Differential Roles of Cardiac Myosin-Binding Protein C and Cardiac Troponin I in the Myofibrillar Force Responses to Protein Kinase A Phosphorylation

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Abstract—The heart is remarkably adaptable in its ability to vary its function to meet the changing demands of the circulatory system. During times of physiological stress, cardiac output increases in response to increased sympathetic activity, which results in protein kinase A (PKA)-mediated phosphorylations of the myofilament proteins cardiac troponin (cTnI) and cardiac myosin-binding protein (cMyBP-C). Despite the importance of this mechanism, little is known about the relative contributions of cTnI and cMyBP-C phosphorylation to increased cardiac contractility. Using engineered mouse lines either lacking cMyBP-C (cMyBP-C−/−) or expressing a non-PKA phosphorylatable cTnI (cTnIala2), or both (cMyBP-C−/−/cTnIala2), we investigated the roles of cTnI and cMyBP-C phosphorylation in the regulation of the stretch-activation response. PKA treatment of wild-type and cTnIala2 skinned ventricular myocardium accelerated stretch activation such that the response was indistinguishable from stretch activation of cMyBP-C−/− or cMyBP-C−/−/cTnIala2 myocardium; however, PKA had no effect on stretch activation in cMyBP-C−/− or cMyBP-C−/−/cTnIala2 myocardium. These results indicate that the acceleration of stretch activation in wild-type and cTnIala2 myocardium is caused by phosphorylation of cMyBP-C and not cTnI. We conclude that the primary effect of PKA phosphorylation of cTnI is reduced Ca2+ sensitivity of force, whereas phosphorylation of cMyBP-C accelerates the kinetics of force development. These results predict that PKA phosphorylation of myofibrillar proteins in living myocardium contributes to accelerated relaxation in diastole and increased rates of force development in systole. (Circ Res. 2007;101:503-511.)

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

Enhanced cardiac contractile performance in response to increased circulatory demands is achieved in part through positive inotropy and lusitropy in response to increased sympathetic tone, resulting in increased stroke work during systole and earlier relaxation to optimize diastolic filling. Underscoring the importance of β-adrenergic stimulation in myocardial function, chronic hyperactivation of β-adrenergic pathways or blunting of the β-adrenergic response has been implicated in end-stage human heart failure. At the level of the myofilament, the force at a given level of Ca2+ and the rate at which force is developed depend on properties that are intrinsic to the contractile proteins, such as protein isoforms, and on factors that affect protein function, such as phosphorylation status. β-Adrenergic stimulation effects on the heart are mediated via cAMP activation of protein kinase A (PKA), which in the myofilament, principally targets the thin filament protein cTnI and the thick filament protein cMyBP-C. In skinned myocardium, phosphorylation of cTnI and cMyBP-C is associated with increased rates of cross-bridge cycling and decreased Ca2+ sensitivity of force (reviewed elsewhere), which together with altered Ca2+ handling (reviewed elsewhere) would be expected to contribute to increased twitch force, decreased twitch duration, and increased rates of relaxation. Despite the physiological importance of the inotropic response in living myocardium, the respective contributions of cTnI and cMyBP-C to PKA-induced changes in myofibrillar contraction are not well understood.

The observation that PKA accelerates force development in skinned myocardium may have important implications for cardiac function in vivo. During both the isovolumic and ejection phases of systole, the left ventricle undergoes torsional deformation as the apex twists counterclockwise relative to the base. Because the timing of electrical and mechanical activation varies across the ventricular wall, the earliest activated regions of the wall (endocardium) contract and stretch regions that are activated later (epicardium). It has also been observed that later in systole, late-activating epicardial fibers forcibly stretch the early-activating endocardial fibers. The strain of endocardial fibers during systolic ejection is thought to result from higher force production by the epicardial fibers, possibly as a result of greater levels of myosin regulatory light chain phosphorylation. Stretch results in a delayed force response (stretch activation), which,
when appropriately timed, could significantly increase force generation during late systole and thereby contribute to late ejection.

Previous studies have shown that stretch activation is an intrinsic property of myocardium and that the rate and amplitude of the delayed development of force following stretch varies with the level of Ca$^{2+}$ activation, suggesting that the response to stretch is regulated on a beat-to-beat basis and contributes to myocardial power generation during ejection. In this regard, we have recently shown that phosphorylated cTnI (cTnIala2), and we have crossed a knock-in mouse that expresses mutant cTnI that is not phosphorylated by PKA. In this regard, we have recently shown that myocardial stretch activation in mice lacking cMyBP-C (cMyBP-C$^{-/-}$) differs dramatically from wild-type (WT) controls. The rates of force decay and delayed force development were accelerated, resulting in overall acceleration of the stretch-activation response. We reasoned that the accelerated stretch-activation response in cMyBP-C$^{-/-}$ myocardium disrupts the timing of force generation such that the delayed increase in force attributable to stretch activation occurs prematurely, which truncates the period of ejection and reduces stroke volume, both of which have been observed in vivo. Later, we observed that PKA treatment accelerated stretch activation in WT myocardium, such that it became indistinguishable from that of cMyBP-C$^{-/-}$ myocardium, but PKA treatment did not alter the stretch-activation response of cMyBP-C$^{-/-}$ myocardium. These results suggest that the effect of PKA phosphorylation to accelerate stretch activation in WT myocardium involves cMyBP-C. However, recent studies suggest that the cTnI phosphorylation state may affect the rate of cross-bridge cycling and thereby modulate the rate of force development in vivo. Thus, there is not a consensus regarding the roles of cMyBP-C and cTnI in the PKA-mediated contractile response of myocardium.

The purpose of this study was to examine the respective effects of PKA-mediated phosphorylations of cMyBP-C and cTnI on the stretch-activation responses of murine skinned myocardium. In addition to the cMyBP-C$^{-/-}$ mouse, we used a knock-in mouse that expresses mutant cTnI that is not phosphorylated by PKA (cTnIala2), and we have crossed cMyBP-C$^{-/-}$ with cTnIala2 mice to produce a line that has no functional PKA target sites on either protein (cMyBP-C$^{-/-}$/cTnIala2). Our results show that the primary effect of PKA phosphorylation of cTnI is to reduce the Ca$^{2+}$ sensitivity of force, whereas cMyBP-C phosphorylation plays the dominant role in PKA modulation of the rate of cross-bridge cycling and force development.

