Distinct sarcomeric substrates are responsible for protein kinase D-mediated regulation of cardiac myofilament Ca" sensitivity and crossbridge cycling

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Running title: PKD regulation of cardiac myofilament function

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Protein kinase D (PKD), a serine/threonine kinase with emerging cardiovascular functions, phosphorylates cardiac troponin I (cTnI) at Ser22/Ser23, reduces myofilament Ca" sensitivity and accelerates crossbridge cycle kinetics. Whether PKD regulates cardiac myofilament function entirely through cTnI phosphorylation at Ser22/Ser23 remains to be established. To determine the role of cTnI phosphorylation at Ser22/Ser23 in PKD-mediated regulation of cardiac myofilament function, we used transgenic mice that express cTnI in which Ser22/Ser23 are substituted by non-phosphorylatable Ala (cTnI-Ala2). In skinned myocardium from wild-type (WT) mice, PKD increased cTnI phosphorylation at Ser22/Ser23 and decreased the Ca" sensitivity of force. In contrast, PKD had no effect on the Ca" sensitivity of force in myocardium from cTnI-Ala2 mice, in which Ser22/Ser23 were unavailable for phosphorylation. Surprisingly, PKD accelerated crossbridge cycle kinetics similarly in myocardium from WT and cTnI-Ala2 mice. Since cardiac myosin-binding protein C (cMyBP-C) phosphorylation underlies PKA-mediated acceleration of crossbridge cycle kinetics, we explored whether PKD phosphorylates cMyBP-C at its PKA sites, using recombinant C1C2 fragments with or without site-specific Ser/Ala substitutions. Kinase assays confirmed that PKA phosphorylates Ser273, Ser282 and Ser302, and revealed that PKD phosphorylates only Ser302. Furthermore, PKD phosphorylated Ser302 selectively and to a similar extent in native cMyBP-C of skinned myocardium from WT and cTnI-Ala2 mice, and this phosphorylation occurred throughout the C-zones of sarcomeric A-bands. In conclusion, PKD reduces myofilament Ca" sensitivity through cTnI phosphorylation at Ser22/Ser23, but accelerates crossbridge cycle kinetics by a distinct mechanism. PKD phosphorylates cMyBP-C at Ser302, which may mediate the latter effect.

Protein kinase D (PKD) is a serine/threonine kinase whose cardiovascular functions are becoming increasingly recognized (1). With regard to cardiac biology, Olson and colleagues were the first to show that neurohormonal activation of PKD in rat cardiomyocytes leads to the phosphorylation and nuclear export of class II histone deacetylase (HDAC) isoforms, such as HDAC5 (2), thereby initiating transcriptional reprogramming towards hypertrophy (2,3). Recently, the same group has shown that PKD is necessary for the full manifestation of pathologic cardiac remodelling following chronic pressure overload or neurohormonal stimulation in mice (4). Concurrently, work in our laboratory has provided evidence that PKD regulates cardiac myofilament function. We have identified several sarcomeric proteins, including the inhibitory subunit of cardiac troponin (cTnI) and cardiac myosin-binding protein C (cMyBP-C), as potential PKD substrates, and have shown that PKD induces cTnI dual phosphorylation at Ser22/Ser23, reduces myofilament Ca" sensitivity and accelerates isometric crossbridge cycle kinetics in rat permeabilized (“skinned”) ventricular myocytes (5). Subsequently, we reported that increased expression of PKD in intact rat ventricular myocytes by adenoviral gene transfer potentiates endothelin-1-induced cTnI phosphorylation at Ser22/Ser23, decreases myofilament Ca" sensitivity and abolishes the positive inotropic
response to this stimulus (6). Nevertheless, any causal link between PKD-mediated cTnI phosphorylation at Ser22/Ser23 and the regulation of myocardial contraction through reduced myofilament Ca\(^{2+}\) sensitivity and/or accelerated crossbridge cycle kinetics remained to be established.

In the present study, we have investigated the causal role of cTnI phosphorylation at Ser22/Ser23 in PKD-mediated regulation of myofilament Ca\(^{2+}\) sensitivity and crossbridge cycle kinetics, using myocardial preparations from transgenic mice that express cTnI containing substitutions of Ser22 and Ser23 by non-phosphorylatable Ala, on a cTnI-null background. Our data reveal that PKD decreases myofilament Ca\(^{2+}\) sensitivity through cTnI phosphorylation at Ser22/Ser23, but accelerates crossbridge cycle kinetics through a distinct mechanism. Our data also identify Ser302 in cMyBP-C as a novel PKD phosphorylation site that is likely to participate in PKD-mediated regulation of crossbridge cycle kinetics.

