Protein Kinase A–Mediated Acceleration of the Stretch Activation Response in Murine Skinned Myocardium Is Eliminated by Ablation of cMyBP-C

Julian E. Stelzer, Jitandrakumar R. Patel, Richard L. Moss

Abstract—β-Adrenergic agonists induce protein kinase A (PKA) phosphorylation of the cardiac myofilament proteins myosin binding protein C (cMyBP-C) and troponin I (cTnI), resulting in enhanced systolic function, but the relative contributions of cMyBP-C and cTnI to augmented contractility are not known. To investigate possible roles of cMyBP-C in this response, we examined the effects of PKA treatment on the rate of force redevelopment and the stretch activation response in skinned ventricular myocardium from both wild-type (WT) and cMyBP-C null (cMyBP-C<sup>−/−</sup>) myocardium. In WT myocardium, PKA treatment accelerated the rate of force redevelopment and the stretch activation response, resulting in a shorter time to the peak of delayed force development when the muscle was stretched to a new isometric length. Ablation of cMyBP-C accelerated the rate of force redevelopment and stretch activation response to a degree similar to that observed in PKA treatment of WT myocardium; however, PKA treatment had no effect on the rate of force development and the stretch activation response in null myocardium. These results indicate that ablation of cMyBP-C and PKA treatment of WT myocardium have similar effects on cross-bridge cycling kinetics and suggest that PKA phosphorylation of cMyBP-C accelerates the rate of force generation and thereby contributes to the accelerated twitch kinetics observed in living myocardium during β-adrenergic stimulation. (Circ Res. 2006;99:884-890.)

Key Words: cross-bridge kinetics  ■  β-adrenergic agonists  ■  positive inotropy  ■  contractile protein function

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β-Adrenergic stimulation of myocardium is associated with increased twitch force, decreased twitch duration, and increased rates of force relaxation in vivo, in part, because of phosphorylation of Ca<sup>2+</sup> handling proteins and myofilament proteins by the intracellular messenger cAMP which causes the activation of protein kinase A (PKA) in the cell. In skinned myocardium, PKA phosphorylations of the thin filament cardiac protein troponin I (cTnI) and the thick filament protein cardiac myosin binding protein-C (cMyBP-C) is associated with decreased Ca<sup>2+</sup> sensitivity of force and increased rates of cross-bridge cycling and (reviewed in<sup>2,3</sup>), although the latter has not been consistently observed.<sup>4–6</sup> However, despite the physiological importance of the inotropic response in myocardium, the possible contributions of cMyBP-C to PKA-induced changes in myofibrillar contraction are still not well understood, although most previous studies have implicated cTnI phosphorylation as having a predominant role.

We have recently shown that myocardium from a mutant mouse lacking cMyBP-C (cMyBP-C<sup>−/−</sup>) exhibits a significant acceleration of cross-bridge cycling kinetics, which was evident as an accelerated rate of force redevelopment<sup>7</sup> and a shorter time to the peak of delayed force development following a rapid stretch to a new length (stretch activation).<sup>8</sup> The latter was an intriguing observation because the stretch activation response is a prominent intrinsic functional feature of myocardium<sup>9,10</sup> and is thought by some to contribute to systolic function in vivo.<sup>11–13</sup> It has been proposed that stretch activation is initiated during diastolic ventricular filling<sup>14</sup> when small numbers of attached cross-bridges are stretched, perhaps as a result of rapid increases in myocyte sarcomere length as chamber dimensions expand.<sup>15</sup> Stretch of myocytes to longer sarcomere lengths augments their force producing capabilities and increases force at the beginning of systole, thereby enhancing myocardial oscillatory work generation.<sup>14,16</sup> However, there is also evidence that stretch activation is initiated during systole as myocytes may be actively stretched during the systolic phase of the cardiac cycle. Because the timing of electrical and mechanical activation in the heart varies across the ventricular wall, regions of the ventricular wall that are activated early in the cardiac cycle (ie, endocardium) contract first and stretch regions that are activated later (ie, epicardium),<sup>17,18</sup> Later in systole, the late-activating epicardial fibers forcibly stretch the early-activating endocardial fibers,<sup>19–20</sup> possibly because epicardial fibers produce more force as a result of increased regulatory light chain phosphorylation.<sup>12</sup> Stretch of the endocardial fibers presumably results in delayed development of force...
(stretch activation) that would increase or at least sustain force generation during late systole. Therefore, extra force produced by stretch, whether originating during diastolic filling or systole, could substantially enhance cardiac function during the later phases of systolic ejection.

