Acceleration of Crossbridge Kinetics by Protein Kinase A Phosphorylation of Cardiac Myosin Binding Protein C Modulates Cardiac Function

Carl W. Tong, Julian E. Stelzer, Marion L. Greaser, Patricia A. Powers, Richard L. Moss

Abstract—Normal cardiac function requires dynamic modulation of contraction. β1-Adrenergic–induced protein kinase (PKA) phosphorylation of cardiac myosin binding protein (cMyBP-C) may regulate crossbridge kinetics to modulate contraction. We tested this idea with mechanical measurements and echocardiography in a mouse model lacking 3 PKA sites on cMyBP-C, ie, cMyBP-C(t3SA). We developed the model by transgenic expression of mutant cMyBP-C with Ser-to-Ala mutations on the cMyBP-C knockout background. Western blots, immunofluorescence, and in vitro phosphorylation combined to show that non–PKA-phosphorylatable cMyBP-C expressed at 74% compared to normal wild-type (WT) and was correctly positioned in the sarcomeres. Similar expression of WT cMyBP-C at 72% served as control, ie, cMyBP-C(tWT). Skinned myocardium responded to stretch with an immediate increase in force, followed by a transient relaxation of force and finally a delayed development of force, ie, stretch activation. The rate constants of relaxation, \( k_{d1} \) (s-1), and delayed force development, \( k_{d2} \) (s-1), in the stretch activation response are indicators of crossbridge cycling kinetics. cMyBP-C(t3SA) myocardium had baseline \( k_{d1} \) and \( k_{d2} \) similar to WT myocardium, but, unlike WT, \( k_{d1} \) and \( k_{d2} \) were not accelerated by PKA treatment. Reduced dobutamine augmentation of systolic function in cMyBP-C(t3SA) hearts during echocardiography corroborated the stretch activation findings. Furthermore, cMyBP-C(t3SA) hearts exhibited basal echocardiographic findings of systolic dysfunction, diastolic dysfunction, and hypertrophy. Conversely, cMyBP-C(tWT) hearts performed similar to WT. Thus, PKA phosphorylation of cMyBP-C accelerates crossbridge kinetics and loss of this regulation leads to cardiac dysfunction. (Circ Res. 2008;103:974-982.)

Key Words: cMyBP-C ■ phosphorylation ■ contraction kinetics

The strength and kinetics of cardiac contraction vary on a beat-to-beat basis as a way to match cardiac output to the circulatory demands of the body. Reduced capacity to modulate contraction has long been recognized as a key feature of dysfunction in heart failure and more recently in hypertrophic cardiomyopathy (HCM). This study explores the possibility that phosphorylation of cardiac myosin binding protein (cMyBP-C) modulates contraction in skinned and living myocardium.

MyBP-C is a component of the thick filament in striated muscle and is evident as 7 to 9 bands at 43-nm intervals within the center of each half-thick filament in the A-band. Its location at every third crossbridge crown, ie, every 42.9 nm suggests that cMyBP-C has a regulatory role with respect to thick filament activity. Unlike the skeletal muscle isoform, cMyBP-C is readily phosphorylated by protein kinase (PK)A, calcium calmodulin kinase (CaMK)II, and PKC. Phosphorylation of cMyBP-C may promote actin–myosin interactions by either relieving a structural constraint on myosin to allow closer proximity with actin or reducing the binding of cMyBP-C to the S2 domain of myosin to allow greater flexibility of the rod that links the myosin head to the thick filament. Thus, phosphorylation of cMyBP-C may affect cardiac function by regulating crossbridge kinetics.

Evidence suggests that cMyBP-C and its phosphorylation status are critical to regulation of cardiac function. For example, mutations of cMyBP-C comprise the most prevalent cause of familial hypertrophic cardiomyopathy. Phosphorylation of cMyBP-C is reduced in human heart failure and atrial fibrillation. In animal models, increased cMyBP-C phosphorylation has been associated with reduced infarct size, whereas decreased phosphorylation is associated with dysfunction in failure and with either dysfunction or preservation of function in ischemia.

A previously developed cMyBP-C knockout (KO) mouse provided initial indications concerning the roles of cMyBP-C phosphorylation in the heart. For example, skinned myocardium from cMyBP-C KO hearts exhibits constitutively accelerated crossbridge kinetics similar to wild-type (WT) hearts after treatment with PKA, suggesting that phosphorylation of cMyBP-C accelerates crossbridge kinetics in response to β1-adrenergic stimulation. Hearts from cMyBP-C
KO mice exhibit hypertrophy, diastolic dysfunction, and systolic dysfunction.22 In contrast, PKA accelerates crossbridge kinetics in skinned myocardium from mouse hearts expressing mutant non–PKA-phosphorylatable cardiac tropo-
in (cTn)I, ie, cTnIala2.24 Unlike cMyBP-C KO hearts, cTnIala2 hearts exhibit normal function.24 These results narrow the mechanism of acceleration of crossbridge kinetics by PKA phosphorylation to cMyBP-C and suggest that cMyBP-C phosphorylation is important for normal cardiac function.

We hypothesized that PKA phosphorylation of cMyBP-C in response to β1-adrenergic stimulation accelerates crossbridge kinetics in vivo, whereas the loss of this regulatory mechanism leads to cardiac dysfunction. To test this idea, we transgenically expressed constitutively non–PKA-phosphorylatable cMyBP-C on the cMyBP-C–null background. We found that skinned myocardium from these hearts no longer exhibits acceleration of contraction kinetics in response to treatment with PKA. Furthermore, hearts with non–PKA-phosphorylatable cMyBP-C exhibit hypertrophy, diastolic dysfunction, systolic dysfunction, and reduced contractile reserve.

**Materials and Methods**

**Mouse Lines**

N-terminal myc-tagged WT or non–PKA-phosphorylatable cMyBP-C was expressed on the cMyBP-C–null background. Gautel et al25 previously identified three PKA phosphorylation sites in human cMyBP-C at Ser273, Ser282, and Ser302; the homologous sites in mouse cMyBP-C are Ser273, Ser282, and Ser302. Here, all 3 residues were mutated to alanine (Figure 1A), and the α-myosin heavy chain promoter was used to promote expression of the mutant transgene in the FVB/N mouse strain. FVB/N mice expressing the transgene were bred with the previously generated cMyBP-C KO mouse (E129X1 strain)22 to ensure expression of the exogenous transgene without competition from native cMyBP-C. WT cMyBP-C was also expressed in cMyBP-C KO mice to serve as a control. t3SA denotes transgenic expression of the Ser-to-Ala mutant cMyBP-C and tWT denotes transgenic expression of WT cMyBP-C. The protocols for care and use of animals were approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health.

**Myofibril Preparation**

Myofibril homogenates were prepared from hearts for subsequent analysis using electrophoresis, Western blotting, immunofluorescence, and in vitro PKA phosphorylation (see the online data supplement, available at http://circres.ahajournals.org).

**Western Blots**

Western blots were used to determine total expression of transgenes using a rabbit polyclonal anti–cMyBP-C antibody22 at a dilution of 1:10 000 and a mouse monoclonal anti-myc (Upstate 05-419 clone 9E10) antibody at 1:250 dilution (see the online data supplement).

**Immunofluorescence**

Immunofluorescence labeling of myofibrils was used to verify incorporation of transgenic cMyBP-C into the thick filament. Rabbit anti–cMyBP-C polyclonal antibody22 at a dilution of 1:10 000 and a mouse monoclonal anti-myc (Upstate 05-419 clone 9E10) antibody at 1:250 dilution were paired with Alexa-Fluor647 (Molecular Probes) goat anti-rabbit secondary antibody at 1:250. Mouse monoclonal anti-myc (Upstate 05-419 clone 9E10) antibody at 1:250 was paired with Alexa-Fluor488 (Molecular Probes) goat anti-mouse secondary antibody at 1:250. (See the online data supplement.)

**PKA Phosphorylation of Myofibrillar Proteins**

Exogenous catalytic subunit of PKA (Sigma P2645) was used to phosphorylate cMyBP-C and cTnI in isolated myofibrils and in the preparations used for mechanical measurements (see the online data supplement). The ratio of enzyme to total myofibrillar protein (wt/wt) was 0.05 (1 U/µL PKA according to Sigma specifications for activity).