**EXPERIMENTAL PROCEDURES:**

Expanded methodology is provided in the Online Supplement.

**Preparation of mouse cardiac trabeculae**

Transgenic mice that express cTnI in which Ser22 and Ser23 are replaced by two non-phosphorylatable Ala residues (cTnI-Ala\(_2\)), on a cTnI-null background, have been described previously (7-9). Both cTnI-Ala\(_2\) mice and wild-type (WT) control mice were given the \(\beta\)-adrenoceptor antagonist propranolol (0.5 g/L) in their drinking water for 3 days prior to sacrifice, to minimize PKA-mediated protein phosphorylation during anesthesia and heart excision. Excised mouse hearts were frozen and stored in liquid N\(_2\), until they were thawed in relaxing solution. Trabeculae were then dissected from the right ventricle, skinned with Triton X-100 and stored in relaxing solution containing 50% glycerol at -20°C for up to 3 days (10,11), until they were used for the assessment of myofilament function.

**Assessment of myofilament function**

Mechanical function of skinned mouse cardiac trabeculae was assessed using equipment and protocols similar to those described previously for skinned myocyte preparations (5,12). Individual trabeculae were attached to a force transducer and a high-speed length controller at each end (Figure 1A) and the sarcomere length was set to ~2.2 \(\mu\)m in relaxing solution (18°C). The skinned trabecula was then transferred into a Ca\(^{2+}\) activation solution and Ca\(^{2+}\)-activated force was measured as the steady-state force achieved during the contraction minus the passive force recorded in relaxing solution. At steady-state force, crossbridge cycle kinetics were assessed by performing a release-restretch maneuver, using a modification of a previously described method (5,13). The muscle was rapidly slackened to ~80% length for 20 ms, to forcibly detach crossbridges during Ca\(^{2+}\) activation, and then restretched to its initial length (Figure 1B). Data obtained during force redevelopment following the release-restretch protocol were fitted to a single exponential curve to determine the rate of force redevelopment (\(k_r\)), as the index of crossbridge cycle kinetics. After the release-restretch protocol, the trabecula was returned to relaxing solution, and the sequence was then repeated in Ca\(^{2+}\) activation solutions containing progressively greater [Ca\(^{2+}\)] (\(p\)Ca range 9.0-4.5) (Figure 1C). Following measurements of Ca\(^{2+}\)-activated force and \(k_r\) in the basal state, the trabecula was incubated in relaxing solution containing PKD, PKA or no kinase (time-matched control) and the phosphatase inhibitor calyculin A, for 30 min at room temperature. Subsequently, the trabecula was transferred back into relaxing solution and the Ca\(^{2+}\)-activated force and \(k_r\) measurements were repeated. Therefore, each trabecula served as its own control, allowing paired comparison of pre- and post-kinase data.

**Assessment of myofilament protein phosphorylation**

Following the dissection of trabeculae, myocytes were mechanically dissociated from the remaining ventricular tissue by homogenization in a Waring blender and skinned for 15 min at 4°C (5). The skinned myocytes were then incubated in relaxing solution containing PKD, PKA or no kinase (time-matched control) and the phosphatase inhibitor calyculin A, for 30 min at room temperature. Subsequently, Laemmli sample buffer was added and myofilament phosphorylation status determined by SDS/PAGE and immunoblot analysis, using phospho-specific antibodies for cTnI and cMyBP-C (6). In complementary experiments, recombinant N-terminally His6-tagged cMyBP-C C1C2 fragments (14), in WT form or containing a Ser/Ala mutation at Ser273, Ser282 or Ser302, were incubated in kinase solution containing PKD or PKA for 20 min at 30°C, with phosphorylation status again assessed.
by SDS/PAGE and immunoblot analysis with phospho-specific antibodies.

**Immunolabelling and confocal microscopy**

Skinned myocytes were centrifuged onto polylysine-coated glass slides and fixed with 4 % paraformaldehyde. Unspecific binding sites were then blocked by incubation in 5 % non-specific goat serum, followed by incubation first with primary antibodies (rabbit anti-cMyBP-C [pSer302 or total] and mouse anti-α-actinin; overnight at 4°C) and then with secondary antibodies (goat Cy5-conjugated anti-rabbit and Cy3-conjugated anti-mouse; 4 h at room temperature). After final washing, the slides were mounted with coverslips in the presence of n-propyl gallate as an anti-fading reagent (15), and imaged on a Leica TCS SP5 confocal microscope equipped with a 63X/1.4NA oil immersion lens, using appropriate excitation and emission wavelengths.

**Data analysis**

All data are given as mean ± SEM. Statistical comparisons were by paired or un-paired Student’s t test, as appropriate, when comparing data between two groups, or by analysis of variance (ANOVA) followed by the Bonferroni test, when comparing data between multiple groups. P<0.05 was considered significant.

