the thick filament upon phosphorylation of cMyBP-C, as shown before [27] and in the present study. A possible mechanism is that cMyBP-C physically constrains the cross-bridge, thereby restricting the volume it explores or at least making large excursions of the cross-bridge heads less likely. A portion of the C-terminal domains of cMyBP-C lies along the thick filament backbone, parallel to titin [20], and a portion of the N-terminal domains could extend away from the backbone surface to interact with the S2 region of myosin proximal to the RLCs and/or with the RLCs or titin. Two points of contact between cMyBP-C and myosin could be envisioned to constrain the movements of myosin heads and so reduce the likelihood of cross-bridge binding to actin. Unresolved is whether the N-terminal domains of MyBP-C bind to actin *in vivo* versus binding to myosin S2 or RLC, since binding to all three targets has been demonstrated *in vitro* [19, 29, 48]. Similarly, changes in interfilament lattice spacing (*IFS*) could alter the radial or azimuthal orientation of cross-bridges relative to actin and thereby alter the probability of interaction between myosin and actin. Thus, phosphorylation-mediated variations in *IFS* could improve or impair the alignment of cross-bridges relative to actin in various contractile and physiological states.

We have shown previously that PKA phosphorylation causes cross-bridge movement in WT trabeculae, but does not induce further movement in cMyBP-C^{-/-} trabeculae [27]. With respect to cross-bridge cycling kinetics, PKA accelerates k_{tr} and kinetic parameters of the stretch activation response in WT myocardium, but no further acceleration is observed in cMyBP-C^{-/-} myocardium [10, 27]. Stretch activation studies of skinned myocardium from cMyBP-C(tWT) and cMyBP-C(t3SA) demonstrate that kinetic parameters of force development are slowed compared to the cMyBP-C null myocardium, and force development only in cMyBP-C(tWT) myocardium is accelerated by PKA [11]. The idea that cMyBP-C and its phosphorylation affect a specific step during the power-stroke of myosin and influence the weak-to-strong binding transition of the cycling cross-bridge pool (for review, [49]) is supported by other investigators [50, 51]. Removal of the cMyBP-C constraint appears to accelerate both rates of cross-bridge attachment and detachment. Thus, when cross-bridges are released and cycling kinetics are accelerated, the Ca²⁺-sensitivity of force may decrease because of reduced dwell time of strong-binding cross-bridges, consistent with earlier reports [13, 14, 50]). Reduced dwell time would diminish cooperative activation by strong binding cross-bridges, and thus there would be less activation of the thin filament at submaximal [Ca²⁺]_i to cause the apparent decrease of Ca²⁺-sensitivity.

Our experiments yielded similar increases in $I_{1,1}/I_{1,0}$ ratios in cMyBP-C(t3SD), PKA-phosphorylated WT and cTnI_{A1a2} myocardium, all of which had phosphorylated or phosphomimetic cMyBP-C; however, these ratios were less than we reported earlier for cMyBP-C^{-/-} knockout myocardium [34]. This comparison suggests that phosphorylation of cMyBP-C does not completely relieve the cMyBP-C constraint on myosin, which can only be accomplished by ablation of the protein. Any number of as-yet untested mechanisms might account for a residual constraint of myosin, including persistent lower-affinity interactions with cMyBP-C or even an allosteric effect on myosin orientation, mobility or rigidity due to C-terminal interactions with the thick filament backbone or titin. Different values of $I_{1,1}/I_{1,0}$ in cMyBP-C^{-/-} vs. cMyBP-C-phosphorylated myocardium are also consistent with the idea that phosphorylation does not induce a complete dissociation of cMyBP-C from S2 (or possibly actin) but more likely produces a subtler conformational change in cMyBP-C that alters its interfaces with one or more binding partners. Changes in binding could also affect the cross-bridge angle of attachment or nucleotide state-associated conformations of S1 in the same crown of myosin heads or even in neighboring crowns. Whatever the mechanism, increased myosin head proximity to actin mediated by PKA phosphorylation of cMyBP-C appears to accelerate cooperative cross-bridge recruitment to force generating states and, in turn, accelerates cross-bridge interaction kinetics during submaximal activation.