We have previously proposed that the accelerated rates of force redevelopment and the stretch activation response with cMyBP-C ablation were attributable to a relieving of the cMyBP-C–mediated constraint on myosin thereby allowing increased interaction with actin. Previous studies have suggested that phosphorylation of cMyBP-C increases the radial dispersion of cross-bridges away from the thick filament backbone, which would increase the likelihood of cross-bridge binding to actin and could contribute to the enhanced systolic function seen with β-adrenergic stimulation in vivo. Based on these earlier experiments, ablation or phosphorylation of cMyBP-C might be expected to similarly accelerate the kinetics of cross-bridge cycling. To explore this idea we examined the effects of PKA treatment on the stretch activation responses of skinned myocardium from wild-type (WT) and cMyBP-C+/− mice. Our results suggest that PKA-mediated phosphorylation of cMyBP-C contributes to faster contraction kinetics in β-agonist–stimulated myocardium by accelerating the kinetics of cross-bridge interaction with actin.

Materials and Methods

Transgenic Animals

cMyBP-C null (cMyBP-C−/−) mice were generated as previously described, and adult (3 to 6 months of age) WT (SV/129 strain) mice were used as controls. All procedures involving animal care and handling were performed according to institutional guidelines and were reviewed and approved by the Medical School Animal Care and Use Committee.

Solutions

Solution compositions were calculated using the computer program Sartorius. All solutions contained (in mmol/L): 100 N,N-bis(2 hydroxy-ethyl)-2-aminoethanesulfonic acid (BES), 15 creatine phosphate, 5 dithiothreitol, 1 free Mg2+, and 4 MgATP. pCa 9.0 solution contained 7 EGTA and 0.02 CaCl2; pCa 4.5 contained 7 EGTA and 0.02 CaCl2; and preactivating solution contained 0.07 EGTA. Ionic strength of all solutions was adjusted to 180 mmol/L with potassium propionate. Solutions containing different amounts of [Ca2+] in were prepared by mixing appropriate volumes of pCa 9.0 and pCa 4.5.

Skinned Myocardial Preparations

Skinned ventricular myocardium was prepared as previously described. Following injection (IP) of 5000 U heparin/kg body weight, mice were anesthetized with inhaled isoflurane (15% isoflurane in mineral oil). The hearts were excised, and right and left ventricles were dissected in room-temperature relaxing solution (in mmol/L: 100 KCl, 20 imidazole, 7 MgCl2, 2 EGTA, and 4 MgATP; pH 7.0) and then rapidly frozen in liquid nitrogen. To prepare skinned myocardium, the frozen ventricles were thawed and homogenized for ~2 seconds in relaxing solution using a Polytron, which yielded multicellular preparations of 100 to 250 μm x 600 to 900 μm. The homogenate was centrifuged at 120g for 1 minute, and the pellet was washed with fresh relaxing solution and resuspended in relaxing solution containing 250 μg/mL saponin and 1% Triton X-100. After 30 minutes, the skinned preparations were washed with fresh relaxing solution and then dispersed in relaxing solution in a glass dish. The dish was kept on ice except during the selection of preparations for mechanical experiments.

Apparatus and Experimental Protocols

Skinned preparations with well-defined, unbranched edges were transferred from the Petri dish to a stainless steel experimental chamber containing relaxing solution. The ends of the preparation were attached to the arms of a motor (model 312B, Aurora Scientific) and force transducer (model 403, Aurora Scientific), as described earlier. The chamber assembly was placed on the stage of an inverted microscope fitted with a ×40 objective and a closed-circuit television camera (model WV-BL600, Panasonic). Bitmap images of the preparations were acquired using an AGP 4X/2X graphics card and associated software (ATI Technologies) and were used to assess mean sarcomere length (SL) during each experiment. Changes in force and motor position were sampled (16-bit resolution, DAP5216a, Microstar Laboratories) at rates ≥2 Hz using SLControl software and saved to computer files for later analysis.