**RESULTS:**

**Effects of PKD on cTnI phosphorylation in WT and cTnI-Ala₂ myocardium**

We have shown previously that PKD phosphorylates cTnI at Ser22/Ser23 in both skinned and intact rat ventricular myocytes (5,6). In the present study, we examined PKD-mediated phosphorylation of cTnI in skinned ventricular myocytes from WT and cTnI-Ala₂ mice, by immunoblot analysis with a previously characterized phospho-specific antibody that recognizes dually phosphorylated (pSer22/23) cTnI (5). PKD induced a significant increase in cTnI phosphorylation at Ser22/Ser23 in WT myocytes and the magnitude of such phosphorylation was comparable to that achieved by PKA, an established cTnI kinase that targets the same sites (Figure 2A). Furthermore, the extent of cTnI phosphorylation observed in response to either kinase was not in excess of that observed in response to the endogenous sympathetic neurotransmitter norepinephrine in intact ventricular myocytes (Online Supplement Figure 1A). As expected, the pSer22/23 phospho-specific cTnI antibody failed to detect any phosphorylation in myocytes from cTnI-Ala₂ mice even after incubation with PKD or PKA (Figure 2B), confirming the utility of this mouse model in delineating the mechanistic roles of Ser22/Ser23 phosphorylation in the regulation of cardiac myofilament function.

**Effects of PKD on the Ca²⁺ sensitivity of force development in WT and cTnI-Ala₂ myocardium**

To determine the role of cTnI phosphorylation at Ser22/Ser23 in PKD-mediated regulation of myofilament Ca²⁺ sensitivity, Ca²⁺-activated force development was studied in skinned ventricular trabeculae from WT and cTnI-Ala₂ mice, before and after incubation with PKD. In WT trabeculae, PKD-mediated phosphorylation significantly decreased the Ca²⁺ sensitivity of force development, as indicated by a rightward shift of the force-pCa relationship and a significant reduction in pCa₅₀ after incubation with PKD (Figure 3A). In contrast, in trabeculae from cTnI-Ala₂ mice, incubation with PKD did not significantly reduce the Ca²⁺ sensitivity of force development (Figure 3B), indicating that PKD-mediated myofilament Ca²⁺ desensitization requires cTnI phosphorylation at Ser22/Ser23. Relative to PKD, incubation with PKA had a qualitatively similar impact on the Ca²⁺ sensitivity of force development in WT trabeculae (Figure 3A). However, although the PKA-mediated decrease in myofilament Ca²⁺ sensitivity was also attenuated in cTnI-Ala₂ trabeculae, the reduction in pCa₅₀ remained statistically significant (Figure 3B). Importantly, in time-matched control experiments, no significant reduction in the Ca²⁺ sensitivity of force development was observed in trabeculae from WT and cTnI-Ala₂ mice that were incubated for an equivalent time in the absence of either kinase (Online Supplement Table 1). Over the course of the experimental protocol (approximately 180 min), maximum force declined slightly (by ~ 10% in all groups) and the steepness of the force-pCa relationship (Hill coefficient) did not change in trabeculae from WT and cTnI-Ala₂ mice that were incubated with PKD, PKA or no kinase (Online Supplement Table 2).

**Effects of PKD on crossbridge cycle kinetics in WT and cTnI-Ala₂ myocardium**

We have shown previously that PKD-mediated phosphorylation accelerates crossbridge cycle kinetics in rat skinned ventricular myocytes (5). To determine the role of cTnI phosphorylation at Ser22/Ser23 in PKD-mediated acceleration of
crossbridge cycle kinetics, the rate of force redevelopment \( (k_r) \) after a release-restretch maneuver (Figure 1B) was determined at each \( p_{Ca} \) in skinned trabeculae from WT and cTnI-Ala2 hearts, before and after incubation with PKD. Since the level of active force is a major determinant of \( k_r \) and changes in myofilament \( Ca^{2+} \) sensitivity alter active force at each \( p_{Ca} \), we plotted the relationship between force and \( k_r \) in order to examine phosphorylation-induced changes in \( k_r \) (16). Following incubation of trabeculae with PKD, \( k_r \) was increased at submaximal forces, such that \( k_r \) at 50% maximal force was significantly greater post-PKD than pre-PKD, with little difference between the responses of trabeculae from WT mice (Figure 4A) and those from cTnI-Ala2 mice (Figure 4B). These findings indicate that PKD-mediated acceleration of crossbridge cycle kinetics does not require cTn phosphorylation at Ser22/Ser23 but occurs through a distinct mechanism. Qualitatively and quantitatively similar results were obtained in muscle preparations incubated with PKA, which increased \( k_r \) at 50% maximal force significantly in trabeculae from both WT and cTnI-Ala2 mice (Figures 4A and 4B). In time-matched control experiments, there was no significant change in \( k_r \) at 50% maximal force in trabeculae from WT and cTnI-Ala2 mice that were incubated for an equivalent time in the absence of either kinase (Online Supplement Table 1). As seen with maximum force, there was a small decline in maximum \( k_r \) (\( k_r \) at \( p_{Ca} \) 4.5) in trabeculae from WT and cTnI-Ala2 mice over the course of the experiment, but the extent of this did not differ between groups incubated with PKD, PKA or no kinase (Online Supplement Table 2).