**Materials and Methods**

An expanded Materials and Methods section is included in the online data supplement at [http://circres.ahajournals.org](http://circres.ahajournals.org).

**Transgenic Animals**

cMyBP-C-null (cMyBP-C$^{-/-}$) mice and cTnIala2 mice in which Ser23/24 were converted to alanines were generated previously. cMyBP-C$^{-/-}$/cTnIala2 mice were generated by breeding homozygous male and female cMyBP-C$^{-/-}$ mice with homozygous male and female cTnIala2 mice. All procedures involving animal care and handling were reviewed and approved by the UW Medical School Animal Care and Use Committee.

**Figure 1.** Stretch-activation response in murine myocardium. The force transient shown (bottom) is typical of the stretch-activation responses of WT myocardium following a stretch of 1% of muscle length (top). Once a steady-state isometric force of ~50% of maximal was achieved in the presence of Ca$^{2+}$, the muscle was stretched and then held at the longer length, as described under Materials and Methods. The recorded variables are labeled on the force recording and described in the text.

**Apparatus and Mechanical Experiments**

Skinned ventricular myocardium was prepared and attached to a motor and force transducer as described previously. Force–pCa relationships on skinned myocardium were constructed by varying the amount of activating Ca$^{2+}$ in solutions, and stretch activation measurements were performed by imposing a rapid stretch of 1% of muscle length on maximally and submaximally activated fibers before and after PKA treatment.

**Determination of Protein Phosphorylation**

Myofibrillar protein analysis was performed by SDS-PAGE using 10 or 12.5% Tris-HCl Criterion Precast gel (Bio-Rad) followed by SYPRO-Ruby (Molecular Probes) staining as previously described. Detection of phosphorylated proteins was achieved with Pro-Q Diamond staining (Molecular Probes). A UVP BioImaging System (UVP Inc) was used to quantify the relative abundance of total myofibrillar proteins and phosphoproteins.

**In Vivo Cardiac Function**

Noninvasive transthoracic echocardiography was performed as described previously. Anesthetized mice were probed with a Visual Sonics 770 ultrasonograph with a 30-MHz transducer (RMV 707B) (Visual Sonics, Toronto, Canada) to acquire 2D M-mode and Doppler images of the left ventricle. All echocardiography parameters were measured over at least 3 consecutive cardiac cycles.

**Results**

**Responses of WT and Mutant Myocardium to Stretch**

The recordings in Figure 1 exemplify stretch activation in WT Ca$^{2+}$-activated myocardium developing prestretch isometric force that was ~50% maximal. The amplitudes corresponding to phases 2 and 3 were normalized to prestretch isometric force to allow comparisons of stretch-activation responses at different levels of activation. P2 values...
in cMyBP-C<sup>−/−</sup> and cMyBP-C<sup>−/−</sup>/cTn<sub>ala2</sub> myocardium differed significantly from WT and cTn<sub>ala2</sub> myocardium (Figure 2A), in that the former were almost always less than the isometric force baseline at all levels of activation (Figure 3A). The apparent rate constant of force decay (k<sub>rel</sub>) in phase 2 is determined by the rates of detachment and reattachment of strongly bound cross-bridges, and at submaximal levels of activation, k<sub>rel</sub> was significantly greater in cMyBP-C<sup>−/−</sup> and cMyBP-C<sup>−/−</sup>/cTn<sub>ala2</sub> myocardium compared with WT and cTn<sub>ala2</sub> myocardium (Figure 4A).

Cross-bridge recruitment (force, P<sub>3</sub>) following stretch is related to prestretch isometric force. Because the number of cross-bridges recruited by stretch progressively decreases as activation is increased (because fewer cross-bridges are in weakly bound or unbound states), values of P<sub>3</sub> normalized to prestretch force also decreased as a function of increased activation. However, no differences in the normalized amplitude of P<sub>3</sub> were seen among the mouse lines studied here, suggesting that the numbers of cross-bridges recruited by stretch at a given activation level were similar. The trough-to-peak excursion of the phase 3 delayed force transient (P<sub>d</sub>) was proportionally greater in cMyBP-C<sup>−/−</sup> and cMyBP-C<sup>−/−</sup>/cTn<sub>ala2</sub> myocardium because of greater cross-bridge detachment (more negative P<sub>2</sub> values) during phase 2 compared with WT and cTn<sub>ala2</sub> myocardium (Figure 3B).

The rate at which stretch cooperatively recruits cross-bridges into force-generating states can be estimated from the apparent rate constant of phase 3 force development (k<sub>df</sub>). Increases in activation accelerate k<sub>df</sub> (Figure 4C) and reduce the delayed force attributable to stretch activation (P<sub>3</sub>) (as a fraction of prestretch force) because a high proportion of cross-bridges are initially bound to the thin filament, thereby reducing the number available for recruitment following stretch. Delayed force development following stretch in WT and cTn<sub>ala2</sub> myocardium occurred as a biexponential process yielding fast and slow rate constants (k<sub>1</sub> and k<sub>2</sub>) and their corresponding amplitudes (a and b) (Table I in the online data supplement). In contrast, delayed force development in both cMyBP-C<sup>−/−</sup> and cMyBP-C<sup>−/−</sup>/cTn<sub>ala2</sub> myocardium occurred as a single exponential process, corresponding to the fast rate of force development in WT and cTn<sub>ala2</sub> myocardium (supplemental Table I). In the present