Rate of Force Redevelopment

The rate constant of force redevelopment (ktr) in skinned myocardium was assessed using a modification of the experimental protocol originally described by Brenner and Eisenberg. Each skinned preparation was transferred from relaxing to activating solutions of varying free [Ca2+] (pCa 6.2 to 4.5) and allowed to generate steady-state force. The myocardial preparation was rapidly (<2 ms) slackened by 20% of its original length, resulting in a rapid reduction of force to near 0, ie, <5% of steady isometric force. This was followed by a brief period of unloaded shortening (10 ms), after which the preparation was rapidly restretched to its original length. A ktr-relative force (P/Po) relationship was obtained by initially activating the skinned myocardium in solution of pCa 4.5 and then in a series of submaximally activating solutions between pCa 6.2 and 5.4, followed by expressing submaximal forces as a fraction of maximum force. For PKA experiments, myocardial preparations were incubated for 30 minutes (22°C) in a solution of pCa 9.0 with 1 U PKA/μL and when incubation was complete, experiments to assess the rates of force redevelopment during maximum and submaximal Ca2+ activations were repeated. The apparent rate constant of force redevelopment (ktr) was estimated by linear transformation of the half-time of force redevelopment, ie, ktr=0.693/t1/2, as described previously.

Stretch Activation Experiments

At the beginning of the experiment fiber length was set to a sarcomere length of ~2.1 μm for measuring initial isometric force and for subsequent imposition of stretch. The preparations were first activated at pCa 4.5 to establish maximum force (Pm). Ca2+-activated forces (P) at maximum and submaximal pCa values were determined as the difference between total force in the activating solution and resting force measured in solution of pCa 9. Fibers were initially activated at pCa 5.75 to yield a submaximal force of ~50% maximal and when the fiber achieved steady-state force a rapid stretch of 1% of fiber length was imposed and held for 5 seconds before returning the fiber to pCa 9.0. Next, preparations were incubated for 30 minutes (22°C) in a solution of pCa 9.0 with 1 U PKA/μL. When incubation with PKA was complete the stretch activation protocol was repeated with care taken to match the pretreatment isometric force. Because PKA treatment decreased the Ca2+ sensitivity of force in both WT and cMyBP-C−/− myocardium it was necessary to use a pCa solution with a slightly higher [Ca2+] (pCa 5.65) to match the pretreatment baseline isometric force. The high-speed (>10 muscle lengths sec−1) stretches imposed in these experiments was designed to minimize changes in cross-bridge populations during stretch, such that the initial increase in force was presumably attributable to the elastic strain of the cross-bridges bound before the stretch was imposed. The method used for measuring the stretch activation variables have been described in detail. Briefly, amplitudes were measured as follows:
• $P_1$, measured from prestretch steady-state force to the peak of phase 1
• $P_2$, measured from prestretch steady-state force to the minimum force decay
• $P_3$, measured from prestretch steady-state force to the peak value of delayed force
• $P_{30}$, difference between $P_1$ and $P_2$

All amplitudes ($A$) were normalized to $Ca^{2+}$-activated prestretch isometric force to allow comparisons between different groups. Apparent rate constants were derived for phase 2 (per second, $k_{a2}$) from the force decay following the peak of phase 1, and for phase 3 (per second, $k_{a3}$) from the point of force reuptake following phase 2 to the completion of delayed force development.