4.2 Mechanical effects of PKA in WT and cTnI_{A1a2} myocardium reveal mechanistic insights

The rate constant of force redevelopment, k_{tr} , provides a measure of the sum of the rate constants of cross-bridge attachment (f_{app}) and detachment (g_{app}) in the transition from weak- to strong-binding states [52-54]. However, by itself the measurement of k_{tr} does not definitively yield the values of either f_{app} or g_{app} . Within this framework, we conclude that the cMyBP-C phosphorylation-mediated decrease in the Ca²⁺-sensitivity of force is due to a decrease in submaximal force resulting from decreased dwell time, since resting force and maximum Ca²⁺-activated force are unchanged by phosphorylation. The impact of Ca²⁺ on f_{app} , and therefore $f_{app}/(f_{app}+g_{app})$, would be diminished when the cMyBP-C constraint on myosin cross-bridge disposition and kinetics is alleviated by phosphorylation. The decreased dwell time diminishes cooperative activation by strongly bound cross-bridges resulting in less thin filament activation.

cMyBP-C and RLC phosphorylation [32] each induce a movement of myosin heads toward actin under resting conditions and each accelerates cross-bridge cycling at sub-maximal

Ca^{2+} activations. Despite these similarities, cMyBP-C and RLC phosphorylation have opposite effects on the Ca^{2+} -sensitivity of force in skinned myocardium. The simplest explanation for differential effects on Ca^{2+} -sensitivity of force, yet similar acceleration of overall cycling kinetics (k_{tr}), is that cMyBP-C phosphorylation accelerates both f_{app} and g_{app} , whereas RLC phosphorylation accelerates f_{app} but either does not accelerate g_{app} , does not increase it to the same degree as cMyBP-C phosphorylation, or actually reduces g_{app} . While there are no direct results to account for these differential effects of protein phosphorylation on g_{app} , it is conceivable that phosphorylations of cMyBP-C and RLC affect myofilament or cross-bridge structure differently.

Ventricular structure and systolic function were improved with constitutively phosphorylated mimetic cMyBP-C(t3SD) compared to the dephosphorylated cMyBP-C(t3SA). Echocardiography parameters were also restored in cMyBP-C(t3SD) similar to *in vivo* measurements of normally phosphorylated cMyBP-C(tWT) hearts (Table 1). Thus, changes in thick filament structure, such as the release of “phosphorylated-like” cross-bridges could lead to protection of the heart from hypertrophy and systolic dysfunction that develops with constitutive dephosphorylation [11] or genetic ablation [31], despite reduced capacity to modulate PKA sites. In addition to changes in myosin structure, differential development of left ventricular hypertrophy could also be influenced by effects of unique phosphorylation motif structures formed by each of the cMyBP-C(t3SA) and cMyBP-C(t3SD) mutations.

cMyBP-C is highly phosphorylated in non-failing hearts of both mouse and human ([55, 56]), and a fourth PKA phosphorylation site has been identified in the motif [57]. Thus, additional Ser307 mutations in phosphomimetic mouse models will allow us to further resolve the role of cMyBP-C phosphorylation in myocardium. Future studies will be directed to determine if PKA treatment has any further effects on myofilament mass distribution in the phosphomimetic myocardium.

In summary, we observe movement of cross-bridge mass, acceleration of cross-bridge cycling kinetics, and reduced Ca^{2+} -sensitivity of force in skinned myocardium in which cMyBP-C is phosphorylated by PKA and in which Ser²³ and Ser²⁴ on the N-terminal extension of cTnI are replaced with Ala and therefore unphosphorylated. Furthermore, $I_{1,1}/I_{1,0}$ ratios in PKA-treated cTnI_{Ala2} myocardium and in phosphomimetic cMyBP-C(t3SD) myocardium were similar to values observed in PKA-treated WT myocardium, suggesting that phosphorylation of cMyBP-C involves *inter-* or *intra-* molecular charge-mediated disruption of the cMyBP-C inhibition, which by itself can influence the disposition of cross-bridges with respect to the actin filament. Our results from mechanical experiments suggest that in murine myocardium, PKA phosphorylation of cTnI and cMyBP-C each contribute to the reduction in the Ca^{2+} -sensitivity of force, but only phosphorylation of cMyBP-C modulates the kinetics of force development. Ultimately, the fine-tuning of the strength and speed of force development by cMyBP-C phosphorylation can be attributed to its ability to alter the availability of the cross-bridge head to actin, thereby modulating initial cross-bridge binding and the extent and rate of subsequent cooperative recruitment of cross-bridges to force generating states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