Figure 2. Effects of PKA treatment on the stretch-activation responses of WT and mutant myocardium. Force transients following a stretch of 1% of muscle length were recorded at [Ca<sup>2+</sup>], yielding a prestretch isometric force of ~50% maximal before (black traces) and following (red traces) PKA treatment in WT (A), cTn<sub>ala2</sub> (B), cMyBP-C<sup>−/−</sup> (C), and cMyBP-C<sup>−/−</sup>/cTn<sub>ala2</sub> (D) myocardium. These representative transients are normalized to prestretch isometric force corresponding to the force baseline, which is arbitrarily set at 0.
study, there were no differences in $k_{df}$ at maximal activation among the mouse lines studied, but $k_{df}$ was significantly faster at submaximal levels of activation in cMyBP-C/H11002/H11002 and cMyBP-C/H11002/H11002/cTnIala2 compared with WT and cTnIala2 myocardium (Figure 4C). Because the slow phase of force development is thought to manifest cooperative cross-bridge recruitment,16 which acts to slow the overall rate of force development,15 its absence in cMyBP-C/H11002/H11002 and cMyBP-C/H11002/H11002/cTnIala2 myocardium suggests that ablation of cMyBP-C either accelerates or eliminates the cooperative recruitment of cross-bridges into force-generating states. As a result, the overall rate of stretch activation is faster in myocardium from these mouse lines.

Effects of PKA Treatment on Stretch Activation in WT and Mutant Myocardium

To investigate the respective roles of cMyBP-C and cTnI in the PKA-induced acceleration of stretch activation, skinned preparations from WT, cMyBP-C⁻/⁻, cTnIala2, and cMyBP-C⁻/⁻/cTnIala2 myocardium were treated with PKA. Because the amplitudes and rates of the phases of the stretch-activation response in mouse myocardium are activation dependent,16 the pCa of the activating solutions was adjusted to ensure that prestretch isometric force in all myocardial preparations was similar both before and after treatment with PKA. For example, to achieve a force of $\approx 50\%$ maximal, WT fibers were activated in solution of pCa 5.75 before treatment with PKA and in solution of pCa 5.65 following treatment with PKA. PKA treatment of WT and cTnIala2 myocardium significantly altered the stretch-activation response (Figure 2A and 2B) in that cross-bridge detachment during phase 2 was accelerated, causing $P_2$ to fall below prestretch isometric force (Figure 3C) and thereby increasing the overall amplitude of phase 3 ($P_{df}$) (Figure 3D). PKA also accelerated both $k_{rel}$ (Figure 4B) and $k_{df}$ (Figure 4D) such that the overall rate of delayed force development was accelerated. The acceleration of $k_{df}$ following PKA treatment in WT and cTnIala2 myocardium was caused by the elimination of the slow rate process ($k_2$) rather than acceleration of the fast rate constant $k_1$, ie, delayed force developed as a single rate process similar to $k_1$ for cMyBP-C⁻/⁻ and cMyBP-C⁻/⁻/cTnIala2 myocardium (supplemental Table I).
In contrast to the effects of PKA treatment on WT and cTnIala2 myocardium, PKA had no apparent effects on stretch activation in cMyBP-C/H11002/H11002/H11002 (Figure 2C) or cMyBP-C/H11002/H11002/H11002/cTnIala2 myocardium (Figure 2D), suggesting that the acceleration of stretch activation following treatment with PKA is attributable to the phosphorylation of cMyBP-C and not cTnI.

**Effects of cMyBP-C and cTnI Phosphorylation on Force–pCa Relationships**

Figure 5 presents an example of phosphoprotein analysis using SYPRO-Ruby and Pro-Q Diamond staining and shows that ablation of cMyBP-C did not change phosphorylation of cTnI in hearts from null mice22,30 and that replacement of endogenous cTnI with mutant cTnIala2 did not change cMyBP-C phosphorylation.31 Ablation of cMyBP-C, replacement of cTnI with cTnIala2, or both (cMyBP-C/H11002/H11002/H11002/cTnIala2) had little effect on the Ca$^{2+}$ sensitivity of force, Ca$^{2+}$-independent force at pCa 9.0, or maximum force at pCa 4.5 when compared with WT controls (supplemental Table II). Figure 5 demonstrates that PKA phosphorylated both cTnI and cMyBP-C in WT myocardium, phosphorylated only cMyBP-C in cTnIala2 myocardium, and only cTnI in cMyBP-C/C$^{-/-}$ myocardium. No phosphorylation of cMyBP-C or cTnI was detected in PKA-treated cMyBP-C/H11002/H11002/H11002/cTnIala2 myocardium, confirming the absence of PKA target sites on cMyBP-C and cTnI in these mice (Figure 5).

PKA treatment significantly reduced the Ca$^{2+}$ sensitivity of force in WT and cMyBP-C/H11002/H11002/H11002 myocardium, phosphorylated only cMyBP-C in cTnIala2 myocardium, and only cTnI in cMyBP-C/C$^{-/-}$ myocardium. No phosphorylation of cMyBP-C or cTnI was detected in PKA-treated cMyBP-C/C$^{-/-}$/cTnIala2 myocardium, confirming the absence of PKA target sites on cMyBP-C and cTnI in these mice (Figure 5).

PKA treatment significantly reduced the Ca$^{2+}$ sensitivity of force in WT and cMyBP-C/C$^{-/-}$ myocardium but produced no significant changes in cTnIala2 or in cMyBP-C/C$^{-/-}$/cTnIala2 myocardium. Although the small reduction in the Ca$^{2+}$ sensitivity of force in PKA-treated cTnIala2 myocardium did not reach statistical significance, a small decrease might be expected because of acceleration of the rates of cross-bridge cycling by cMyBP-C phosphorylation, in that increased rates would reduce the time cross-bridges spend in force-producing states. In any case, the contribution of cMyBP-C phosphorylation to decreased Ca$^{2+}$ sensitivity appears to be minor, and, overall, our results support the idea that decreased Ca$^{2+}$ sensitivity of force with PKA phosphorylation is mainly attributable to phosphorylation of cTnI, confirming earlier conclusions.3,4,32