### Determination of Myofibrillar Protein Phosphorylation

Myofibrillar proteins of WT and cMyBP-C−/− myocardium were separated by SDS-PAGE using 12.5% Tris-HCl Criterion Precast gel (Bio-Rad, Hercules, Calif). To detect phosphorylated proteins, the gels were stained with Pro-Q Diamond following the protocol of the vendor (Molecular Probes). Briefly, the gels were (1) fixed in 10% glacial acetic acid/50% methanol (1.5 hour; 3 solution changes); (2) washed with $dH_2O$ (1 hour; 6 solution changes); (3) stained with Pro-Q Diamond (1.5 hour); and (4) destained with Pro-Q Diamond destaining solution (Molecular Probes) overnight. To detect myofibrillar proteins, the gels were stained with SYPRO Ruby (Molecular Probes; 3 hours) and destained with 7% glacial acetic acid/10% methanol (2 hours; 4 solution changes). Both the phosphoproteins and myofibrillar proteins were detected using UVP BioImaging System (UVP, Inc, Upland, Calif) and quantified using LaserPix (Bio-Rad). To quantify protein phosphorylation levels, different volumes (4, 6, 8, and 10 $\mu$L of −PKA and +PKA samples prepared from WT and cMyBP-C−/− skinned myocardium were loaded on the same gel. After gel electrophoresis, the gels were stained with SYPRO Ruby to detect proteins and with Pro-Q Diamond to detect phosphoproteins (Figure I in the online data supplement, available at http://circres.ahajournals.org). The area of protein and phosphoprotein bands was multiplied by mean optical density and plotted versus volume loaded (supplemental Figures II and III), and then a first order linear regression was fitted to the data points to determine the slope of the relationship between optical density and volume loaded.

### Data Analysis

Cross-sectional areas of skinned preparations were calculated by assuming that the preparations were cylindrical and by measuring the width of the mounted preparation. Submaximal $Ca^{2+}$-activated force ($F$) was expressed as a fraction of the force ($F_P$) generated at pCa 4.5, i.e., $P/F$. Rate constants of force decay ($k_{a2}$) were obtained by fitting a single exponential to the time course of decay, i.e., $y=a(1-\exp(-k_{a2}x))$, where $a$ is the amplitude and $k_{a2}$ is the rate constant of decay. Rate constants of delayed force development were obtained by a double exponential fit, $y=a \cdot \exp(-k_{a1}x)+b \cdot \exp(-k_{a2}x)$, where $a$ is the amplitude of the first exponential phase that rises with rate constant $k_{a1}$ and $b$ is the amplitude of the second exponential phase rising with rate constant $k_{a2}$ or were estimated by linear transformation of the half-time of force redevelopment, i.e., $k_{a2}=\ln(0.5)/X(f(t))^{-1}$. All data are reported as means±SEM. Comparisons of stretch activation variables between WT and cMyBP-C−/− groups measured before and after treatment with PKA were done using an unpaired $t$ test, and comparisons within the same group before and after treatment with PKA was performed using a paired $t$ test. Significance was set at $P<0.05$.

### Results

### Responses of WT and cMyBP-C−/− Myocardium to Stretch

Figure 1 presents typical stretch activation responses (normalized to prestretch isometric force) of half-maximally $Ca^{2+}$-activated WT and cMyBP-C−/− skinned myocardium following rapid stretches of 1% of initial muscle length. As reported previously,8 cMyBP-C−/− myocardium exhibits a stretch activation response that is significantly faster than in WT, ie, the apparent rate constants derived from the phase 2 force decay ($k_{a2}$) and phase 3 delayed force development ($k_{a3}$) are increased, as shown in Tables 1 and 2. The minimum force ($P_3$) reached at the end of the phase 2 force decay in cMyBP-C−/− myocardium is less than in WT myocardium, indicating greater cross-bridge detachment during phase 2. In contrast, the amplitude of delayed force development ($P_3$) does not differ from WT, indicating that similar numbers of cross-bridges were recruited by stretch. Because phase 3 delayed force development in WT myocardium is a biexponential process at half-maximal activation, we also analyzed delayed force development using a double-exponential fit, which yielded fast and slow rate constants ($k_{a1}$ and $k_{a2}$) and their corresponding amplitudes ($a$ and $b$), as shown in Table 1. In contrast, delayed force development in cMyBP-C−/− myocardium occurs as a single exponential process, corresponding to the faster rate constant (Table 1), i.e., the slower phase of force development, likely representing cooperative cross-bridge recruitment,15 is absent so that force develops at an overall accelerated rate.