**PKD-mediated phosphorylation of recombinant cMyBP-C C1C2 domain**

Recent evidence indicates that cMyBP-C phosphorylation is predominantly responsible for PKA-mediated acceleration of crossbridge cycle kinetics in mouse myocardium (9,17). PKA is known to phosphorylate cMyBP-C at three sites, namely Ser273, Ser282 and Ser302, within the conserved linker region between its immunoglobulin I-like C1 and C2 domains (14). Although we have shown previously that PKD phosphorylates a recombinant cMyBP-C fragment that contains this linker region (5), the specific PKD-targeted residue(s) have not been identified. To determine if PKD phosphorylates cMyBP-C at any of its established PKA phosphorylation sites, we performed in vitro kinase assays using recombinant cMyBP-C C1C2 fragments, in WT form or containing a Ser273Ala, Ser282Ala or Ser302Ala mutation, with phosphorylation detected using recently-developed phospho-specific cMyBP-C antibodies (18). Experiments with PKA-mediated phosphorylation confirmed the specificity of the pSer273, pSer282 and pSer302 phospho-specific cMyBP-C antibodies, with a robust signal detected with all three antibodies when WT C1C2 fragment was used as substrate and a loss of signal with each antibody only upon substitution of the pertinent Ser residue by Ala (Figure 5A). Interestingly, following PKD-mediated phosphorylation, WT C1C2 showed no increased signal with the pSer273 and pSer282 phospho-specific cMyBP-C antibodies, but a strongly increased signal with the pSer302 phospho-specific cMyBP-C antibody (Figure 5B). Furthermore, this signal was lost only upon targeted substitution of Ser302 by Ala in the substrate protein (Figure 5B). These findings indicate that PKD selectively phosphorylates Ser302 in the recombinant cMyBP-C C1C2 fragment.

**PKD-mediated phosphorylation of native cMyBP-C in WT and cTnI-Ala2 myocardium**

We next determined the pattern of PKD-mediated phosphorylation of native full-length cMyBP-C in skinned ventricular myocytes from WT and cTnI-Ala2 mice, using the pSer273, pSer282 and pSer302 phospho-specific cMyBP-C antibodies. In these experiments, PKD catalyzed significant phosphorylation of native cMyBP-C at Ser302, but not at Ser273 or Ser282, with no difference in the magnitude of the PKD-mediated Ser302 phosphorylation between myocytes from WT mice (Figure 6A) and those from cTnI-Ala2 mice (Figure 6B). In contrast, PKA led to significant phosphorylation of native cMyBP-C at all 3 sites, once again with comparable responses seen in myocytes from WT mice (Figure 6A) and cTnI-Ala2 mice (Figure 6B). These findings confirm that Ser302 in cMyBP-C is a novel target for PKD-mediated phosphorylation, and additionally indicate that cMyBP-C phosphorylation by PKD (selectively at Ser302) or by PKA (at Ser273, Ser282 and Ser302) is unaffected by Ala substitutions of Ser22/Ser23 in cTnI. Furthermore, the extent of cMyBP-C phosphorylation at Ser302 in response to either kinase was not in excess of that observed in response to norepinephrine in intact ventricular myocytes (Online Supplement Figure 1B).
Finally, we determined the sarcomeric localization of Ser302 phosphorylation in skinned myocytes from WT and cTnI-Ala2 mice, by immunolabelling and confocal microscopy. As expected, total cMyBP-C was detected as a doublet located either side of the M-line in the C-zones of the sarcomeric A-band, in myocytes from WT mice (Figure 7A, left panel). An identical pattern was observed in myocytes from cTnI-Ala2 mice (Figure 7A, right panel), confirming that Ala substitutions of Ser22/Ser23 in cTnI do not disrupt sarcomeric structure. Importantly, the pSer302 phospho-specific cMyBP-C antibody showed only weak labelling in control myocytes, but revealed a pattern that was identical to that seen with the total cMyBP-C antibody upon PKD- or PKA-mediated phosphorylation, with comparable images observed in myocytes from WT and cTnI-Ala2 mice (Figure 7B). Taken together, these experiments indicate that PKD phosphorylates cMyBP-C at Ser302, such phosphorylation occurs throughout the C-zones of sarcomeric M-bands, and Ala substitutions of Ser22/Ser23 in cTnI do not interfere with cMyBP-C phosphorylation by PKD or PKA. These findings raise the possibility that the common ability of PKD and PKA to accelerate the crossbridge cycle rate in skinned trabeculae from both WT and cTnI-Ala2 mice may arise from their common ability to induce cMyBP-C phosphorylation at Ser302.