**Figure 4.** Effect of activation and PKA treatment on stretch $k_{rel}$ and $k_{df}$. $k_{rel}$ and $k_{df}$ values were calculated from the force responses to stretches of 1% of muscle length at different levels of activation. A, $k_{rel}$ values before PKA treatment in WT (filled circles), cTnIala2 (filled triangles), cMyBP-C/C$^{-/-}$ (filled squares), and cMyBP-C/C$^{-/-}$/cTnIala2 (filled diamonds) myocardium. B, $k_{rel}$ values following PKA treatment in WT (open circles), cTnIala2 (open triangles), cMyBP-C/C$^{-/-}$ (open squares), and cMyBP-C/C$^{-/-}$/cTnIala2 (open diamonds) myocardium. C, $k_{df}$ values before PKA treatment in WT (filled circles), cTnIala2 (filled triangles), cMyBP-C/C$^{-/-}$ (filled squares), and cMyBP-C/C$^{-/-}$/cTnIala2 (filled diamonds) myocardium. D, $k_{df}$ values following PKA treatment in WT (open circles), cTnIala2 (open triangles), cMyBP-C/C$^{-/-}$ (open squares), and cMyBP-C/C$^{-/-}$/cTnIala2 (open diamonds) myocardium. Data are means±SEM from 8 to 10 myocardial preparations.
Echocardiography

Morphological data in supplemental Table III show that both cMyBP-C\textsuperscript{H11002}/cMyBP-C\textsuperscript{H11002}/cTnI\textsubscript{ala2} mice exhibited significant left ventricular hypertrophy. Echocardiographic indices show that ablation of cMyBP-C had profound effects on in vivo diastolic and systolic function, as indicated by reduced endocardial fraction shortening, ejection fraction, and prolonged isovolumic relaxation time in cMyBP-C\textsuperscript{H11002}/cMyBP-C\textsuperscript{H11002} and cMyBP-C\textsuperscript{H11002}/cMyBP-C\textsuperscript{H11002}/cTnI\textsubscript{ala2} mice (supplemental Table III). In contrast, cTnI\textsubscript{ala2} mice showed left ventricular morphology and in vivo cardiac function similar to WT mice (supplemental Table III).

Discussion

The goal of this work was to determine the respective roles of cMyBP-C and cTnI in PKA-mediated myocardial inotropy. We took advantage of existing (cMyBP-C\textsuperscript{H11002}/cMyBP-C\textsuperscript{H11002} and cTnI\textsubscript{ala2}) and novel (cMyBP-C\textsuperscript{H11002}/cMyBP-C\textsuperscript{H11002}/cTnI\textsubscript{ala2}) transgenic mouse lines to investigate the effects of phosphorylation of cMyBP-C and cTnI, the main targets of PKA in the myofilaments, on force and the kinetics of force development in skinned myocardium. Our results show that the acceleration of stretch activation caused by treatment with PKA is primarily a consequence of cMyBP-C phosphorylation, whereas the decrease in Ca\textsuperscript{2+} sensitivity of force is mainly attributable to phosphorylation of cTnI. The acceleration of stretch activation observed here in skinned myocardium would predict increases in the rates and amplitude of systolic force and pressure generation resulting from β-adrenergic stimulation in vivo.

Stretch activation is an intrinsic property of cardiac muscle\textsuperscript{17}; however, until recently, little was known about its functional role in the heart. Stretch activation has been postulated to be important for oscillatory power generation during systolic ejection.\textsuperscript{12,14,18} Consistent with this idea, the kinetics of stretch activation appear to vary with heart rate in mammals.\textsuperscript{17} We recently showed that the rate of delayed force development following stretch varies with the level of Ca\textsuperscript{2+} activation,\textsuperscript{16} which determines the number of cross-bridges available for cooperative recruitment following stretch and consequently the ability of myocardium to perform oscillatory work. For optimal cardiac function, force development and pressure generation during systole must occur with precise timing. Thus, any disruption in the kinetics of the stretch-activation response could alter cardiac function because of premature or late stretch activation, resulting in reduced oscillatory power.\textsuperscript{12,18,19} In this regard, mutations in sarcomeric proteins that slow\textsuperscript{33} or accelerate\textsuperscript{18,19} oscillatory work in myocardium also impair cardiac function.

We have recently shown that ablation of cMyBP-C dramatically accelerates cross-bridge cycling kinetics by eliminating or significantly accelerating cooperative cross-bridge recruitment\textsuperscript{19} and ultimately results in cardiac dysfunction and hypertrophy. The mechanism by which ablation of cMyBP-C accelerates cross-bridge kinetics appears to be similar to that of cMyBP-C phosphorylation,\textsuperscript{22} i.e., an increase in the proximity of myosin heads to actin\textsuperscript{34,35} caused by disruption of cMyBP-C binding to the S2 domain of myosin,\textsuperscript{36-38} which increases the probability of cross-bridge binding to actin and accelerates the transition(s) to force-generating states. Because force development in myocardium is a highly cooperative process,\textsuperscript{39} even a small change in the disposition of myosin heads resulting from ablation or phos-
phorylation of cMyBP-C could significantly alter the rates of cross-bridge attachment or detachment in response to stretch. In cMyBP-C<sup>-/-</sup> or cMyBP-C<sup>-/-</sup>/cTnI<sub>ala2</sub> myocardium, chronic ablation of cMyBP-C accelerates stretch activation and leads to cardiac dysfunction, whereas reversible phosphorylation of cMyBP-C during β-adrenergic stimulation in the healthy heart provides a mechanism by which pump function can be transiently enhanced to match circulatory demands.