### Effects of PKA Treatment on the Stretch Activation Responses of WT and cMyBP-C−/− Myocardium

To investigate the possible roles of cMyBP-C in the effects of phosphorylation on the stretch activation response, skinned preparations of both WT and cMyBP-C−/− myocardium were treated with PKA. Because the amplitudes and apparent rate constants of the phases of the stretch activation response in mouse myocardium are activation dependent,15,29 the pCa of the activating solutions was adjusted to ensure that prestretch isometric force was $\approx 50\%$ of maximal in WT and cMyBP-C−/− myocardium both before and after treatment with PKA. This was achieved by activating fibers at pCa 5.75 before PKA treatment and at pCa 5.65 following PKA treatment.
The decrease in the Ca$^{2+}$ sensitivity of force with PKA treatment in this study was similar in WT and cMyBP-C$^{-/-}$ myocardium and is not consistent with a recent report$^{30}$ suggesting that the absence of cMyBP-C in a genetically altered mouse model significantly diminishes the decrease in the Ca$^{2+}$ sensitivity of force with PKA treatment. Although a direct comparison between the results obtained in this study and that of Cazorla et al$^{30}$ is not possible because of inherent differences between the 2 mouse models (ie, the presence of slow skeletal TnI in mutant myocardium in Cazorla et al$^{30}$), our data suggest that cTnI phosphorylation is the main contributor to the PKA-mediated decrease in Ca$^{2+}$ sensitivity of force.$^{3}$

As shown in Figure 2A, PKA treatment of WT myocardium significantly accelerated $k_{11}$ and $k_{s1}$ (Table 1 and 2), such that $P_3$ fell below pre-stretch isometric force levels, although the amplitude of the delayed force development ($P_3$) was unaffected (Table 2). Following PKA treatment, delayed force development in WT myocardium occurred as a single exponential process with a single rate constant ($k_1$) that was similar to that of cMyBP-C$^{-/-}$ myocardium (Table 1). In contrast to its effects in WT myocardium, PKA treatment of cMyBP-C$^{-/-}$ myocardium did not significantly alter the stretch activation response (Figure 2B), suggesting that PKA-mediated acceleration of the stretch activation response in WT myocardium involves phosphorylation of cMyBP-C. This observation was confirmed in experiments in which the rate constant of force development ($k_1$), an alternative measure of cross-bridge cycling kinetics, was characterized in WT and cMyBP-C$^{-/-}$ myocardium. Before treatment with

### Table 1. Effect of PKA Phosphorylation on the Phase 3 Delayed Force Rise in WT and cMyBP-C$^{-/-}$ Myocardium

<table>
<thead>
<tr>
<th>Group</th>
<th>$P_3$ ($P_3/P_0$)</th>
<th>$P_2$ ($P_2/P_0$)</th>
<th>$k_{s1}$ (sec$^{-1}$)</th>
<th>$a$</th>
<th>$k_1$ (sec$^{-1}$)</th>
<th>$b$</th>
<th>$k_0$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−PKA</td>
<td>0.081±0.009</td>
<td>0.059±0.007</td>
<td>17.4±1.5</td>
<td>0.63±0.04</td>
<td>28.7±2.2</td>
<td>0.37±0.03</td>
<td>3.9±0.9</td>
</tr>
<tr>
<td>+PKA</td>
<td>0.079±0.008</td>
<td>0.118±0.011*</td>
<td>29.6±2.1*</td>
<td>1.00*</td>
<td>30.1±2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cMyBP-C$^{-/-}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−PKA</td>
<td>0.080±0.008</td>
<td>0.117±0.010*</td>
<td>31.3±1.7*</td>
<td>1.00*</td>
<td>30.8±2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+PKA</td>
<td>0.081±0.009</td>
<td>0.118±0.011</td>
<td>30.7±2.2</td>
<td>1.00</td>
<td>30.3±2.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means±SEM from 10 cardiac preparations. In each case, data were obtained following stretches of 1% of muscle length at an activation level of ~50% of maximal. The amplitudes are normalized to pre-stretch isometric force and the apparent rate constants for the phase 3 delayed force transient were obtained either from conversion of half-times of force recovery ($k_0$) or by fitting a double exponential to each record, $y=a \cdot \exp(-k_1 \cdot x)+b \cdot \exp(-k_0 \cdot x)$, where $a$ is the amplitude of the first exponential phase that rises with rate constant $k_1$, and $b$ is the amplitude of the second exponential phase that rises with rate constant $k_0$, as explained in the text. *Significantly different from WT-PKA ($P<0.05$).