DISCUSSION:

Multiple kinase pathways regulate cardiac myofilament function through the phosphorylation of sarcomeric proteins (19). Although much of the focus to date has been on PKA-mediated phosphorylation, other kinases such as protein kinase C isoforms, protein kinase G and Ca²⁺/calmodulin-dependent protein kinase have also been shown to play important regulatory roles (19). Previously, we have shown that PKD phosphorylates cTnI at Ser22/Ser23 (5,6), an observation that was subsequently confirmed by others (20), and reported that such phosphorylation is associated with reduced myofilament Ca²⁺ sensitivity (5,6) and accelerated crossbridge cycle kinetics (5). In the present study, we have used myocardial preparations from transgenic mice that express cTnI containing Ala substitutions of Ser22/Ser23, in order to explore the mechanistic importance of Ser22/Ser23 phosphorylation in PKD-mediated regulation of myofilament function. Our data provide the first direct evidence that cTnI phosphorylation at Ser22/Ser23 is responsible for PKD-mediated desensitization of myofilaments to Ca²⁺, but not for PKD-mediated acceleration of crossbridge cycle kinetics. Furthermore, we identify Ser302 in cMyBP-C as a novel PKD substrate and show that PKD phosphorylates Ser302 without affecting the phosphorylation status of the other known PKA target sites in cMyBP-C, at Ser273 and Ser282. These findings suggest that cMyBP-C phosphorylation at Ser302 may be a critical mechanism through which the crossbridge cycle rate is regulated.

Role of cTnI phosphorylation in PKD-mediated regulation of myofilament function

In the present study, we found that both PKD and PKA decreased myofilament Ca²⁺ sensitivity and accelerated crossbridge cycle kinetics in skinned trabeculae from WT mice (Figure 3A). These findings are consistent with our earlier work with PKD in rat skinned ventricular myocytes (5) and comparable studies with PKA by multiple laboratories in rat (21-23) and mouse (16,17,24) skinned myocardial preparations. The PKD-mediated reduction in myofilament Ca²⁺ sensitivity was abolished in skinned trabeculae from cTnI-Ala2 mice (Figure 3B), indicating that cTnI phosphorylation at Ser22/Ser23 is the molecular mechanism underlying this response. The PKA-mediated reduction in myofilament Ca²⁺ sensitivity was also attenuated in skinned trabeculae from cTnI-Ala2 mice, which is consistent with recent evidence from the Moss laboratory, who used skinned myocardium from the same mouse model (9). Notably, the small PKA-mediated reduction of pCa₅₀ (from 5.83±0.03 pre-PKA to 5.77±0.02 post-PKA) in cTnI-Ala₂ myocardium was statistically significant (Figure 3B), whereas the comparable effect reported by Stelzer et al. (9) was not (pCa₅₀ reduced from 5.77±0.03 pre-PKA to 5.73±0.03 post-PKA). This divergence may reflect a contribution from PKA-mediated phosphorylation of sarcomeric targets other than Ser22/Ser23 in cTnI under our experimental conditions, or other differences between the two studies, such as a higher pre-PKA pCa₅₀ in our work (5.83±0.03 versus 5.77±0.03 in Stelzer et al. (9)) or the use of distinct myocardial preparations (trabeculae versus mechanically-isolated multicellular preparations). In contrast to its marked effect on the PKD-mediated reduction in myofilament Ca²⁺ sensitivity, Ala substitution of Ser22/Ser23 in cTnI had no impact on PKD-mediated acceleration of crossbridge cycle kinetics (Figure 4), indicating that phosphorylation of these sites is not the mechanism through which the latter effect is...
achieved. PKA-mediated acceleration of crossbridge cycle kinetics was also of similar magnitude in skinned trabeculae from WT and cTnI-Ala2 mice (Figure 4), which is consistent with the findings of Stelzer et al. (9) and again points to an underlying mechanism that is distinct from cTnI phosphorylation at Ser22/Ser23.