In submaximally activated WT and cTnI<sub>ala2</sub> myocardium, the acceleration of phase 3–delayed force development by treatment with PKA is attributable to the elimination of the slower rate constant of force development (k<sub>s</sub>) related to cooperative cross-bridge recruitment, such that phase 3 proceeds with a single fast rate constant of force development similar to k<sub>f</sub> observed in cMyBP-C<sup>-/-</sup> and cMyBP-C<sup>-/-</sup>/cTnI<sub>ala2</sub> myocardium (supplemental Table I). On the other hand, in maximally activated WT and cTnI<sub>ala2</sub> myocardium, PKA treatment did not alter the rate of delayed force development (Figure 4C and 4D). During maximal Ca<sup>2+</sup> activations a large fraction of cross-bridges is strongly bound to actin, leaving relatively few myosin heads available for recruitment by stretch, whereas at low levels of activation, fewer cross-bridges are bound to actin, leaving more cross-bridges for recruitment by stretch. Therefore, PKA phosphorylation of cardiac myofilaments either accelerates or eliminates cooperative recruitment of cross-bridges by stretch (supplemental Table I). Accelerated cross-bridge recruitment with PKA treatment would be expected to contribute to accelerated force development in the rising phase of the myocardial twitch. In cMyBP-C<sup>-/-</sup> and cMyBP-C<sup>-/-</sup>/cTnI<sub>ala2</sub> hearts, the chronic acceleration of the stretch-activation response effectively detunes stretch activation so that the timing of delayed force development occurs too early to sustain ejection in late systole, thus significantly diminishing ejection fraction (supplemental Table III).

Although the acceleration of cross-bridge recruitment and delayed force development with β-adrenergic stimulation would be expected to accelerate force development during systolic ejection in vivo, optimization of stroke volume also requires that ventricular filling is maintained during diastole by accelerating the rates of cross-bridge detachment and force relaxation. In this study, PKA phosphorylation of WT and cTnI<sub>ala2</sub> myocardium produced a dramatic acceleration in the rate of cross-bridge detachment (k<sub>rel</sub>) following stretch (Figure 4E). Genetic ablation or acute biochemical extraction of cMyBP-C has been shown to increase shortening velocity, presumably because of increased rates of cross-bridge detachment. Thus, it appears that ablation or phosphorylation of cMyBP-C result in similar accelerations in the rates of cross-bridge detachment, perhaps because of a decrease in a putative internal viscous load normally provided by cMyBP-C, which acts to slow the speed of shortening. PKA phosphorylation of WT and cTnI<sub>ala2</sub> myocardium also increased the number of cross-bridges that detach following stretch, which is evident in more negative values of P<sub>f</sub> (Figure 3). Because more negative values of P<sub>f</sub> have been interpreted as indicating greater reversal of force-producing steps in response to stretch, cMyBP-C phosphorylation appears to accelerate cross-bridge reverse transitions from strongly bound to weakly bound states so that force relaxation is accelerated. Davis et al have proposed that reversal of force-producing steps following stretch may improve contractile efficiency in myocardium because cross-bridges could conceivably detach from actin and quickly reattach without consuming ATP.

The mechanism by which PKA phosphorylation enhances cross-bridge detachment is not known for certain but might be attributable to increased myosin head flexibility and range of movement caused by relief of spatial constraints imposed on the S2 domain of the myosin molecule. The effect of cMyBP-C to slow the rates of cross-bridge cycling may appear to be deleterious to contractile function; however, slowed rates of cross-bridge detachment may be beneficial in vivo by prolonging systolic ejection and perhaps increasing contractile efficiency by minimizing ATP utilization. Conversely, ablation of cMyBP-C accelerates cross-bridge detachment and significantly truncates the period of systolic ejection such that less blood is pumped with each beat. Myocardial force generation is proportional to the number of attached cross-bridges interacting with the thin filament and the amount of time those cross-bridges remain attached. Therefore, cMyBP-C–induced phosphorylation increases the number of detached cross-bridges following stretch (as indicated by decreased P<sub>f</sub>) and accelerates the apparent rate of cross-bridge detachment (as indicated by accelerated k<sub>rel</sub>) following stretch, such that the number of strongly bound cross-bridges is diminished and the time cross-bridges remain in strongly attached states is reduced. Such a mechanism would be beneficial during β-adrenergic stimulation in vivo in accelerating force relaxation following ejection and thereby enhancing diastolic filling.

cTnI plays an important role in the regulation of contraction by interacting with actin to inhibit actomyosin ATPase activity at low cytoplasmic Ca<sup>2+</sup>, whereas PKA phosphorylation of cTnI at Ser23/24 alters its interaction with troponin C and reduces Ca<sup>2+</sup>-binding affinity. In the present study, the substitution of Ser23/24 residues for nonphosphorylatable Ala23/24 in cTnI (cTnI<sub>ala2</sub> and cMyBP-C<sup>-/-</sup>/cTnI<sub>ala2</sub>) myocardium was observed in cMyBP-C<sup>-/-</sup> and cMyBP-C<sup>-/-</sup>/cTnI<sub>ala2</sub> hearts, the chronic acceleration of the stretch-activation response effectively detunes stretch activation so that the timing of delayed force development occurs too early to sustain ejection in late systole, thus significantly diminishing ejection fraction (supplemental Table III).
contractility and cardiac output. The lack of cMyBP-C19–22 and decreased levels of cMyBP-C phosphorylation45,46 in myocardium have been implicated in cardiac dysfunction. Here we show that ablation of cMyBP-C causes severe hypertrophy and impaired in vivo systolic and diastolic function in both cMyBP-C+/− and cMyBP-C−/−/cTnIΔalp mice. Furthermore, ablation of cMyBP-C has been shown to significantly attenuate the cardiac responsiveness to β-adrenergic agonist stimulation, suggesting that cMyBP-C plays an important role in the contractile response to β-adrenergic stimulation in vivo.47 Myocardial mechanical data demonstrate that the acceleration of cross-bridge cycling in myocardium lacking cMyBP-C accelerates stretch activation during submaximal contractions, which would reduce stretch-induced oscillatory power in late systole and prematurely terminate ejection. Thus, we propose that in healthy myocardium, cMyBP-C normally acts to slow the rates of cross-bridge attachment and transition to force-generating states, so that the peak of force generation is delayed, and also slows cross-bridge detachment and force relaxation by stabilizing force-generating states. The delayed force development in early systole will also delay the stretch-activation of force in late systole, thereby prolonging the period of ejection. However, during adrenergic stimulation, phosphorylation of cMyBP-C accelerates the rates of cross-bridge cycling and force generation such that the timing of the stretch-activation response matches the increased heart rate and contributes to the accelerated rate of twitch force generation. In this scheme, the main effect of PKA phosphorylation of cTnl is to decrease the Ca2+-binding affinity of troponin, which causes an earlier onset of relaxation, whereas the main effect of cMyBP-C phosphorylation is to accelerate stretch activation early in systole because of closer juxtaposition of myosin heads to actin. Together, PKA-mediated phosphorylations of cTnl and cMyBP-C tune myofibrillar contraction to accelerate systolic ejection and optimize the durations of systolic ejection and diastolic filling.