### Table 2. Effect of PKA Phosphorylation on Phase 1 and 2 Stretch Activation Parameters in WT and cMyBP-C$^{-/-}$ Myocardium

<table>
<thead>
<tr>
<th>Group</th>
<th>$P_1$ ($P_1/P_0$)</th>
<th>$P_2$ ($P_2/P_0$)</th>
<th>$k_{rel}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−PKA</td>
<td>0.251±0.015</td>
<td>0.022±0.005</td>
<td>228±13</td>
</tr>
<tr>
<td>+PKA</td>
<td>0.245±0.017</td>
<td>−0.039±0.006*</td>
<td>343±15*</td>
</tr>
<tr>
<td>cMyBP-C$^{-/-}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−PKA</td>
<td>0.243±0.016</td>
<td>−0.037±0.006*</td>
<td>366±15*</td>
</tr>
<tr>
<td>+PKA</td>
<td>0.241±0.015</td>
<td>−0.037±0.007</td>
<td>357±16</td>
</tr>
</tbody>
</table>

Data are means±SEM, from 10 cardiac preparations. Data were obtained following a stretch of 1% of muscle length at an activation level of ~50% of maximal. The amplitudes are normalized to pre-stretch isometric force and the apparent rate constant of force decay ($k_{rel}$) was obtained by fitting a single exponential to the time course of decay, ie, $y=a \cdot \exp(-k_{rel} \cdot x)$, where $a$ is the amplitude of the single exponential phase and $k_{rel}$ is the rate constant of decay, as explained in the text. *Significantly different from WT-PKA ($P<0.05$).
The phosphorylation states of myofibrillar proteins from WT and cMyBP-C−/− mice were analyzed before treatment with PKA. PKA phosphorylated only cTnI in cMyBP-C−/− myocardium but phosphorylated both cTnI and cMyBP-C in WT myocardium before treatment with PKA. PKA phosphorylated both cTnI and cMyBP-C in WT myocardium but phosphorylated only cTnI in cMyBP-C−/− myocardium. Thus, the observed acceleration of \( k_{\text{rel}} \) and \( k_{\text{df}} \) in WT myocardium was associated with phosphorylation of both cTnI and cMyBP-C, whereas the lack of effect of PKA in cMyBP-C−/− myocardium was associated with phosphorylation of only cTnI.

**Discussion**

The long-term goal of this work is to determine the role of cMyBP-C in PKA-mediated myocardial inotropy. Here we present evidence that PKA-mediated phosphorylation of cMyBP-C accelerates the stretch activation response and the rates of cross-bridge cycling in WT myocardium. These results suggest that cMyBP-C is a significant regulator of the kinetics of force development in myocardium and modulates the rate of pressure development during systolic ejection in vivo.

Previous studies have shown that mutations of sarcomeric proteins in mouse myocardium that alter the stretch activation response can impair oscillatory power generation during systole. From these studies, it appears that both the timing (ie, time to peak delayed force development, \( k_{\text{rel}} \)) and amplitude (ie, additional force recruited by stretch, \( P_3 \)) of the stretch activation response may contribute to modulation of systolic function. Paradoxically, despite systolic dysfunction and severe ventricular hypertrophy in cMyBP-C−/− mice,23,33 the initial rise in pressure during systole in vivo is normal.33 This can be explained by our previous observations that cMyBP-C−/− skinned myocardium exhibits accelerated stretch activation kinetics and elimination or acceleration of cooperative activation of the thin filament, leading to early cessation of systole and diminished ejection fraction. The chronic acceleration of the stretch activation response in cMyBP-C−/− effectively detunes the stretch activation mechanism, so that the timing of delayed force development occurs too early and does not contribute to force generation late in systole thereby diminishing oscillatory power generation.