**Comparison of Phosphorylation Levels**

For each phosphorylation reaction, gel (10% polyacrylamide) lanes were loaded with different volumes of myofibrils at known concen-
trations. Pro-Q Diamond (Molecular Probes, phosphoprotein stain) and Sypro-Ruby (Molecular Probes, total protein stain) were used to compare phosphorylation levels of cMyBP-C and cTnI. Slopes of Pro-Q intensity versus protein load and slopes of Sypro-Ruby intensity versus protein load were calculated for each experiment. Double ratio “PL(experiment)/WTbaseline)” of Pro-Q/protein slope of Sypro/Protein slope of an experiment in comparison to WT baseline provided a semiquantitative assessment (see the online data supplement).

\[
\frac{PL_{\text{experiment}}}{WT_{\text{baseline}}} = \frac{[\text{ProQ_slope(experiment)}]/[\text{ProQ_slope(WTbaseline)}]}{[\text{Sypro_slope(experiment)}]/[\text{Sypro_slope(WTbaseline)}]}
\]

**Stretch Activation and Force Versus pCa Measurements**

Stretch activation was assessed in skinned myocardium23 with or without exogenous PKA (Sigma P2645) to quantify the effects of PKA phosphorylation of regulatory proteins on crossbridge kinetics. This was done by setting pCa (pCa = -log10([Ca^2+])) to achieve half-maximal force, setting sarcomere length at 2.1 μm, and applying a rapid stretch of 1% of fiber length (see the online data supplement). Force values were measured at various pCa to characterize force–pCa relationships.26

**Heart and Body Weight Determinations**

Heart weight/body weight ratios were determined as an indicator of cardiac hypertrophy. Once anesthetized, mice (2 to 6 months of age) were weighed to determine total body weight. Hearts were then removed, all major vessels and connective tissues were dissected away, and the heart including the atrial appendage was carefully blotted and weighed.

**Echocardiography**

Echocardiography was done using a Visualsonics Vevo 770 system with a 30-MHz probe to record intact in vivo cardiac structure and function using M-mode, B-mode, blood flow Doppler, tissue Doppler, and ECG-gated reconstruction of left ventricle changes during a cardiac cycle (EKV mode). Dobutamine (10 μg/g) was administered by IP injection to elicit a maximum β1 response that was sustained for 15 minutes27 to maximize in vivo PKA phosphorylation of regulatory proteins on crossbridge kinetics. Without exogenous PKA (Sigma P2645) to quantify the effects of PKA activity was similar between experiments; consequently, the lack of phosphorylation of mutant cMyBP-C could not be attributed to low PKA activity.

**Results**

**cMyBP-C Transgene Expression**

Double positive staining of anti-cMyBP-C and anti-myc Western blots verified expression of transgenes that were introduced into the KO mice (Figure 1B). WT myocardium showed positive staining for anti-cMyBP-C but not anti-myc, whereas KO showed no positive staining. The mouse lines 309 (t3SA) and 519 (WT) chosen for these experiments had closely matched expression of transgenic cMyBP-C, ie, 74%±2 and 72%±3 of control, respectively (Figure 1C).

**Incorporation of cMyBP-C**

The expressed cMyBP-C appeared to be appropriately positioned in both cMyBP-C(t3SA) and cMyBP-C(tWT) myocardium. Immunofluorescent labeling of myofibrils with anti-cMyBP-C/Alexa-Fluor 488 and anti-myc/Alexa-Fluor 647 showed that anti-cMyBP-C and anti-myc fluorescence images yielded matches for both cMyBP-C(t3SA) and cMyBP-C(tWT) but not in KO myocardium (Figure 2A through 2D).

**PKA Phosphorylation of cMyBP-C and cTnI**

ProQ Diamond phosphoprotein staining showed that PKA did not phosphorylate the mutant cMyBP-C in cMyBP-C(t3SA) myocardium but phosphorylated cMyBP-C in both cMyBP-C(tWT) and WT myocardium (Figure 3 and Table 1). Conversely, PKA treatment increased phosphorylation of cTnI in all lines to essentially equal levels (Figure 3). The similar PKA phosphorylation of cTnI in all mouse lines showed that PKA activity was similar between experiments; consequently, the lack of phosphorylation of mutant cMyBP-C could not be attributed to low PKA activity.
Stretch Activation of Myocardium

Stretch activation at 50% maximal force in cMyBP-C(t3SA) skinned myocardium differed from that in both WT and cMyBP-C(tWT) myocardium (Figure 4A and 4B and Table 1) but only after PKA treatment. Before PKA treatment, cMyBP-C(t3SA) myocardium exhibited similar rates of force decay (k_rel) and delayed force development (k_df) as in WT and cMyBP-C(tWT) myocardium (Table 1). Unlike WT and cMyBP-C(tWT), PKA treatment had no effect on either phase of the stretch activation response in cMyBP-C(t3SA) in that the rate constants k_rel and k_df were unchanged after PKA treatment. Thus, the acceleration of contraction kinetics in WT myocardium requires phosphorylation of cMyBP-C but does not involve cTnI.

As shown in the recording in Figure 4C, cMyBP-C(t3SA) myocardium exhibited slower rates of force decay and delayed force development in comparison to KO myocardium both before and after PKA treatment (Table 1). As previously reported,23 contraction kinetics in KO were unaffected by PKA (Table 1).

Table 1. Effects of PKA Treatment on Skinned Myocardium

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cMyBP-C(tWT)</th>
<th>cMyBP-C(t3SA)</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stretch activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>k_rel (sec⁻¹)</td>
<td>230±12†</td>
<td>232±19†</td>
<td>222±15†</td>
<td>338±20*</td>
</tr>
<tr>
<td>k_rel (sec⁻¹)+PKA</td>
<td>330±12†</td>
<td>303±19†</td>
<td>220±21†</td>
<td>327±28</td>
</tr>
<tr>
<td>k_df (sec⁻¹)</td>
<td>18.2±1.7†</td>
<td>21.2±1.1†</td>
<td>22.5±1.6†</td>
<td>29.9±1.7*</td>
</tr>
<tr>
<td>k_df (sec⁻¹)+PKA</td>
<td>29.1±1.6†</td>
<td>29.4±1.4†</td>
<td>22.5±1.6†</td>
<td>29.6±1.6</td>
</tr>
</tbody>
</table>

| Force–pCa         |          |              |               |          |
| n                | 8        | 8            | 8             | 8        |
| pCa50            | 5.76±0.02| 5.75±0.02    | 5.76±0.02     | 5.73±0.02|
| pCa50+PKA        | 5.65±0.03†| 5.65±0.02†  | 5.68±0.03†    | 5.65±0.02†|

| cMyBP-C phosphorylation |          |              |               |          |
| n                | 8        | 4            | 4             | 4        |
| PL, experiment/WTbaseline| 1    | 0.71±0.03*  | 0.42±0.11*    | NA       |
| PL, (experiment+PKA)/WTbaseline| 2.97±0.25†| 2.42±0.40†  | 0.48±0.08*    | NA       |

Data are means±SEM. See the online data supplement for calculation of k_rel, k_df, and PL (experiment/WTbaseline). For force–pCa relationships, normalized force vs pCa data points were fitted to Hill’s equation:

\[
\frac{[Ca^{2+}]^n}{[K_{50}]^n+[Ca^{2+}]^n}
\]

where \(K_{50}\) is \([Ca^{2+}]\) at 50% force, \(N\) is the Hill coefficient, and \(pCa_{50} = -\log_{10}[K_{50}]\). NA indicates not applicable. *Significantly different from WT with the same PKA treatment (ie, with or without), \(P<0.05\); †significantly different from its own control without PKA, \(P<0.05\); ‡significantly different from KO with the same PKA treatment (ie, with or without), \(P<0.05\).
cMyBP-C(t3SA) exhibited a small increase in β myosin heavy chain (βMHC) content, i.e., <10% of total MHC, which is significantly less than the 13% to 19% βMHC in KO myocardium (Figure I in the online data supplement). All 4 lines of mice showed similar levels of titin phosphorylation (supplemental Figure II). Thus, neither βMHC expression nor titin phosphorylation can account for the observed differences in kinetics.

**Force–pCa Relationships**
Myocardium from all 4 lines exhibited similar steady-state force responses to variations in activating calcium, both at baseline and following treatment with PKA (Table 1). Figure 4D shows force–pCa relationships for cMyBP-C(t3SA) and cMyBP-C(tWT) myocardium. PKA treatment decreased pCa50 within each line; however, there were no differences between the lines either at baseline or following PKA treatment (see supplemental Table II). Thus, the PKA-induced decrease in Ca$^{2+}$ sensitivity of force does not involve phosphorylation of cTnI but instead is attributable to phosphorylation of cTnL.

**Heart Weight/Body Weight Ratio**
Both cMyBP-C(t3SA) and cMyBP-C(tWT) mice exhibited a generally normal physical appearance and no evidence of early mortality. However, cMyBP-C(t3SA) and KO mice had significantly increased heart weight/body weight ratios compared to ratios for WT and cMyBP-C(tWT) mice, indicating cardiac hypertrophy (Table 2).