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Disclosures

None.

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Materials and Methods

Transgenic Animals

Adult male and female SV/129 mice (3-6 months of age) were used in this study. cMyBP-C null (cMyBP-C<sup>−/−</sup>) mice and cTnI<sub>ala2</sub> mice in which ser<sup>23/24</sup> were converted to alanine were generated as previously described. Superscript 1,2 cMyBP-C<sup>−/−</sup>/cTnI<sub>ala2</sub> mice were generated by breeding homozygous male and female cMyBP-C<sup>−/−</sup> mice with homozygous male and female cTnI<sub>ala2</sub> mice to produce F1 mice that were heterozygous for both cMyBP-C and cTnI loci and subsequent breedings of these offspring produced the desired cMyBP-C<sup>−/−</sup>/cTnI<sub>ala2</sub> genotype. As in the case of cMyBP-C<sup>−/−</sup> hearts, ablation of cMyBP-C in cMyBP-C<sup>−/−</sup>/cTnI<sub>ala2</sub> hearts was associated with significant hypertrophy. All procedures involving animal care and handling were performed according to institutional guidelines and were reviewed and approved by the University of Wisconsin Medical School Animal Care and Use Committee.

Apparatus and Experimental Protocols

Solution compositions for mechanical experiments were calculated using the computer program of Fabiato<sup>3</sup> and stability constants listed by Godt & Lindley<sup>4</sup> corrected to pH 7.0 and 22°C. All solutions contained (in mM) 100 N,N-bis (2 hydroxy-ethyl)-2-aminoethanesulfonic acid (BES), 15 creatine phosphate, 5 dithiothreitol, 1 free Mg<sup>2+</sup>, and 4 MgATP. pCa 9.0 solution contained 7 EGTA and 0.02 CaCl<sub>2</sub>; pCa 4.5 contained 7 EGTA and 0.07 CaCl<sub>2</sub>; and pre-activating solution contained 0.07 EGTA. Ionic strength of all solutions was adjusted to 180 mM with potassium propionate. Solutions containing different amounts of Ca<sup>2+</sup> free were prepared by mixing appropriate volumes of solutions of pCa 9.0 and pCa 4.5.

Skinned multicellular ventricular myocardium for mechanical experiments was prepared as previously described. Following skinning, the ends of the ventricular preparations were
attached to the arms of a position motor and force transducer as previously described. Motor position and force signals were sampled using SL Control software and saved to computer files for later analysis.

**Force-pCa Relationships**

Methods for obtaining and analysis of force-pCa relationships are described in detail elsewhere. Briefly, each myocardial preparation was allowed to develop steady force in solutions of varying free [Ca$^{2+}$]. The difference between steady-state force and the force baseline obtained after the 20% slack step was measured as the total force at that free [Ca$^{2+}$]. Active force was then calculated by subtracting Ca$^{2+}$-independent force in solution of pCa 9.0 from the total force and was normalized to the cross-sectional area of the preparation, which was calculated from the width of the preparations assuming a cylindrical cross-section. Force-pCa relationships were constructed by expressing submaximal force (P) at each pCa as a fraction of maximal force (P$_o$) determined at pCa 4.5, i.e., P/P$_o$. The apparent cooperativity in the activation of force development was inferred from the steepness of the force-pCa relationship and was quantified using a Hill plot transformation of the force-pCa data. The force-pCa data were fit using the equation, P/P$_o$ = [Ca$^{2+}$]$^{n}$/(k$^n$ + [Ca$^{2+}$]$^n$), where $n$ is the Hill coefficient, and $k$ is the [Ca$^{2+}$] required for half-maximal activation (i.e., pCa$_{50}$).

**Stretch Activation Experiments**

For stretch activation experiments, fiber length in relaxing solution was adjusted to achieve a sarcomere length of ~2.1 µm for measurement of initial isometric force and for subsequent imposition of stretch. To evoke stretch activation a rapid stretch (~10 muscle lengths s$^{-1}$) of 1% of muscle length was imposed on fibers that were activated to develop either maximal force (P$_o$) or submaximal forces of ~50% and ~25% P$_o$. Next, preparations were incubated for
30 minutes (22°C) in a solution of pCa 9.0 with 1U PKA/µl. Following incubation with PKA, the stretch activation protocols were repeated at each level of activation, taking care to match pre-treatment submaximal forces by adjusting [Ca^{2+}]_{free}. Since PKA treatment decreased the Ca^{2+} sensitivity of force in both WT and cMyBP-C^{-/-} myocardium it was necessary to use solution with a slightly higher [Ca^{2+}]_{free}. The method used for measuring the stretch activation variables have been described in detail. The amplitudes of the phases of the stretch activation responses were measured as follows and are shown in Figure 1:

- P₁, measured from pre-stretch steady-state force to the peak of phase 1,
- P₂, measured from pre-stretch steady-state force to the minimum force value at the end of phase 2,
- P₃, measured from pre-stretch steady-state force to the peak value of delayed force, and
- Pdf, difference between P₃ and P₂.

All amplitudes were normalized to the pre-stretch Ca^{2+} activated force to allow comparisons between preparations and activation levels. Apparent rate constants were calculated for phase 2 (k_{rel}, s^{-1}) between the peak of phase 1 and the minimum of phase 2 and for phase 3 (k_{df}, s^{-1}) from the beginning of force re-uptake following phase 2 to the completion of delayed force development.