Role of cMyBP-C phosphorylation in PKD-mediated regulation of myofilament function

cMyBP-C is localized to the crossbridge-containing C-zones of cardiac sarcomeric A-bands, where it interacts with multiple other proteins, including myosin subfragment-2 (S2) at its N-terminus and titin at its C-terminus, and has been ascribed roles in both physiology and disease (25). Recent studies by the Moss laboratory in two different genetically-modified mouse models, one with targeted deletion of the cMyBP-C gene Mybpc3 (9) and the other expressing cMyBP-C in which three Ser residues (Ser273, Ser282 and Ser302) were replaced by non-phosphorylatable Ala (17), have provided robust evidence that cMyBP-C phosphorylation plays an essential role in PKA-mediated acceleration of crossbridge cycle kinetics. In this context, our previous work has shown that PKD can phosphorylate a recombinant cMyBP-C fragment (5), which included the PKA phosphorylation sites at Ser273, Ser282 and Ser302 that were originally identified by Gautel et al. in human cMyBP-C (14). However, our subsequent studies suggested that Ser282 in native cMyBP-C of rat myocytes was unlikely to be a PKD target (6). Our present findings provide novel evidence that PKD phosphorylates the recombinant cMyBP-C C1C2 fragment at Ser302 and, unlike PKA, induces no detectable phosphorylation at Ser273 or Ser282 (Figure 5). Furthermore, PKD induces selective phosphorylation of Ser302 also in native cMyBP-C that is incorporated into the sarcomeric lattice of mouse skinned myocytes (Figure 6). These findings identify Ser302 in cMyBP-C as a novel PKD substrate. In the light of this finding, it is pertinent to note that, of the three PKA sites in cMyBP-C, only the Ser302 motif (297LeuLysLysArgAspSer302) conforms to the optimal PKD phosphorylation motif, which favours an aliphatic amino acid (Leu, Val or Ile) at the -5 position and a basic amino acid (Arg or Lys) at the -3 position, relative to the targeted Ser residue (26). Our observation that PKD-mediated Ser302 phosphorylation appears to occur throughout the C-zones of sarcomeric A-bands (Figure 7) suggests that it may have functional consequences. Indeed, the quantitatively and spatially similar effects of PKD and PKA on cMyBP-C phosphorylation at Ser302 in skinned myocytes from both WT and cTnI-Ala2 mice, despite their markedly different effects on cMyBP-C phosphorylation at Ser273 and Ser282 in these preparations, suggests that this may be the mechanism underlying their common ability to accelerate crossbridge cycle kinetics in the presence and absence of cTnI phosphorylation at Ser22/Ser23. It also suggests that the two proteins are phosphorylated independently of each other.

PKA-mediated phosphorylation of cMyBP-C is believed to accelerate crossbridge cycle kinetics by relieving a tether-like constraint imposed on myosin heads by cMyBP-C binding to myosin, which then increases their proximity to, and probability of interaction with, actin (27). An alternative mechanism, the switch of an actin binding site, was also recently proposed (28); however, this interaction would need to be significantly weaker in order not to impede actomyosin filament sliding, which is in agreement with the strict myosin filament localisation of N-terminal cMyBP-C fragments in cardiomyocytes (29). The relative importance of the individual PKA phosphorylation sites at Ser273, Ser282 and Ser302 in regulating cMyBP-C binding to myosin (or actin) is unknown, although there is evidence that suggests an ordered phosphorylation of these sites by PKA, with Ser282 phosphorylation occurring before phosphorylation of the other sites (14,30). The N-terminal myosin-binding region of cMyBP-C, which interacts with myosin at its S2 segment in a phosphorylation-regulated manner, comprises the immunoglobulin I-like domains C1 and C2 connected by a presumably flexible linker that contains Ser273, Ser282 and Ser302. Based on their structural analyses of the myosin S2 interactions of cMyBP-C domains C1 and C2 (31,32), Ababou et al. have recently proposed a model in which the C1-C2 linker extends towards the C-terminus of S2, to reach the area around residue 930 (32). In this model, the phosphorylation sites at Ser273, Ser282 and Ser302 appose negatively-charged patches on the surface of S2, such that their phosphorylation would lead to charge repulsion and destabilization of the interaction (32). In the context of our present findings regarding the potential importance of Ser302 phosphorylation in regulating crossbridge cycle kinetics, it is intriguing to note that a Glu924Lys mutation in the S2 region that was proposed to appose Ser302, which is associated with familial hypertrophic cardiomyopathy,
abolishes C1C2 binding to S2 (33), without affecting the binding of either the C1 or the C2 domain alone (32). It appears therefore that the interaction between this region of S2 and the Ser302-containing region of the C1-C2 linker in cMyBP-C may be critical in maintaining the binding of the cMyBP-C N-terminus to myosin, such that interference with this interaction (for example, as a result of S2 mutation or cMyBP-C phosphorylation at Ser302) may be sufficient to have structural and functional consequences, providing a likely mechanism for PKD-mediated acceleration of crossbridge cycle kinetics. Further investigation is clearly required to test this hypothesis. It will also ultimately be necessary to determine the atomic structure of the phosphorylated linker, which was recently proposed to adopt a compact conformation (34). Of broader potential physiological significance, a series of studies by Sadayappan et al. (18,35,36) in genetically modified mice that express cMyBP-C with non-phosphorylatable or phospho-mimetic amino acid substitutions at Ser273, Ser282 and Ser302 suggest that the functional importance of cMyBP-C phosphorylation extends beyond the regulation of crossbridge cycle kinetics, for example to determining post-ischemic cardiac function. Once again, further investigation is required to determine the contributions of individual phosphorylation sites, including Ser302, to such functional consequences of cMyBP-C phosphorylation.