The abbreviations used are:

BDM	2,3-butanedione monoxime
cMyBP-C	cardiac myosin binding protein-C
cMyBP-C^{-/-}	cardiac specific cMyBP-C homozygous knockout mouse
cMyBP-C(t3SA)	mouse with cardiac specific transgenic expression of mutant cMyBP-C with <i>Ala-for-Ser</i> mutations in the 3 PKA sites
cMyBP-C(t3SD)	mouse with cardiac specific transgenic expression of mutant cMyBP-C with <i>Asp-for-Ser</i> mutations in the 3 PKA sites
cMyBP-C(t3WT)	mouse with cardiac specific transgenic expression of wild-type cMyBP-C on the cMyBP-C ^{-/-} background
cTnC	cardiac troponin C
cTnI	cardiac troponin I
<i>d</i>_{1,0}	lattice spacing
<i>F</i>	unitary cross-bridge force
<i>f</i>_{app}	rate constant of cross-bridge attachment
<i>f</i>_{app}/(<i>f</i>_{app}+<i>g</i>_{app})	fraction of cycling cross-bridges bound to actin
Fn-3	fibronectin-III
<i>g</i>_{app}	rate constant of cross-bridge detachment
<i>I</i>_{1,1}/<i>I</i>_{1,0}	intensity ratio
<i>IFS</i>	inter-filament spacing
Ig-I	immunoglobulin-I
<i>k</i>_{tr}	rate of force redevelopment
LV	left ventricular
LVIDd	LV inner diameter at end diastole
pCa	-log [Ca ²⁺] _{free}
pCa₅₀	pCa value at which isometric force is half-maximal
ΔpCa₅₀	change in pCa ₅₀
PKA	Protein kinase A
PWd	LV posterior wall thickness at end diastole
RLC	regulatory light chain
S1	myosin subfragment-1

S2	myosin subfragment-2
SR	sarcoplasmic reticulum
TnIAla₂	mouse with cardiac specific transgenic expression of mutant cTnI with <i>Ala-for-Ser</i> mutations in the 2 PKA sites)

RESEARCH HIGHLIGHTS

Background: Sympathetically-mediated phosphorylation of muscle proteins modulate cardiac contractility to help meet circulatory demands.

Result: Myosin binding protein-C restricts myosin access to actin and its phosphorylation moves myosin closer to actin, thereby speeding contraction, as evidenced here in X-ray diffraction patterns from demembranated cardiac trabeculae.

Conclusion: The charge associated with phosphorylation can release this constraint on myosin to allow for rapid heart pumping and filling.

Significance: Myosin binding protein-C phosphorylation helps to maintain healthy contractile function and is a potential target of therapy in myocardial disease.

Fig. 1.

(A) Representative x-ray pattern from skinned cTnI_{Ala2} mouse myocardium with labeled 1,0 (*inner two spots*, arising from thick filament-associated mass) and 1,1 (*outer two spots*, arising from thick and thin filament-associated mass) equatorial spots [32, 34]. (B) Bar graph representations of the ratio of intensities of the 1,1 and 1,0 equatorial x-ray reflections ($I_{1,1}/I_{1,0}$) from skinned WT and cTnI_{Ala2} myocardium untreated (control) and treated with PKA in relaxing solution (pCa 9.0). *Significant differences between untreated (i.e., unphosphorylated) and PKA treatment (i.e., phosphorylated) ($p < 0.05$). The measurements in WT myocardium were collected during the same experimental sessions as cTnI_{Ala2}, but were reported earlier [32]. (C) Bar graph representations of $I_{1,1}/I_{1,0}$ from cMyBP-C(t3SA), cMyBP-C(tWT), and cMyBP-C(t3SD) skinned myocardium in pCa 9.0. For reference, $I_{1,1}/I_{1,0}$ from cMyBP-C^{-/-} null myocardium reported earlier using the same experimental conditions was greater than WT myocardium at 0.40 ± 0.06 and did not change with PKA treatment [27].