**Determination of Myofibrillar Protein Phosphorylation**

Myofibrillar proteins of WT and mutant myocardium were separated by SDS-PAGE using 10 or 12.5% Tris-HCl Criterion Precast gel (BioRad) as previously described. To detect phosphorylated proteins, the gels were stained with Pro-Q Diamond following the protocol of the vendor (Molecular Probes). Briefly, the gels were (a) fixed in 10% glacial acetic acid/50% methanol (1.5 hr; 3 solution changes), (b) washed with dH₂O (1 hr; 6 solution changes), (c)
stained with Pro-Q Diamond (1.5 hr), and (d) destained with Pro-Q Diamond destaining solution (Molecular Probes) overnight. To detect myofibrillar proteins, the gels were stained with SYPRO Ruby (Molecular Probes; 3 hr) and destained with 7% glacial acetic acid/10% methanol (2 hr; 4 solution changes). Gels of phosphoprotein and total myofibrillar proteins were scanned using a UVP BioImaging System (UVP, Inc.) and quantified using LaserPix (BioRad). The skinned myocardial preparations loaded on the gels were prepared from a total of three hearts from each mouse line. To quantify protein phosphorylation levels, different volumes (in the range of 3 to 10 μL) of –PKA and +PKA samples prepared from WT and mutant 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 and the area and mean raw optical density of protein and phosphoprotein bands were determined using LabWorks analysis software (UVP BioImaging System, CA). The product of the area and mean raw optical density vs volume loaded were generated and a first order linear regression was fitted to the data points to determine the slope of the relationship between optical density and volume loaded as described previously.8

**Echocardiography**

Transthoracic echocardiography was performed by using a Visual Sonics 770 ultrasonograph with a 30-MHz transducer (RMV 707B) (Visual Sonics, Toronto) as described previously.1,9 Mice were lightly anesthetized with isoflurane (1%) and maintained on a heated platform. Two-dimensionally guided M-mode images of the LV and Doppler studies were acquired at the tip of the papillary muscles. LV mass-to-body ratio (LV/BW), LV dimension in diastole (LVDd), left ventricular end-systolic internal dimension (LVIDs), thickness of the
posterior wall in diastole (LVPWd) and systole (LVPWs), and isovolumic relaxation time (IVRT) were recorded. Endocardial fractional shortening (EnFS) was calculated as (LVDd-LVDs)/LVDd x 100, where LVDs is LV dimension in systole. Ejection fraction (EF) was calculated as (end diastolic volume – end systolic volume/end diastolic volume) x 100. All parameters were measured over at least three consecutive cycles.

**Statistical Analysis**

Cross-sectional areas of skinned preparations were calculated by measuring the width of the mounted preparation and assuming a cylindrical cross-section. Sub-maximal Ca$^{2+}$-activated force (P) was expressed as a fraction of the force ($P_o$) generated at pCa 4.5, i.e., $P/P_o$. Rate constants of force decay ($k_{rel}$) were obtained by fitting a single exponential to the time course of decay, i.e., $y = a \cdot (1 - \exp(-k_{rel} \cdot x))$, where $a$ is the amplitude and $k_{rel}$ is the rate constant of decay. Rate constants for delayed force development were obtained by a double exponential fit, $y = a \cdot \exp(-k_1 \cdot x) + b \cdot \exp(-k_2 \cdot x)$, 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 rising with rate constant $k_2$, or as a single composite rate constant estimated by linear transformation of the half-time of force redevelopment, i.e., $[k_{df} = -\ln 0.5 \times (t_{1/2})^{-1}]$.

All data are reported as means ± SEM. Comparisons of force-pCa relationships and stretch activation variables between groups both before and after treatment with PKA, and echocardiography variables were done using a one-way analysis of variance (ANOVA) or a student’s t-test. Significance level was set at $p < 0.05$. 
References


Figure Legend

**Figure 1S. The slopes of protein and phosphoprotein determined from plots of area*mean raw optical density vs volume loaded for WT myocardium.** Different volumes of -/+ PKA treated skinned myocardial samples prepared from three hearts of WT mice 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 mean raw optical density (OD) of MyBP-C, TnT, and cTnI bands were determined and plotted against volume (uL) loaded. Regression lines were fitted to the data points and the resultant slope for proteins and phosphoproteins is shown in the top and bottom panel, respectively. Each bar represents the mean and the error bar the SEM.

**Figure 2S. The slopes of protein and phosphoprotein determined from plots of area*mean raw optical density vs volume loaded for MyBP-C−/− myocardium.** Different volumes of -/+ PKA treated skinned myocardial samples prepared from three hearts of MyBP-C−/− mice 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 mean raw optical density (OD) of TnT and cTnI bands were determined and plotted against volume (uL) loaded. Regression lines were fitted to the data points and the resultant slope for proteins and phosphoproteins is shown in the top and bottom panel, respectively. Each bar represents the mean and the error bar the SEM.

**Figure 3S. The slopes of protein and phosphoprotein determined from plots of area*mean raw optical density vs volume loaded for cTnIala2 myocardium.** Different volumes of -/+ PKA treated skinned myocardial samples prepared from three hearts of cTnIala2 mice 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 mean raw optical density (OD) of MyBP-C and TnT bands were determined and plotted against volume (uL) loaded. Regression lines were fitted to the data points and the resultant slope for proteins and phosphoproteins is shown in the top and bottom panel, respectively. Each bar represents the mean and the error bar the SEM.