**Echocardiography**
Echocardiography demonstrated similar hypertrophy and severe global dysfunction in cMyBP-C(t3SA) and KO hearts (Table 2 and Figure 5). Dramatically increased posterior wall thickness during diastole with normal left ventricular (LV) inner diameter indicated hypertrophy. Increased IVRT indicates a longer time for LV relaxation before the start of filling during diastole, implying impaired relaxation. The ratio of the blood flow Doppler peak (E) at mitral inflow to the tissue movement Doppler peak (e') at the lateral mitral annulus (E/e') provided another index of diastolic function. The
increased E/e' ratio indicated that the rates of myocardial relaxation were slowed, and there was diastolic dysfunction in these hearts. Reduced fractional shortening (FS) under basal conditions indicated systolic dysfunction in both cMyBP-C(t3SA) and KO hearts.

Dobutamine treatment showed that cMyBP-C(t3SA) and KO hearts have different levels of reduced contractile reserve (Table 2). Dobutamine increased FS and EF in WT and cMyBP-C(tWT) hearts to the same maximal level. However, dobutamine increased FS and EF of cMyBP-C(t3SA) hearts to lower levels than either WT or cMyBP-C(tWT) hearts, which indicates reduced contractile reserve. KO hearts exhibited smaller increases in both EF and FS than did cMyBP-C(t3SA) hearts, indicating that KO hearts had the lowest contractile reserve of all the lines, i.e., the rank order of contractile reserve was WT>cMyBP-C(tWT)>cMyBP-C(t3SA)>KO.

Table 2. Functional and Physical Measurements of Hearts

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cMyBP-C(tWT)</th>
<th>cMyBP-C(t3SA)</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>412±25</td>
<td>424±57</td>
<td>427±45</td>
<td>453±59</td>
</tr>
<tr>
<td>HR (bpm) with dobutamine</td>
<td>615±31†</td>
<td>673±35†</td>
<td>651±30†</td>
<td>652±23†</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.7±0.1</td>
<td>4.0±0.1</td>
<td>4.0±0.1</td>
<td>4.2±0.2</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.6±0.2‡</td>
<td>3.0±0.2</td>
<td>3.2±0.1</td>
<td>3.5±0.2*</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>0.8±0.1‡</td>
<td>1.0±0.1‡</td>
<td>1.5±0.1*</td>
<td>1.3±0.1*</td>
</tr>
<tr>
<td>PWs (mm)</td>
<td>1.0±0.1†</td>
<td>1.2±0.1†</td>
<td>1.6±0.1*</td>
<td>1.5±0.1*</td>
</tr>
<tr>
<td>IVRT (ms)</td>
<td>15.3±1.3‡</td>
<td>16.0±1.0‡</td>
<td>22.5±1.6*</td>
<td>24.8±1.4*</td>
</tr>
<tr>
<td>E/e'</td>
<td>10.7±1.7‡</td>
<td>18.1±2.1‡</td>
<td>30.1±3.9*</td>
<td>30.7±2.8*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>30.3±2.8‡</td>
<td>24.6±2.5</td>
<td>20.5±1.3*</td>
<td>17.2±1.2*</td>
</tr>
<tr>
<td>FS (%) with dobutamine</td>
<td>55.3±5.0††</td>
<td>47.3±3.5‡†</td>
<td>38.1±1.7‡†</td>
<td>24.1±2.2††</td>
</tr>
<tr>
<td>EF (%)</td>
<td>49.8±3.2‡</td>
<td>46.1±3.0</td>
<td>38.6±2.7</td>
<td>35.2±3.0*</td>
</tr>
<tr>
<td>EF (%) with dobutamine</td>
<td>91.0±1.0††</td>
<td>88.6±1.8†‡</td>
<td>74.7±3.0†‡</td>
<td>55.8±5.7†‡</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>5.3±0.1‡</td>
<td>5.8±0.2‡</td>
<td>7.3±0.1*</td>
<td>7.2±0.3*</td>
</tr>
</tbody>
</table>

HR indicates heart rate; LVIDd, LV inner diameter in diastole; LVIDs, LV inner diameter in systole; PWd, posterior wall thickness in diastole; PWS, posterior wall thickness in systole; FS, fractional shortening; EF, ejection fraction; IVRT, isovolumetric relaxation time; E/e', ratio of peak blood inflow Doppler at mitral valve to peak tissue movement Doppler at mitral valve annulus. *P<0.05 vs WT; ‡P<0.05 vs KO; †P<0.05 after dobutamine treatment in comparison to baseline.

Figure 5. Echocardiography revealed hypertrophy in cMyBP-C(t3SA) and KO hearts. Two-dimensional echocardiography in the parasternal long axis allowed qualitative assessment of the entire LV. In diastole, the LV posterior walls of cMyBP-C(t3SA) and KO myocardium exhibited increased thickness. All hearts showed similar LV inner diameter in diastole. Higher resolution M-mode measurements at the level of the papillary muscle corroborated findings of these 2D views (Table 2).
Discussion
We have transgenically expressed non–PKA-phosphorylatable cMyBP-C on a cMyBP-C KO background to study the functional effects of PKA phosphorylation of cMyBP-C in living myocardium. Stretch activation studies on skinned myocardium from cMyBP-C(t3SA) mice demonstrated the loss of PKA mediated acceleration of contraction kinetics. In vivo echocardiography showed hypertrophy, diastolic dysfunction, systolic dysfunction, and reduced dobutamine augmentation of systolic function in cMyBP-C(t3SA) hearts. Thus, our data indicated that PKA phosphorylation of cMyBP-C in response to β1-adrenergic stimulation accelerates crossbridge kinetics, and loss of this regulatory mechanism contributes to cardiac dysfunction.

Stretch activation measurements showed that PKA phosphorylation of cMyBP-C accelerated crossbridge kinetics, a phenomenon that does not involve cTnI. After an initial elastic response to stretch, the concurrent processes of crossbridge detachment, crossbridge attachment, and reversal of power stroke all contribute to the phases of force decay and delayed force development, with detachment and attachment comprising the dominant contributions.30–32 The rate constants of force decay (k_d) and delayed force development (k_u) manifest crossbridge turnover kinetics. WT and cMyBP-C(tWT) myocardium showed increased k_u and k_d following PKA treatment, with corresponding increases in phosphorylation of cMyBP-C. In contrast, cMyBP-C(t3SA) myocardium did not show any changes in k_u or k_d in response to PKA treatment because of the failure of PKA to phosphorylate the mutant cMyBP-C.

Previous work by Stelzer et al24 showed that the PKA acceleration of k_u was absent in cMyBP-C–null myocardium, but baseline kinetics in null myocardium were accelerated to levels observed in WT myocardium following PKA treatment. This left open the possibility that crossbridge cycling kinetics were saturated in null myocardium, such that if cTnI phosphorylation had an effect on kinetics, it would not be observed in this preparation. The present results eliminate this possibility because the baseline (pre-PKA) kinetics of cMyBP-C(t3SA) myocardium were similar to WT and cMyBP-C(tWT) myocardium, and yet cMyBP-C(t3SA) myocardium exhibited no change in k_u on treatment with PKA.

Echocardiography during dobutamine augmentation provided evidence of enhancement of global function attributable to PKA phosphorylation of cMyBP-C. Dobutamine stimulated β1-adrenergic activity in vivo to cause PKA phosphorylation of contractile proteins including cMyBP-C. Dobutamine also increases the amplitude and accelerates the kinetics of calcium transients.33 In this study, dobutamine increased EF and FS of cMyBP-C(t3SA) hearts to a lesser extent than in WT or cMyBP-C(tWT) hearts. Dobutamine augmentation of systolic function in cMyBP-C(t3SA) hearts can be attributed to enhanced calcium transients, whereas the lesser effects of dobutamine on cMyBP-C(t3SA) can be attributed to the absence of acceleration of crossbridge kinetics resulting from lack of PKA phosphorylation of cMyBP-C.

Considerable evidence supports the idea that the global cardiac dysfunction observed in cMyBP-C(t3SA) hearts is related to the inability to modulate crossbridge kinetics as a means to vary cardiac performance. For example, the nonselective β-blocker propranolol reduces phosphorylation of cMyBP-C in rats34 and depresses the positive force frequency response of intact papillary muscle from mice.35 In cMyBP-C(t3SA) myocardium, the inability to accelerate crossbridge kinetics with β1-adrenergic stimulation could provide the stimulus for compensatory hypertrophy and further dysfunction. Work by Perrino et al36 showing that either chronic or intermittent pressure overload can cause hypertrophy and dysfunction in mice is consistent with this hypothesis. Increased LV thickness with normal LV inner cavity dimension seen on echocardiography of cMyBP-C(t3SA) mice and the increased heart weight to body weight ratios provided evidence for hypertrophy. Increased IVRT and E/e’ ratio indicated diastolic dysfunction, whereas reduced FS indicated systolic dysfunction. Thus, cMyBP-C(t3SA) hearts showed global dysfunction that is attributed directly or via compensatory hypertrophy to the inability to accelerate crossbridge kinetics in response to β-adrenergic agonists.