Our results here suggest that cMyBP-C phosphorylation enhances systolic function by accelerating cross-bridge turnover kinetics and the stretch activation response of myocardium, ie, by a mechanism similar to that proposed for cMyBP-C ablation.8 In terms of possible structural mechanisms, the removal of cMyBP-C−/− or its phosphorylation have been proposed to increase the proximity of myosin to actin, perhaps as a result of disrupted binding of cMyBP-C to the S2 domain of myosin35 and thereby increase the probability of cross-bridge formation and accelerate the transition(s) to force generating states. However, unlike the chronic acceleration of stretch activation in cMyBP-C−/− myocardium, which leads to systolic dysfunction, the reversible acceleration of kinetics by PKA phosphorylation of cMyBP-C in WT myocardium provides a regulatory mechanism to enhance cardiac function only in response to increased circulatory demand. In this regard, the significant acceleration in the overall rate of the stretch activation response in PKA treated WT myocardium would be expected to augment oscillatory power generation during systole and contribute to accelerated twitch kinetics with \( \beta \)-adrenergic stimulation in vivo.

Applying a rapid stretch to isometrically contracting myocardium results in a concomitant increase in force attributable to strain of attached cross-bridges, which is followed by detachment of strained cross-bridges and a decrease in force and finally by the attachment of new cross-bridges and redevelopment of force to greater than prestretch isometric levels. The acceleration of \( k_{\text{rel}} \) in PKA-treated WT myocardium is attributable to an acceleration of cross-bridge detach-
and is consistent with previous reports that show that both cMyBP-C ablation and PKA phosphorylation accelerate shortening velocity in myocardium, presumably because of increased rates of cross-bridge detachment. The decreased amplitude of P₂ with PKA phosphorylation in WT myocardium suggests greater cross-bridge detachment following stretch or perhaps an increased reversibility of force-producing steps in response to stretch. Taken together, these results are consistent with the idea that cMyBP-C phosphorylation or lack of cMyBP-C in myocardium increases cross-bridge compliance and decreases cross-bridge stiffness, thereby enhancing strain dependent cross-bridge detachment. Such a mechanism would be expected to be beneficial in accelerating force relaxation and diastolic filling during β-adrenergic stimulation in vivo.

Treatment of WT myocardium with PKA did not alter the amplitude of P₁, suggesting that the number of cross-bridges recruited by stretch did not change, however, the rate constant of delayed force development (k_rel) was dramatically accelerated, suggesting that the rates of force-generating transitions were accelerated. Just as in the case of cMyBP-C ablation, PKA phosphorylation of WT myocardium dramatically accelerated or eliminated the slow phase of delayed force development (thought to represent cross-bridge recruitment) (Table 1), so that the overall rate of phase 3 was accelerated and peak delayed force was achieved earlier. The PKA-mediated acceleration of the overall rate of the stretch activation response in WT myocardium is consistent with accelerated rates of force redevelopment (k_tr) (Figure 3) because k_rel is thought to be the sum of the forward (f_app) and reverse (g_app) rate constants describing the transitions between force-generating and non-force-generating states, and, thus, acceleration of k_rel is attributable to an increase in one or both rate constants. In this case, accelerated k_rel with PKA phosphorylation of WT myocardium was associated with the acceleration of k_rel and k_g, suggesting that both the rates of cross-bridge detachment and recruitment were accelerated.