Relative roles of PKD versus PKA in myofilament phosphorylation

In the present study, we treated WT and cTnI-Ala2 mice with propranolol, to minimize PKA-mediated protein phosphorylation arising from sympathetic activation during anesthesia and surgical excision of the heart. Such an approach has been used previously by us (5) and by other investigators (23,37) in exploring the functional consequences of kinase-mediated myofilament protein phosphorylation in skinned preparations. Performing experiments using hearts from untreated mice would not provide useful information on the functional consequences of PKD-mediated phosphorylation of sites that are targeted also by PKA, since those sites would be already occupied. Thus, the use of propranolol prevents the very high levels of PKA-induced phosphorylation that are an artefact of the anesthesia and dissection procedure and which cannot be present in the resting animal (otherwise sympathetic stimulation would have no effect on the heart).

As we have suggested recently (1), PKD-mediated phosphorylation of substrate proteins that are targeted also by PKA at the same site(s) is likely to be of greater functional significance in settings where the PKA pathway is down-regulated, such as in heart failure. Other observations that suggest a greater role for PKD in the heart failure setting include our recent finding that PKA inhibits PKD activation (38) and reports that PKD expression is increased in failing myocardium, as recently reviewed (1).

In conclusion, our study provides direct evidence that PKD decreases myofilament Ca2+ sensitivity as a consequence of cTnI phosphorylation at Ser22/Ser23, but accelerates crossbridge cycle kinetics through a distinct mechanism. It also identifies Ser302 in cMyBP-C as a novel PKD phosphorylation site, which provides a potential mechanism through which PKD may regulate crossbridge cycle kinetics. These observations add to the growing evidence that PKD-mediated pathways regulate cardiac myofilament function through coordinated phosphorylation of multiple sarcomeric proteins.

REFERENCES:

FOOTNOTES:
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FIGURE LEGENDS:

Figure 1. Ca\(^{2+}\) activated force and force redevelopment in mouse skinned trabeculae. (A) A skinned trabecula, attached at either end to a force transducer and a high-speed length controller. (B) Representative recording of force redevelopment after a release-restretch protocol at pCa 5.61. (C) Representative recording of Ca\(^{2+}\)-activated force in bath solutions at pCa 5.81, 5.61 and 5.29.

Figure 2. Effects of PKD and PKA on cTnI phosphorylation at Ser22/Ser23 in skinned ventricular myocytes from (A) WT and (B) cTnI-Ala\(^{2}\) mice. The representative immunoblots show PKD- or PKA-mediated cTnI phosphorylation as detected by a phospho-specific pSer22/23 cTnI antibody, with protein loading illustrated by Coomassie staining of the membranes. The bar charts show quantitative data from such experiments using multiple skinned myocyte preparations (4 hearts per group). cTnI phosphorylation is expressed relative to a common positive control sample included in each experiment. *\(P < 0.05\) versus no-kinase control (con).

Figure 3. Effects of PKD and PKA on the Ca\(^{2+}\) sensitivity of force development in skinned ventricular trabeculae from (A) WT and (B) cTnI-Ala\(^{2}\) mice. The mean force-pCa curves show data obtained before (pre; open circles) and after (post; filled circles) incubation of trabeculae with PKD or PKA, as indicated. Force values were normalized to the maximum force, measured at pCa 4.5. The bar charts show mean pCa\(_{50}\) values obtained before (pre; open bars) and after (post; filled bars) incubation of trabeculae with PKD or PKA, as indicated. *\(P < 0.05\) versus pre-kinase (\(n=7\) per group).