PKA also phosphorylates cTnI in response to β1 stimulation, and previous studies suggest that phosphorylation of cTnI plays a role in PKA acceleration of crossbridge kinetics;37 however, our results cannot be attributed to PKA phosphorylation of cTnI, because PKA treatment phosphorylated cTnI in cMyBP-C(t3SA) and WT myocardium to similar levels. Our observation that PKA treatment caused a similar decrease in calcium sensitivity of force in both cMyBP-C(t3SA) and WT myocardium provided functional verification that PKA similarly phosphorylated cTnI in the 2 mouse lines. In a separate study, PKA treatment accelerated crossbridge kinetics in myocardium expressing native cMyBP-C and exclusively non–PKA-phosphorylatable cTnI; however, PKA did not accelerate crossbridge kinetics in cMyBP-C–null myocardium, despite similar increases in the level of phosphorylation of TnI.24 Thus, in the present study, the acceleration of crossbridge kinetics attributable to PKA treatment of skinned myocardium is mediated by phosphorylation of cMyBP-C, whereas the decrease in Ca²⁺ sensitivity of force is caused by phosphorylation of cTnI.

Our results do not eliminate the possibility that cMyBP-C phosphorylation would affect Ca²⁺ sensitivity of force at a sarcomere length different from that used here, ie, 2.1 μm, because Cazorla et al38 reported length-dependent differences in the effects of PKA on Ca²⁺ sensitivity in WT and null myocardium. Although both types of myocardium exhibited a decrease in Ca²⁺ sensitivity at an sarcomere length of 1.9 μm,38 only WT exhibited this decrease at 2.3 μm,38 leaving open the possibility that cMyBP-C phosphorylation modulates length-dependence of contraction.

Alterations in myofilament responsiveness to calcium during the cardiac cycle may explain why different changes in kinetics in cMyBP-C(t3SA) and KO myocardium led to similar cardiac dysfunction. Initial calcium binding to tropo-

nin converts the thin filament from a “blocked” state to a “closed” state in which crossbridges are more likely to bind.39 Subsequent crossbridge attachment with the ensuing transition from weakly to strongly bound states further activates the thin filament to an “open” state and cooperatively promotes
further crossbridge attachment. Cooperative binding of strongly bound crossbridges maintains the thin filaments in the open state, which allows continued crossbridge attachment and results in a continued increase in force during the initial decay in calcium. The constitutively slower kinetics of cMyBP-C(t3SA) myocardium would reduce the number of crossbridges that bind during a twitch in response to the rise in calcium and resulting from cooperative recruitment. In contrast, the constitutively accelerated kinetics of KO myocardium would reduce the dwell time of strongly bound crossbridges, which would reduce the cooperative recruitment of crossbridges during the initial decline in Ca\(^{2+}\) during the twitch. The shortened ejection time in KO hearts is consistent with this idea. Thus, the slowed kinetics of crossbridge turnover in cMyBP-C(t3SA) myocardium would reduce force generated per unit calcium change during a twitch, whereas the faster kinetics of KO myocardium would abbreviate force generation once Ca\(^{2+}\) begins to decline, both of which would decrease peak myocardial force and lead to pump dysfunction. Conversely, KO myocytes demonstrated prolonged calcium transient decay45; thus, slowed calcium handling likely caused impaired relaxation that was seen by echocardiography of KO hearts.

There are other potential phosphorylation sites on cMyBP-C, but these were not changed by the mutations and did not affect our findings. Yuan et al recently reported additional phosphorylation sites in canine cMyBP-C with murine homologs at Ser284 and Ser307. Basal phosphorylation of these additional sites in vivo could explain the weakly positive Pro-Q-Diamond staining of the mutant cMyBP-C in cMyBP-Ct(t3SA) myofibers. However, the lack of increase in phosphorylation after PKA treatment suggests that these sites are not phosphorylated by PKA.

In an earlier study, Sadayappan et al developed a mouse expressing non–PKA-phosphorylatable cMyBP-C (MyBP-C\(^{H11001}\)). On an essentially null background resulting from the constitutive expression of a truncated variant of cMyBP-C. They observed that LV chamber diameter was dilated at diastole, septal thickness was increased, and systolic function was depressed. In the present work, we show that systolic dysfunction in the presence of a non–PKA-phosphorylatable cMyBP-C is caused by slowing of crossbridge cycling kinetics. Furthermore, we find that expression of a non–PKA-phosphorylatable cMyBP-C results in significant diastolic dysfunction in the absence of adrenergic stimulation, presumably because of slower crossbridge cycling in the absence of baseline phosphorylation of cMyBP-C. Finally, our mutant mouse exhibits substantially blunted contractile reserve in response to adrenergic stimulation as a consequence of the inability to phosphorylate cMyBP-C. There are some differences between our mouse model and the model by Sadayappan et al in terms of background genetics (KO\(^{-}\) versus truncation\(^{46}\)), mutations (MyBP-C\(^{H11001}\)) has additional Thr-to-Ala mutations on Thr272 and Thr281\(^{19}\)), and expression level of mutant cMyBP-C (74% versus 40%\(^{19}\)). Not surprisingly, echocardiography revealed some phenotypic differences between the 2 models, ie, in contrast to MyBP-C\(^{H11001}\) hearts, our cMyBP-C(t3SA) hearts showed profoundly in-

increased LV posterior wall thickness but no LV inner chamber dilation in comparison to WT.

In conclusion, our results show that PKA phosphorylation of cMyBP-C accelerates crossbridge kinetics in myocardium and the loss of this regulatory mechanism leads to hypertrophy, diastolic dysfunction, systolic dysfunction, and reduced contractile reserve.

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Disclosures

None.

References


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Acceleration of crossbridge kinetics by protein kinase A phosphorylation of cardiac myosin binding protein C modulates cardiac function

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ONLINE DATA SUPPLEMENT FOR EXPANDED MATERIAL AND METHODS

Myofibril Preparation

This protocol produces intact myofibrils that can be used for ATPase assay, kinase reactions, immunofluorescence, gel electrophoresis, and western blotting. The base rigor buffer consisted of KCl 60 mmol/L, MgCl₂ 2 mmol/L, and 3-(N-Morpholino) propanesulfonic acid 4-Morpholinepropanesulfonic acid (MOPS) 20 mmol/L at pH=7.4. One can substitute 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) 20 mmol/L pH=7.4 for MOPS. Different steps of the preparation procedure and different follow-on experiments dictated adding other components to the base rigor buffer. All work was done at 4°C. Steps were as follows. (1) Excise heart and place immediately in rigor buffer with 10 mmol/L 2,3-butanedionemonoxime (BDM) followed with gentle compressions and rinse to remove all blood. Krebs Hensleit solution (NaCl 119 mmol/L, glucose 12 mmol/L, KCl 4.6 mmol/L, NaHCO₃ 2.5 mmol/L, KH₂PO₄ 1.2 mmol/L, MgCl₂ 1.2 mmol/L, CaCl₂ 1.8 mmol/L) after bubbling in 95%O₂-5%CO₂ for 30 minutes or relaxing solution (KCl 100 mmol/L, HEPES 20 mmol/L, ethylene glycol tetra acetic acid (EGTA) 2 mmol/L, adenosine triphosphate (ATP) 4 mmol/L, pH=7) was used for this step. The subsequent steps needed to be free of ATP so that crossbridges remain in rigor to maintain sarcomeric integrity. (2) Place washed hearts in 5 ml of rigor buffer with protease inhibitor cocktail (Sigma P8340 at volume:volume ratio of 1:100; P8340 consisted of [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] = AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstatin-A), EGTA 1 mmol/L, and phosphatase inhibitor cocktail (cocktail
PKA phosphorylated cMyBP-C Accelerates Kinetics consisted of okadaic acid 0.1 μmol/L, and Sigma P2850 at volume:volume ratio of 1:100; P2850 consisted of cantharidin, bromotetramisole, and microcystin). Alternatively, okadiac acid at 5 μmol/L can be used. The advantage of okadiac acid is its water solubility and lack of interference with kinase reactions. The phosphatase inhibition cocktail for inhibition of protein phosphatase 1A (PP-1A), protein phosphatase 2A (PP-2A), and protein phosphatase 2B (PP-2B) consisted of okadiac acid 0.5 μmol/L and cyclosporine-A 1 μmol/L. This combination provides ease of use and reduced exposure to okadiac acid. (3) Polytron 20 seconds 3-times to mechanically break the heart to myocyte sized fragments. (4) Place the polytroned solution in a Dounce homogenizer (glass pestle/tube-bulb) and pump for 35-times to further shear tissue. (5) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 10 minutes. (6) Remove supernatant. (7) Re-suspend pellet in 5ml of rigor buffer with 1% Triton-X100 and 1 mmol/L EGTA + protease inhibitor cocktail; all re-suspensions were at about 1:40 for pellet volume to solution volume ratio which is about 5 ml in most cases, (8) Place re-suspension in Dounce homogenizer and pump 5 times. (9) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 5 minutes. (10) Remove supernatant; EGTA was used to chelate intracellular calcium; however, EGTA needs to be removed for protein concentration analysis. (11) Re-suspend pellet in rigor buffer with 1% Triton-X100 + protease inhibitors and incubate for 20 minutes with gentle agitation twice during the extraction time. (12) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 5 minutes. (13) Remove supernatant. (14) Re-suspend in rigor buffer. (15) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 5 minutes. (16) Remove supernatant, (17) Re-suspend in rigor buffer with 1 μg/μl of bovine serum albumin (BSA); this is needed to prevent clumping of myofibrils. (18) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml
Falcon tube at 2500 RPM (1500g) for 5 minutes. (19) Repeat steps 16-18. (20) Add rigor buffer with 1 μg/μl BSA to pellet at about (1:1) ratio for maximum concentration; and measure final volume. This usually yields a final concentration of 6-8 μg/μl. (21) Take 3-samples of 10-μl volume and dilute to 50-μl for Pierce BCA protein assay (23225) and use the average of 3 to estimate concentration. (22) As necessary, place the final preparation in 50% glycerol for long term storage. The freshly prepared myofibrils will retain near full ATPase activity for about 24 hours when stored at 4°C.