The lack of responsiveness of cMyBP-C−/− skinned myocardium to PKA (Figures 2B and 3) despite an increase in cTnI phosphorylation suggests that the effect of PKA to accelerate stretch activation and the rate of force redevelopment in WT myocardium is attributable to cMyBP-C phosphorylation and not cTnI phosphorylation. This conclusion is reinforced by the observations that the levels of basal phosphorylation of cTnI were similar in WT and cMyBP-C−/− myocardium, as were the increases in cTnI phosphorylation by PKA (Figure 4). Our results do not completely rule out a role for cTnI phosphorylation in the regulation of the kinetics of force development, because it is possible that the accelerating effects of cMyBP-C phosphorylation might obscure any further kinetic effects caused by cTnI phosphorylation. However, this possibility seems unlikely because we have previously shown that at similar levels of activation the cross-bridge turnover kinetics of cMyBP-C−/− myocardium can be further accelerated with N-ethylmaleimide myosin subfragment-1 (NEM-S1) incubation, indicating that cross-bridge kinetics in these mice are not saturated.

As our understanding of cMyBP-C function improves, it is becoming clear that cMyBP-C is an important regulator of cardiac contractility and its phosphorylation by PKA is a mechanism that contributes to increased cardiac output in response to β-adrenergic stimulation. Conversely, the lack of cMyBP-C leads to cardiac dysfunction. With regard to the role of cMyBP-C in the regulation of contraction kinetics, we favor a working model of regulation in which PKA phosphorylation of cMyBP-C accelerates the kinetics of cross-bridge cycling and stretch activation (and systolic ejection) by regulating the proximity and interaction of myosin with actin.

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Disclosures
None.

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PKA-MEDIATED ACCELERATION OF THE STRETCH ACTIVATION RESPONSE IN MURINE SKINNED MYOCARDIUM IS ELIMINATED BY ABLATION OF cMyBP-C

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Running title: β-adrenergic stimulation and stretch activation

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Materials and Methods

Figure Legend

Figure 1S. PKA-induced phosphorylation of cMyBP-C and cTnI in WT and cMyBP-C<sup>−/−</sup> myocardium. In three experiments, different volumes (4, 6, 8, and 10 uL) of −PKA and +PKA samples prepared from WT and cMyBP-C<sup>−/−</sup> skinned myocardium were loaded on the same gel. PKA treatment resulted in phosphorylation of cMyBP-C and cTnI in WT myocardium and cTnI in cMyBP-C<sup>−/−</sup> myocardium.

Figure 2S. Optical density of protein and phosphoprotein bands plotted versus volume loaded prior to and following PKA treatment in WT myocardium. Different volumes of −/+ PKA treated skinned myocardial samples isolated from WT hearts were separated by SDS-PAGE and stained with SYPRO-Ruby for total proteins (top panel) and Pro-Q Diamond for phosphoproteins (bottom panel). The area and optical density of cTnI and cMyBP-C bands were determined and plotted against volume loaded. Regression lines were fitted to the data points (mean ± SEM) and the resultant slopes are shown in parentheses.

Figure 3S. Optical density of protein and phosphoprotein bands plotted versus volume loaded prior to and following PKA treatment in cMyBP-C<sup>−/−</sup> myocardium. Different volumes of −/+ PKA treated skinned myocardial samples isolated from cMyBP-C<sup>−/−</sup> hearts were separated by SDS-PAGE and stained with SYPRO-Ruby for total proteins (top panel) and Pro-Q Diamond for phosphoproteins (bottom panel). The area and optical density of cTnI bands were determined and plotted against volume loaded. Regression lines were fitted to the data points (mean ± SEM) and the resultant slopes are shown in parentheses.
<table>
<thead>
<tr>
<th></th>
<th>Phosphoproteins stained with Pro-Q Diamond</th>
<th>Proteins stained with SYPRO Ruby</th>
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<td>WT</td>
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Figure 1S

**Phosphoproteins stained with Pro-Q Diamond**

**Proteins stained with SYPRO Ruby**

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WT (Protein analysis using SyproRuby)

WT (Phosphoprotein analysis using Pro-Q)
**Figure 3S**

**cMyBP-C-/- (Protein analysis using SyproRuby)**

![Graph showing the relationship between volume (uL) and area*mean optical density for cTnI -PKA and cTnI +PKA.]

**cMyBP-C-/- (Phosphoprotein analysis using Pro-Q)**

![Graph showing the relationship between volume (uL) and area*mean optical density for cTnI -PKA and cTnI +PKA.]

- cTnI -PKA (1.42)
- cTnI +PKA (1.87)