Fig. 2.

(A) Bar graph representations of the inter-thick filament spacing (*IFS*) determined from the $d_{1,0}$ lattice spacing [32, 34] in untreated (control) and PKA-treated (phosphorylated) WT and cTnI_{Ala2} myocardium. #Significant differences between untreated WT and cTnI_{Ala2} ($p < 0.05$). The measurements in WT myocardium were collected during the same experimental sessions as cTnI_{Ala2}, but were reported earlier [32]. (B) Bar graph representations of *IFS* in cMyBP-C(t3SA), cMyBP-C(tWT), and cMyBP-C(t3SD) skinned myocardium. *Significant differences between cMyBP-C(t3SA), cMyBP-C(tWT), and cMyBP-C(t3SD) myocardium ($p < 0.05$). For reference, *IFS* from cMyBP-C^{-/-} null myocardium reported earlier using the same experimental conditions was not different from WT myocardium prior to PKA treatment (54 ± 1 nm) and increased with PKA treatment (57 ± 1 nm) [27].

Fig. 3.

(A) Force-pCa relationships established in WT skinned myocardium untreated (closed circle, solid line *fit*) and treated with PKA (closed downward triangle, dotted line). Fitting the mean data with the Hill equation yield pCa_{50} . (B) Force-pCa relationships in $cTnI_{Ala2}$ skinned myocardium untreated (closed circles, solid line *fit*) and treated with PKA (closed downward triangle, dotted line). The measurements in WT myocardium were collected during the same experimental sessions as $cTnI_{Ala2}$, but were reported earlier [32]. For reference, the Ca^{2+} -sensitivity of force in $cMyBP-C^{-/-}$ null myocardium is similar to WT myocardium [10].

Fig. 4.

The rate of force redevelopment (k_{tr}) was measured as described [32] at sub-maximal (pCa 6.0 - 5.5) and maximum (pCa 4.5) $[Ca^{2+}]_{free}$ in untreated and PKA-treated WT and cTnI_{Ala2} skinned myocardial preparations. Effects of cTnI_{Ala2} mutation on (A) k_{tr} -pCa and (B) k_{tr} -relative force relationships were established by comparison of unphosphorylated WT (closed circle) and cTnI_{Ala2} (open circle) myocardium. (C) Effects of PKA phosphorylation on k_{tr} -force relationships were established in unphosphorylated (closed circle) and phosphorylated (closed downward triangle) WT skinned myocardial preparations. (D) Effects of PKA phosphorylation on k_{tr} -force relationships in cTnI_{Ala2} skinned myocardium. The measurements in WT myocardium were collected during the same experimental sessions as cTnI_{Ala2}, but were reported earlier [32]. For reference, the rate of force development in cMyBP-C^{-/-} null myocardium was accelerated compared to WT myocardium and PKA did not further accelerate k_{tr} [10].

Table 1

Summary of echocardiographic data from cMyBP-C(tWT), cMyBP-C(tSA), and cMyBP-C(tSD) anesthetized mice.

Mean $\pm$ SEM	tWT n=14	t3SA, n=13	t3SD, n=12
Heart Rate (beats/minute)	408 $\pm$ 7	426 $\pm$ 9	416 $\pm$ 6
LVIDd (mm)	3.73 $\pm$ 0.09	4.05 $\pm$ 0.09 [#]	3.62 $\pm$ 0.12
PWd (mm)	1.08 $\pm$ 0.07	1.52 $\pm$ 0.07 ^{*,#}	1.04 $\pm$ 0.04
EF (%)	59.8 $\pm$ 2.8	53.3 $\pm$ 1.9 [#]	65.8 $\pm$ 4.1

Abbreviations are: left ventricular (LV) inner diameter at end diastole (LVIDd); LV posterior wall thickness at end diastole (PWd).

* Significant difference (p<0.05) compared to cMyBP-C(tWT);

[#] Significant difference (p<0.05) compared to cMyBP-C(t3SD).