**Western Blots**

Western blots were used to determine total expression of transgenes. Sets of four different amounts of total myofibril homogenate from each mouse were loaded onto polyacrylamide gels. Western blots were done using a rabbit polyclonal anti-cMyBP-C antibody at a dilution of 1:10,000. The slopes of plots of anti-cMyBP-C antibody intensity *versus* total protein load for each cMyBP-C KO mouse with insertion of a transgene was divided by the slope for a normal WT mouse on the same blot to determine the percent expression of mutant (t3SA) or WT (tWT) cMyBP-C. Mouse monoclonal anti-myc (Upstate® 05-419 clone 9E10) antibody at 1:250 dilution was used to verify expression of the transgene.
Exogenous Protein Kinase A Phosphorylation

Exogenous PKA treatment was done to phosphorylate cMyBP-C and cTnI in myofibrils and mechanical preparations. The lyophilized catalytic subunit of PKA was purchased from Sigma (P2645) and kept frozen until use. PKA was thawed on ice on the day of the experiment, subsequently equilibrated to room temperature for 12 minutes, and then re-suspended. All stock solutions and solutions containing myofibrils were equilibrated to room temperature before mixing with PKA. Deviation from the warming and equilibration sequence greatly reduced PKA activity. The protocol for phosphorylating myofibrils involved bathing 100 μg of freshly prepared myofibrils in 50 μl of solution containing PKA catalytic subunit, KCl 60 mmol/L, MgCl₂ 10 mmol/L, and ATP 2 mmol/L at 30°C for 60 minutes. The ratio of enzyme to total myofibrillar protein (weight/weight) was 0.05 (1 unit of PKA/μl according to Sigma specifications for activity). The reaction was stopped with the addition of 4X SDS sample buffer (Tris-HCl 0.25 mol/L pH 8.8, dithiothreitol 0.32 mol/L, sodium dodecyl sulfate 12% weight/V, glycerol 40% V/V, bromophenol blue 0.08% weight/V) which solubilized the proteins. There were no detectable differences in levels of phosphorylation when the reaction was run at 30°C or 22°C. See stretch activation portion for exogenous in vitro PKA phosphorylation of the mechanical preparation.

Pro-Q Diamond Phosphoprotein and Sypro-Ruby Staining

Pro-Q Diamond phosphoprotein stain was used for detection and semi-quantitative estimation of amount of phosphorylated proteins. Sypro-Ruby was used for checking for protein loading on all proteins. Myofibrils were prepared with above protocol with protein concentration estimated
by using Pierce BCA assay. Myofibrils were loaded at total protein amounts of 4 μg, 8 μg, and 12 μg into different lanes of 10% Bio-Rad Criterion polyacrylamide gels. Electrophoresis was done at 175V for 62 minutes. The following steps stained the gel for phosphorylated protein: (1) Remove gel from cassette and wash with de-ionized (ddH₂O) water twice. (2) Rock gel in fixative solution (50% methanol, 10% glacial acetic acid) on an orbital shaker for 3-sessions of 30 minutes each with complete solution exchange between each session. (3) Wash gel with ddH₂O 10-times followed by 6 sessions of 5-minute ddH₂O wash on orbital shaker. (4) From this point onward, care was taken to eliminate potential of photobleaching. All staining and washing were done in a container completely opaque to light. Furthermore, all solution changes took place in a darkened environment. (5) Stain gel with 75 ml of Pro-Q Diamond (Molecular Probes:P-33300) on orbital shaker for 90 minutes with rotating the container by 90° at 45 minute mark. (6) Wash with ddH₂O 2-times. (7) Complete 4-sessions of 25 minutes de-staining with Pro-Q Diamond De-staining solution (Molecular Probes: P-33311) on orbital shaker with complete solution exchange for all sessions; change to new stain-free container after 2nd de-stain session. (8) Complete 4 sessions of 5-minute ddH₂O wash on orbital shaker. (9) Molecular Imager F/X (Bio-Rad) with laser excitation at 532 nm and long pass filter at 555 nm was used to capture Pro-Q Diamond fluorescence image. (10) Post-stain gel with 60 ml of Sypro Ruby (Molecular Probes: S12000) over night. (11) De-stain with Sypro-Ruby de-stain solution (10% methanol, 7% glacial acetic acid) for 4 sessions of 30 minutes each with complete solution exchange for each session. (12) Wash with ddH₂O with 6 sessions of 5 minutes each. (13) UVP EC3 imaging system at UV source with 302 nm excitation filter and long pass 560 nm emission filter was used to capture Sypro-Ruby fluorescence image. (14) As necessary, secondary post-stain with Coomassie blue to provide readily visualized gel.
Phosphorylation Gel Analysis

We developed a method that minimized staining variability, optical to digital capture variability, and loading of protein of interest variability to compare phosphorylation levels of a specific protein across multiple mouse lines and conditions. Bio-Rad Laser-Pix software was used to convert images of stained gels to quantitative values for analysis. Each band of interest has its own background band. Total count intensity of band was calculated as

\[
\text{Total Intensity} = \text{Band Area} \times (\text{Mean Intensity} - \text{Mean Background Intensity})
\]

Value of 0 intensity for 0 protein load was inserted as another data point. Linear regression was done on the bands of interest from lanes with different total myofibril protein loading to calculate the slope of total count/protein load for each mouse and experiment condition. Differences in slopes reflect differences in properties that are a fraction of total amount of protein (e.g. fraction of phosphorylated cMyBP-C, fraction of cMyBP-C versus total protein …etc.). This method eliminates the possibility that total myofibril protein load differences would be interpreted as differences in fractional quantities e.g. fraction of cTnI that is phosphorylated. Calculated P-slope is the total ProQ count versus total myofibril protein load. The calculated L-slope is the total Sypro-Ruby count versus total myofibril protein load. The regression \( r^2 \) values nearly always exceeded 0.97 for slopes calculated from Pro-Q Diamond and Sypro-Ruby staining. P-slope can be described by the following.
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[Equation 2]

\[ PSlope \left( \frac{ProQ}{total \ myofibril \ protein \ loaded} \right) = CFF \left( \frac{ProQFluorescence}{Protein(experiment)P04} \right) \times PPF \left( \frac{Protein(experiment)P04}{Protein(experiment)} \right) \times EPF \left( \frac{Protein(experiment)}{total \ myofibril \ protein \ loaded} \right) \]

CFF is the captured fluorescence factor, PPF is the phosphorylated experimental protein fraction, and EPF is experimental protein fraction from the estimated total myofibril protein. CFF is a function of dye binding efficiency, fluorescence characteristics of the dye, excitation intensity, and collections electro-optics (e.g. photomultiplier gain for BioRad system, optical resolution, and charged couple device (CCD) density per pixel for UVP system). The ratio of the experiment P-slope to the WT baseline P-slope within each gel provided a value that should be free of differences in CFF between gels as the effects of CFF would be cancelled. The following set of equations describes the cancelling process. First, using equation [2], one can write the ratio of P-slopes as:

[Equation 3]

\[ \frac{PSlope_{Experiment}}{PSlope_{WTBaseline}} = \frac{CFF_{Experiment} \times PPF_{Experiment} \times EPF_{Experiment}}{CFF_{WTBaseline} \times PPF_{WTBaseline} \times EPF_{WTBaseline}} \]
Since CFF within the same gel should be the same, then, \( CFF_{WTbaseline} \) cancels \( CFF_{Experiment} \) to yield equation 4.

\[
\frac{PSlope_{Experiment}}{PSlope_{WTBaseline}} = \frac{PPF_{Experiment} \times EPF_{Experiment}}{PPF_{WTBaseline} \times EPF_{WTBaseline}}
\]

Similarly, the ratio of L-Slopes of Sypro-Ruby cancels the CFFs to allow estimation of the ratio of L-slopes as:

\[
\frac{LSlope_{Experiment}}{LSlope_{WTBaseline}} = \frac{EPF_{Experiment}}{EPF_{WTBaseline}}
\]

Thus, equation 4 divided by equation 5 (i.e. P-slope ratio / L-slope ratio) will cancel the EPF of a specific protein to yield the ratio of phosphorylated protein of interest for an experiment to the phosphorylated protein of interest in the WT baseline.

\[
\left[ \frac{PSlope_{Experiment}}{PSlope_{WTBaseline}} \right] \times \left[ \frac{LSlope_{Experiment}}{LSlope_{WTBaseline}} \right] = \frac{PPF_{Exp} \times EPF_{Experiment}}{PPF_{WTBaseline} \times EPF_{WTBaseline}} = \frac{PPF_{Experiment}}{PPF_{WTBaseline}}
\]
In summary, ratio of P-slopes provides independence from variance of fluorescence properties between gels, and combined ratio provides independence from variance of fraction of protein of interest as a part of total myofibril protein between sample preparations. This analysis method works well provided that a stable WT baseline exists. The summary equation from all the above now reduces to the following.

\[
P_L\left(\frac{\text{experiment}}{WT\text{baseline}}\right) = \frac{[\text{ProQ\_slope(experiment)}/\text{ProQ\_}(WT\text{baseline})]}{[\text{Sypro\_slope(experiment)}/\text{Sypro\_slope}(WT\text{baseline})]}
\]

A protocol of ensuring stable WT baseline phosphorylation without using pharmacological intervention was used. The protocol involves: (1) moving the mice from central housing to experiment location the night before the procedure, (2) providing a quiet environment for the over-night stay, and (3) anesthetizing each specific mouse free from sight of surgery and minimizing the scent of blood (ie. surgery done under the hood and changing gloves between each mouse.)

**Dual Wavelengths Immunofluorescence**

Dual wavelengths immunofluorescence of Anti-cMyBP-C and Anti-cMyc was used to demonstrate incorporation of transgenic MyBP-C. Myofibril suspension at a concentration of 2-4 μg/μl was prepared. Unless otherwise noted, all buffer was rigor buffer with 1 μg/μl of BSA. All rocking was done on an orbital shaker. A 50 μl aliquot of myofibril preparation was dropped onto a #1.5 cover slip. A 90 sec wait allowed adherence of myofibrils to the cover slip. Dipping
the cover slip in the rigor buffer washed away the unattached myofibrils. Placing the cover slip with myofibril side down onto 50 μl of 3% goat serum in a Petri dish for 15 minutes blocked non-specific binding sites. Buffer was added until the cover slip floated up, the cover slip was turned over, and the dish rocked gently for 5 minutes to wash away the goat serum. A mixture of primary antibodies was prepared ahead of time. This mixture with final dilutions included polyclonal rabbit anti-cMyBP-C antibody at 1:250 dilution and monoclonal mouse anti-Myc antibody (Upstate 05-419 clone 9E10) at 1:25 dilution. The concentration of primary antibody needed to be optimized for each specific antibody; however, increasing concentration to 40 times needed for Western blots provided a good starting point. Placing the cover slip with myofibril side down onto 50 μl of primary antibody mixture in a Petri dish started the primary antibody binding reactions. This was continued for 60 minutes at 4°C without rocking in a covered Petri dish. Fresh buffer was added until the cover slip floated, the cover slip was inverted, and the dish rocked gently for 5 minutes to wash away the primary antibodies. A mixture of secondary antibodies was prepared ahead of time. All handling procedures involving fluorescence secondary antibodies were done in lowest possible light conditions. The secondary antibodies mixture with final dilutions consisted of Molecular Probes goat anti-rabbit AlexaFluor647® at 1:250 dilution and Molecular Probes goat anti-mouse AlexaFluor488® at 1:250 dilution. The cover slip with myofibril side down was placed onto 50 μl of the secondary antibody mixture in a Petri dish. The incubation was continued for 30 minutes at room temperature without rocking in a covered Petri dish that was completely protected from light. Two 5-minute washes as described earlier removed the secondary antibodies. The cover slip with myofibril side down was placed onto 200 μl of 4% para-formaldehyde and incubated for 10 minutes to fix the samples. Dilution with wash buffer and two 5-minutes washes removed the para-formaldehyde. A drop of
Molecular Probes Prolong Gold (P36930) antifade mounting reagent was placed on a microscope slide. The cover slip with myofibril side down was carefully placed onto the drop of antifade mounting reagent without trapping bubbles. The cover slip was immobilized on the slide with clear finger nail polish application to its 4 corners. After 24 hours of curing, the edges of the cover slip were sealed with clear finger nail polish. A Zeiss LSM 5 Pascal microscope system with Plan Apo-chromatic 100X oil immersion objective was used for imaging. The initial locating of myofibrils and focus adjustments were done using the UV light source under visual guidance. Actual imaging was done using laser excitation, optical filters, and photomultiplier. A HeNe laser with 633 nm excitation and a 650 nm long pass filter generated signals to create images for anti-cMyBP-C immunofluorescence. An argon laser with 488 nm excitation and 505-530 nm band pass filter generated signals to create images for anti-cMyc immunofluorescence.

**Stretch Activation on Skinned Myocardium**

Stretch activation was assessed in skinned myocardium to quantify the effects of PKA phosphorylation of regulatory proteins on crossbridge kinetics. The protocol was as follows: Sarcomere length was set to 2.1 μm, an initial activation was done at pCa 4.5 (pCa = -log₁₀[Ca²⁺]) to establish maximum force, pCa was varied to determine pCa for half-maximal force (i.e. pCa 5.75), and finally a rapid stretch equivalent to 1% of fiber length was applied and held for 5 seconds. PKA phosphorylation was done using 1-unit PKA/μL at 22°C instead of 30°C to prevent potential degradation of skinned myocardium. The PKA phosphorylation solution was washed out by 4-times volume exchange. Phosphate incorporation due to PKA phosphorylation was assessed from similarly treated myofibrils, as described above. The forces
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measured were $P_0$, the pre-stretch baseline; $P_1$, the force difference between $P_0$ and the peak force immediately post-stretch; $P_2$, the force difference between $P_0$ and the minimum at the end of the post-stretch decay; $P_3$, the force difference between $P_0$ and the peak of delayed force development; and $P_{df}$, the force difference between $P_3$ and $P_2$. Rate constants $k_{rel}$ and $k_{df}$ were determined for the $P_1$ to $P_2$ force decay and the $P_2$ to $P_3$ delayed force development (stretch activation), respectively. Fitting $A(1-\exp(-k_{rel}t))$ to the force decay from the peak force immediately post stretch ($P_1$) to the minimum force ($P_2$) yielded rate constant $k_{rel}$, where $A$ is amplitude and $t$ is time. Calculating the inverse of the half-time for delayed force development from $P_2$ yielded rate constant $k_{df}$. Forces were normalized to $P_0$ to allow comparisons between experiments.

**Echocardiography**

Echocardiography was done using a Visualsonics Vevo 770 system with a 30 MHz probe to record intact *in vivo* cardiac structure and function. Left ventricular (LV) chamber dimensions, LV posterior wall (PW) thickness, and fractional shortening (FS) were measured using M-mode in the parasternal short-axis view at the level of the papillary muscle. Ejection fraction (EF) was estimated using Vevo 770 software from the 2-dimensional parasternal long-axis view of the left ventricle in the EKV™ (EKG-based kilohertz visualization) mode. The EKV™ mode reconstructs one averaged cardiac cycle cine with 1000 frames per scan line; thus, the reconstructed cine is averaged from greater than 1000 actual heart beats. Blood flow Doppler and tissue movement Doppler were done in the apical 4-chamber view. Isovolumetric relaxation time (IVRT) was measured from the blood flow Doppler spectra. The ratio of the blood flow Doppler
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peak (E) at mitral inflow to the tissue movement Doppler peak (e’) at the lateral mitral annulus \((E/e’)^4\) provided another index of diastolic function. With the exception of EF (which is already essentially averaged over ~ 1000 cardiac cycles), all measured parameters were averages from at minimum of 3 cardiac cycles. TH performed and CT read all the echocardiography.