Figure 4. Effects of PKD and PKA on the rate of force redevelopment (k\(_{tr}\)) in skinned ventricular trabeculae from (A) WT and (B) cTnI-Ala\(^{2}\) mice. The force-k\(_{tr}\) relationships show data obtained before (pre; open circles) and after (post; filled circles) incubation of trabeculae with PKD or PKA, as indicated. Force values were normalized to the maximum force and k\(_{tr}\) values were normalized to the maximum k\(_{tr}\), both measured at pCa 4.5. The bar charts show mean relative k\(_{tr}\) at 50% maximum force obtained before (pre; open bars) and after (post; filled bars) incubation of trabeculae with PKD or PKA, as indicated. *\(P < 0.05\) versus pre-kinase (\(n=7\) per group).

Figure 5. Phosphorylation by (A) PKA or (B) PKD of recombinant His6-tagged C1C2 fragments of human cMyBP-C, in wild-type form or with a Ser/Ala substitution at Ser273, Ser282 or Ser302. Phosphorylation was detected by immunoblot analysis using phospho-specific pSer273, pSer282 or pSer302 cMyBP-C antibodies, with protein loading illustrated by Coomassie staining of the membranes.

Figure 6. Effects of PKD and PKA on cMyBP-C phosphorylation at Ser273, Ser282 and Ser302 in skinned ventricular myocytes from (A) WT and (B) cTnI-Ala\(^{2}\) mice. The representative immunoblots show PKD- or PKA-mediated cMyBP-C phosphorylation as detected by phospho-specific pSer273, pSer282 and pSer302 cMyBP-C antibodies, with protein loading illustrated by Coomassie staining of the membranes. The bar charts show quantitative data from such experiments using multiple skinned myocyte preparations (4 hearts per group). cMyBP-C phosphorylation is expressed relative to a common positive control sample included in each experiment. *\(P < 0.05\) versus no-kinase control (con).

Figure 7. Confocal microscope images showing the localization of (A) total cMyBP-C and (B) cMyBP-C phosphorylated at Ser302, in skinned myocytes from WT and cTnI-Ala\(^{2}\) (Ala2) mice, following incubation with no kinase (CON), PKD or PKA. Samples were immunolabelled additionally with an α-actinin antibody, to demarcate the Z-discs, and nuclei were stained with DAPI. The images were obtained from perinuclear regions of each sample. In the merged images, red indicates α-actinin labelling, green indicates cMyBP-C (total and pSer302) labelling, and the nuclei are stained blue. Scale bar 10\(\mu\)m.
Figure 1

A. Skinned mouse trabecula

B. Force redevelopment

C. Ca^{2+} activated force
Figure 2

A. cTnI phosphorylation in WT myocardium

IB: pS22/23 cTnI

Coomassie stain

Relative S22/23 phosphorylation

B. cTnI phosphorylation in cTnI-Ala₂ myocardium

IB: pS22/23 cTnI

Coomassie stain

Relative S22/23 phosphorylation
Figure 3

A. Effects of PKD and PKA phosphorylation on Ca\(^{2+}\) sensitivity of force development in WT myocardium

B. Effects of PKD and PKA phosphorylation on Ca\(^{2+}\) sensitivity of force development in cTnI-Ala\(_2\) myocardium
Figure 4

A. Effects of PKD and PKA phosphorylation on $k_r$ in WT myocardium

B. Effects of PKD and PKA phosphorylation on $k_r$ in cTnI-Ala$_2$ myocardium
**Figure 5**

**A. PKA phosphorylation of His\textsubscript{6}-C1C2**

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Coomassie stain

**B. PKD phosphorylation of His\textsubscript{6}-C1C2**

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Coomassie stain
Figure 6

A. cMyBP-C phosphorylation in WT myocardium

IB:pS273 cMyBP-C

Coomassie stain

IB:pS282 cMyBP-C

Coomassie stain

IB:pS302 cMyBP-C

Coomassie stain

C. MyBP-C phosphorylation in cTnI-Ala2 myocardium

IB:pS273 cMyBP-C

Coomassie stain

IB:pS282 cMyBP-C

Coomassie stain

IB:pS302 cMyBP-C

Coomassie stain
A. Localization of total cMyBP-C (labeled with non-phosphospecific antibody)

B. Localization of phosphorylated cMyBP-C (labeled with pS302 phosphospecific antibody)
Distinct sarcomeric substrates are responsible for protein kinase D-mediated regulation of cardiac myofilament Ca\(^{2+}\) sensitivity and crossbridge cycling
Sonya C. Bardswell, Friederike Cuello, Alexandra J. Rowland, Sakthivel Sadayappan, Jeffrey Robbins, Mathias Gautel, Jeffery W. Walker, Jonathan C. Kentish and Metin Avkiran

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