Dobutamine (10 \(\mu\)g/g) was administered by intraperitoneal (IP) injection to elicit a maximum \(\beta_1\) response that was sustained for 15 minutes\(^5\) in order to maximize \textit{in vivo} PKA phosphorylation. Dobutamine was injected IP after the baseline measurements were completed. Post-dobutamine imaging was initiated 8 minutes after IP injection. Phosphoprotein gel staining of contractile proteins from similarly treated mice showed that phosphorylation peaked 10 minutes after injection, followed by a slight decrease in phosphorylation at 15 minutes (data not shown). This post-dobutamine injection time window allowed only EF and FS to be recorded for assessment of dobutamine augmentation.

Mice were maintained in a consistent controlled state. Anesthesia was maintained with continuous inhalation of isoflurane. Body temperature was kept constant at 37\(^\circ\)C using a heat lamp and a heating pad with continuous feedback control. Continuous cardiac monitor followed the heart rate. Anesthesia was adjusted to ensure heart rate did not fall below 400 beats per minute (BPM) and mice did not wake-up during the procedure. All mice reported in the study had complete recovery from procedure.
Mouse Age for β-Myosin Heavy Chain Expression

Mice from all four lines of: (1) normal wild type “WT”, (2) cMyBP-C knock-out “KO”, (3) transgenic expression of non-PKA-phosphorylatable mutant cMyBP-C onto KO background “t3SA”, and (4) transgenic expression of WT cMyBP-C onto KO background “tWT” were aged to 90 ± 7 days at the time of heart extraction to ensure age differences did not contribute to differences in relative β myosin heavy chain (βMHC) expression with respect to (αMHC).

Colloidal Coomassie Staining for MHC

Colloidal coomassie blue (CCB) staining on large format (18 X 16 cm) 6% gel\(^6\) was chosen as the initial assessment of βMHC expression due to its stoichiometric binding to protein, sensitivity, and maximum separation. Myofibril preparations were made from hearts of 4 different mice on each line of mice. Stacking gels contained 3%T, 15%C, 10% glycerol, 0.13 mol/L Tris (pH 6.8) and 0.1% SDS with N-N' diallyltartardiamide (DATD) used as the cross-linker. Separating gels contained 6%T, 2.6%C, 10% glycerol, 0.37 mol/L Tris (pH 8.8) and 0.1% SDS with DATD as the cross-linker. \(\%T\) and \(\%C\) are calculated as the following.

\[
\%T = \left( \frac{\text{acrylamide (mg)} + \text{DATD (mg)}}{\text{volume (ml)}} \right) \times 100
\]
[Equation 9]

\[
\% C = \left( \frac{DATD (mg)}{acrylamide (mg) + DATD (mg)} \right) \times 100
\]

Gel polymerized in room temperature for 1 hour and overnight at 4°C. Electrophoresis was done using a Hoefer SE 600 Vertical Electrophoresis Unit with 0.75 millimeter spacers and a Bio-Rad PowerPac 300 power supply. The running buffer consisted of 25 mmol/L Tris (base), 192 mmol/L glycine and 0.1% SDS, pH 8.3. The gels ran at constant current of 16 mA for 9 hours at 4°C. Coomassie blue staining was done for 16 hours. The composition is as follows: 10% phosphoric acid, 20% methanol, 10% ammonium sulfate, 0.12% G-250 Coomassie. Gel was destained in ddH₂O for minimum of 3 hours or until background became clear. Gels were imaged with UVP EC3 system. The relative proportions of α and β MHC isoforms were determined by densitometric analysis of CCB stained gels using LaserPix software.

**Dual Wavelengths Fluorescent Western Blot for MHC Isoforms**

We adapted a dual wavelengths fluorescence based western blot method to analyze α and β MHC expression to (1) confirm colloidal coomassie findings, (2) reduce the time required from 4 days for colloidal coomassie staining to 1 day for dual wavelength fluorescence western blot, and (3) provide a more easily manipulated final product than large 6% gel for handling and imaging. Aliquots of myofibril preparations from 16 hearts (4 mice from each of 4 lines) were used. Electrophoresis was done on 5% Bio-Rad Criterion gel at 150 volts for 4 hours at 4°C with pre-cooled running buffer. Replacing existing running buffer with pre-cooled running buffer at 4°C
every 80 minutes was necessary to maintain the integrity of gel and to produce sharp bands. Protein bands were transferred to 0.45 μm nitrocellulose membranes under conditions of 55 volts for 90 minutes at 4°C with transfer buffer (20% methanol, 0.1% SDS, 0.3% Tris, 1.4% glycine). PVDF membrane had too much background noise. Using 0.2 μm nitrocellulose membrane captured more protein at the cost of an increase in background noise. Membranes was blocked with Odyssey blocking buffer (Li-Cor 927-4000) for 1-hour at room temperature. Mouse monoclonal anti-total MHC antibody (Chemicon, MAB1552) at 1:100 dilution and rabbit polyclonal anti-βMHC (Sigma/Atlas, HPA00129, affinity isolated) antibody at 1:500 dilution were suspended in Odyssey blocking buffer with 0.1% polyoxyethelene sorbitan monolaurate (Tween 20) and were incubated with the membrane for 1-hour in room temperature with gently orbital shaking. Changing to new container, immediate wash with Tris-buffered saline with Tween “TBST” (NaCl 0.15 mol/L, Tris 0.02 mol/L pH 7.4, 0.05% Tween 20), and followed by 4 session of 10-minute washes with TBST cleared non-specific binding. Secondary fluorescent antibodies consisted of IRDye800CW goat anti-mouse (Li-Cor, 926-32210, cross-adsorbed, 778 nm excitation/795 nm emission) at 1:10,000 dilution and Alexa Fluor 680 goat anti-rabbit (Molecular Probes, A21109, highly cross-adsorbed, 679 nm excitation/702 nm emission) at 1:10,000 dilution. From this point onward, all steps were done in minimal lighting to prevent photo-bleaching. A mixture of secondary antibodies was incubated with the membrane for 50 minutes in room temperature with gentle orbital shaking and completely shielded from all light. All washes were done with TBST. Changing to a new container, an immediate wash followed by one 5 minute wash, 2 times of 30 minutes, and then 3 times of 5 minutes greatly reduced background noise. An Odyssey infrared imaging system (Li-Cor) provided the double wavelength excitation and captured the double wavelength emission image. Anti-total
MHC/IRDye800CW fluorescence reported the amount of total MHC. Anti-βMHC/Alexa Fluor 680 fluorescence reported the amount of βMHC. Quantification was done using the Odyssey software. A dried membrane can be imaged repeatedly over multiple days without significant changes.

**Determination of Titin Phosphorylation**

Cardiac titin phosphorylation of in all 4-lines were done using the previously mentioned methods of myofibril preparation and Pro-Q Diamond phosphoprotein staining with followed by Sypro-Ruby total protein staining. The following protocol was used to produce minimally phosphorylated myofibrils for use as a comparative standard across all gels: lightly anesthetize WT mouse with isoflurane, injection metoprolol (specific β₁ adrenergic blocker) IP at 5 μg/g (drug/body weight) dose, allow mouse to recover for 30 minutes, and then re-anesthetize mouse for removal of heart. The following precautions and modification were used to optimize detection of titin phosphorylation: (1) freeze solubilized myofibril suspension in 50% glycerol to -80°C immediately and use within 3 months, (2) add β-mercaptoethanol to a final concentration of 10 mmol/L in top well of Bio-Rad Criterion cassette just before electrophoresis to minimize protein cross-linking during the migration, (3) use 7.5% gel, (4) use myofibrils from metoprolol treated WT heart (WT<sub>bBlock</sub>) as a comparative baseline so that WT basal phosphorylation could be included in the analysis.

[Equation 10]

\[
PL\left(\frac{\text{experiment}}{WT_{bBlock}}\right) = \frac{[\text{ProQ_slope(experiment)}/\text{ProQ}_-(WT_{bBlock})]}{[\text{Sypro_slope(experiment)}/\text{Sypro_slope}(WT_{bBlock})]}
\]
ONLINE DATA SUPPLEMENT FOR EXPANDED RESULTS

βMHC Expression

Colloidal Coomassie Blue (CCB) staining with corroborating dual wavelength western blotting (2WL-WB) showed that t3SA line has increased βMHC expression of at most ~10% of total MHC (Online Figure I and Online Table I) in comparison to WT at 3-months of age but tWT had similar βMHC expression as WT. Both CCB staining and 2WL-WB showed that comparative expression of βMHC across all four lines is KO > t3SA > tWT=WT (Online Figure I and Online Table I). As supported by similar stretch activation kinetics at basal conditions between WT, tWT, and t3SA, this increase in βMHC expression did not affect function.

Differences in age of mice and methodology can explain the small increase in measured % βMHC from our prior reported value of ~13%\textsuperscript{8} to the current value of ~19%. Mice in this study are about 4-weeks older than in the prior study. CCB and 2WL-WB have different protein binding characteristics than the silver staining used in the prior study.\textsuperscript{8}

Titin Phosphorylation

ProQ-Diamond phosphoprotein staining and subsequent Sypro-Ruby total protein staining to adjust for loading differences showed that basal titin phosphorylation is similar across all 4-lines (WT = KO = t3SA = tWT, see Online Figure II and Online Table I).
Steady State Force/pCa Measurements

Myocardium from all 4-lines showed similar steady state force response to variations in activating calcium at basal conditions and after PKA treatment (see Online Table II). PKA treatment did not change maximum force but did decrease calcium sensitivity across all 4-lines (see Online Table II). Thus the PKA-induced decrease in calcium sensitivity does not involve phosphorylation of cMyBP-C but instead is due to phosphorylation of cTnI.⁹
REFERENCES:


ONLINE DATA SUPPLEMENT FIGURE LEGENDS

Online Figure I: Both colloidal coomassie blue (CCB) staining and dual wavelength fluorescence western blot (2WL-WB) showed corroborating % expression of βMHC of KO > t3SA > tWT=WT. 2 μg and 1.25 μg of myofibril from each heart were loaded onto gels for CCB staining and 2WL-WB respectively. (I.A) Gray scaled image showed CCB staining of both αMHC and βMHC. Red showed anti-total MHC fluorescence. Green showed anti-βMHC fluorescence. Yellow showed matched portion of anti-total MHC attributable to βMHC. (I.B) % βMHC expression was calculated from CCB image. Separately, % βMHC expression was also calculated from anti-total MHC fluorescence image using matching yellow band as confirmatory guide for portion of total MHC attributable to βMHC. n=4 hearts for each line, * denotes p < 0.05 vs. WT, # denotes p < 0.05 vs. KO. (I.C) alternatively, ratio of anti-βMHC fluorescence vs. anti-total MHC fluorescence from was calculated. n=4 hearts for each line, * denotes p < 0.05 vs. WT, # denotes p < 0.05 vs. KO.

Online Figure II: All 4-lines showed similar basal titin phosphorylation. (II.A) Example of ProQ phosphoprotein and Sypro-Ruby total protein staining. Titin appeared as the most consistently phosphorylated protein across all mice on ProQ staining. ProQ staining also verified that t3SA and βBlock (metoprolol) treated WT have decreased cMyBP-C phosphorylation. Sypro-Ruby staining showed similar protein loading on all lines. Sypro-Ruby also verified that KO is missing cMyBP-C. (II.B) n=4 hearts for all lines. There is no detectable difference in titin phosphorylation across all lines (t3SA = tWT = WT = KO).
**Online Table I: Summary of βMHC expression and Titin Phosphorylation Values**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>WT, n=4</th>
<th>KO, n=4</th>
<th>t3SA, n=4</th>
<th>tWT, n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% βMHC by CCB</td>
<td>0#</td>
<td>18.7 ± 1.2*</td>
<td>8.5 ± 1.8*#</td>
<td>0.8 ± 0.8 #</td>
</tr>
<tr>
<td>% βMHC by 2WL-WB</td>
<td>0.9 ± 0.1</td>
<td>19.4 ± 0.7*</td>
<td>9.9 ± 1.3*#</td>
<td>2.4 ± 1.1#</td>
</tr>
<tr>
<td>βMHC IIInt/total MHC IIInt</td>
<td>0.013 ± 0.001</td>
<td>0.109 ± 0.006*</td>
<td>0.038 ± 0.008*#</td>
<td>0.013 ± 0.001#</td>
</tr>
<tr>
<td>% Titin phosphorylation vs. WT treated with βBlock</td>
<td>70.8 ± 16.5</td>
<td>73.9 ± 8.7</td>
<td>63.4 ± 8.6</td>
<td>61.6 ± 9.5</td>
</tr>
</tbody>
</table>

“*” denotes p < 0.05 vs. WT. “#” denotes p < 0.05 vs. KO. Abbreviations are: colloidal coomassie blue (CCB), dual wavelength fluorescence western blot (2WL-WB), Integrated Fluorescence Intensity (IIInt). Metoprolol 5μg/g (drug/body weight) IP was used as βBlock at 30 minutes prior to heart extraction.
**Online Table II: Summary of Steady State Force/pCa Results**

<table>
<thead>
<tr>
<th></th>
<th>WT, n=8</th>
<th>tWT, n=8</th>
<th>t3SA, n=8</th>
<th>KO, n=8</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMax (mN/mm²)</td>
<td>19.26 ± 2.06</td>
<td>18.93 ± 1.76</td>
<td>19.25 ± 1.47</td>
<td>20.01 ± 2.07</td>
</tr>
<tr>
<td>FMax + PKA (mN/mm²)</td>
<td>18.54 ± 2.19</td>
<td>18.24 ± 1.87</td>
<td>18.36 ± 1.33</td>
<td>18.73 ± 1.84</td>
</tr>
<tr>
<td>Fmin (mN/mm²)</td>
<td>0.74 ± 0.41</td>
<td>0.71 ± 0.13</td>
<td>0.69 ± 0.11</td>
<td>0.74 ± 0.13</td>
</tr>
<tr>
<td>Fmin + PKA (mN/mm²)</td>
<td>0.65 ± 0.11</td>
<td>0.63 ± 0.10</td>
<td>0.59 ± 0.08</td>
<td>0.63 ± 0.12</td>
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<tr>
<td>nH</td>
<td>3.80 ± 0.18</td>
<td>3.78 ± 0.23</td>
<td>3.80 ± 0.22</td>
<td>3.76 ± 0.15</td>
</tr>
<tr>
<td>nH + PKA</td>
<td>3.71 ± 0.20</td>
<td>3.86 ± 0.20</td>
<td>3.85 ± 0.19</td>
<td>3.76 ± 0.13</td>
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<tr>
<td>pCa50</td>
<td>5.76±0.02</td>
<td>5.75±0.02</td>
<td>5.76±0.02</td>
<td>5.73±0.02</td>
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<tr>
<td>pCa50 + PKA</td>
<td>5.65±0.03*</td>
<td>5.65±0.02*</td>
<td>5.68±0.03*</td>
<td>5.65±0.02*</td>
</tr>
</tbody>
</table>

"*" denotes p < 0.05 when compared to basal condition without PKA.

For force-pCa relationship, normalized force versus calcium data points were fitted to Hill’s equation

\[
\frac{[Ca^{2+}]}{[K_{50}]}^N + [Ca^{2+}]^N
\]

where K_{50} is [Ca^{2+}] for 50% force, N is the Hill coefficient, and pCa_{50}=-log_{10}[K_{50}].
PKA phosphorylated cMyBP-C Accelerates Kinetics

Online Figure I.A

<table>
<thead>
<tr>
<th>LINE:</th>
<th>WT</th>
<th>KO</th>
<th>t3SA</th>
<th>tWT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse:</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
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<tr>
<td>Colloidal Coomassie</td>
<td>αMHC</td>
<td>βMHC</td>
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<tr>
<td>Anti-total MHC</td>
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<td>Anti-βMHC</td>
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Online Figure I.B

- **% βMHC Expression**
- **Method**: CCB, 2WL-WB
- **Error bars**: +/- 1 SE

Online Figure I.C

- **βMHC Intensity/total MHC intensity**
- **Line**: WT, KO, t3SA, tWT
- **Error bars**: +/- 1 SE
Online Figure II.A

<table>
<thead>
<tr>
<th>Line</th>
<th>t3SA</th>
<th>tWT</th>
<th>WT</th>
<th>KO</th>
<th>WT+βBlock</th>
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<tbody>
<tr>
<td>Load (μg)</td>
<td>4,</td>
<td>8,</td>
<td>12,</td>
<td>4,</td>
<td>8, 12, 4,</td>
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ProQ-Diamond
Titin

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Sypro-Ruby
Titin

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MHC

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cMyBP-C

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Online Figure II.B

![Bar chart showing % Titin Phosphorylation vs. WT treated with βBlock for different lines: t3SA, tWT, WT, KO. Error bars indicate +/- 1 SE.](chart.png)