**Figure 4S. The slopes of protein and phosphoprotein determined from plots of area*mean raw optical density vs volume for cMyBP-C⁻/⁻/cTnI₉₉₂ myocardium.** Different volumes of -/+ PKA treated skinned myocardial samples prepared from three hearts of cMyBP-C⁻/⁻/cTnI₉₉₂ mice 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 mean raw optical density (OD) of TnT bands were determined and plotted against volume (uL) loaded. Regression lines were fitted to the data points and the resultant slope for proteins and phosphoproteins is shown in the top and bottom panel, respectively. Each bar represents the mean and the error bar the SEM.
<table>
<thead>
<tr>
<th>Group</th>
<th>Activation Level (P/P₀)</th>
<th>a</th>
<th>k₁ (s⁻¹)</th>
<th>b</th>
<th>k₂ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PKA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.00</td>
<td>1.00</td>
<td>41.1 ± 2.5</td>
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<tr>
<td>cTnIala₂</td>
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<td>1.00</td>
<td>42.0 ± 2.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cMyBP-C⁻/⁻</td>
<td>1.00</td>
<td>1.00</td>
<td>42.4 ± 2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cMyBP-C⁻/⁻/ cTnIala₂</td>
<td>1.00</td>
<td>1.00</td>
<td>43.9 ± 2.8</td>
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<td>-</td>
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<tr>
<td>WT</td>
<td>0.52 ± 0.02</td>
<td>0.63 ± 0.04</td>
<td>17.9 ± 1.8</td>
<td>0.37 ± 0.03</td>
<td>4.0 ± 0.9</td>
</tr>
<tr>
<td>cTnIala₂</td>
<td>0.54 ± 0.02</td>
<td>0.67 ± 0.04</td>
<td>20.1 ± 1.9</td>
<td>0.33 ± 0.03</td>
<td>4.4 ± 1.1</td>
</tr>
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<td>cMyBP-C⁻/⁻</td>
<td>0.53 ± 0.02</td>
<td>1.00*</td>
<td>31.1 ± 2.2*</td>
<td>-</td>
<td>-</td>
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<tr>
<td>cMyBP-C⁻/⁻/ cTnIala₂</td>
<td>0.55 ± 0.02</td>
<td>1.00*</td>
<td>33.3 ± 2.5*</td>
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<td>-</td>
</tr>
<tr>
<td>+ PKA</td>
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<td></td>
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<tr>
<td>WT</td>
<td>0.24 ± 0.01</td>
<td>0.51 ± 0.03</td>
<td>10.4 ± 1.1</td>
<td>0.49 ± 0.03</td>
<td>2.1 ± 0.5</td>
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<td>cTnIala₂</td>
<td>0.27 ± 0.02</td>
<td>0.54 ± 0.03</td>
<td>12.2 ± 1.3</td>
<td>0.46 ± 0.03</td>
<td>2.4 ± 0.5</td>
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<td>cMyBP-C⁻/⁻</td>
<td>0.28 ± 0.02</td>
<td>1.00*</td>
<td>20.5 ± 1.6*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cMyBP-C⁻/⁻/ cTnIala₂</td>
<td>0.28 ± 0.02</td>
<td>1.00*</td>
<td>22.5 ± 1.7*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1S. Activation dependence of phase 3 delayed force development in WT and mutant skinned myocardium.
Rate constants were calculated from force transients in response to stretches of 1% of muscle length at each of the indicated levels of activation (adjusted by varying free [Ca\(^{2+}\)]). Data are reported as means ± SEM from 8-10 preparations. As described in Material and Methods, the apparent rate constants for delayed force recovery were obtained by fitting each record with a double exponential equation, 
\[
y = a \cdot \exp(-k_1 \cdot x) + b \cdot \exp(-k_2 \cdot x),
\]
where ‘a’ is the amplitude of the first exponential phase with rate constant \(k_1\) and ‘b’ is the amplitude of the second exponential phase with rate constant \(k_2\).

*Significantly different from WT and cTnI\(\text{ala2}\), \(p < 0.05\).

**Significantly different from - PKA control, \(p < 0.05\).
Table 2S. Force development in skinned myocardium from WT and mutant mice

<table>
<thead>
<tr>
<th>Group</th>
<th>$P_{\text{rest}}$ (mN mm$^{-2}$)</th>
<th>$P_0$ (mN mm$^{-2}$)</th>
<th>$n_H$</th>
<th>pCa$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>- PKA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.7 ± 0.2</td>
<td>20.4 ± 2.0</td>
<td>4.0 ± 0.3</td>
<td>5.76 ± 0.02</td>
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<tr>
<td>cTnI$_{ala2}$</td>
<td>0.8 ± 0.2</td>
<td>21.7 ± 2.4</td>
<td>4.1 ± 0.3</td>
<td>5.77 ± 0.03</td>
</tr>
<tr>
<td>cMyBP-C$^{-/-}$</td>
<td>0.8 ± 0.2</td>
<td>21.1 ± 2.3</td>
<td>3.5 ± 0.3</td>
<td>5.74 ± 0.02</td>
</tr>
<tr>
<td>cMyBP-C$^{-/-}$/cTnI$_{ala2}$</td>
<td>0.9 ± 0.2</td>
<td>22.4 ± 2.2</td>
<td>3.5 ± 0.3</td>
<td>5.78 ± 0.03</td>
</tr>
<tr>
<td><strong>+ PKA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.8 ± 0.2</td>
<td>19.2 ± 2.2</td>
<td>3.9 ± 0.3</td>
<td>5.65 ± 0.02*</td>
</tr>
<tr>
<td>cTnI$_{ala2}$</td>
<td>0.9 ± 0.2</td>
<td>20.4 ± 2.3</td>
<td>3.9 ± 0.3</td>
<td>5.73 ± 0.03</td>
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<tr>
<td>cMyBP-C$^{-/-}$</td>
<td>0.9 ± 0.2</td>
<td>21.8 ± 2.4</td>
<td>3.5 ± 0.3</td>
<td>5.66 ± 0.02*</td>
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<tr>
<td>cMyBP-C$^{-/-}$/cTnI$_{ala2}$</td>
<td>0.7 ± 0.2</td>
<td>21.9 ± 2.2</td>
<td>3.4 ± 0.3</td>
<td>5.77 ± 0.03</td>
</tr>
</tbody>
</table>

All values are expressed as means ± SEM from 6 skinned myocardial preparations. $P_0$, maximal Ca$^{2+}$-activated force at pCa 4.5; $P_{\text{rest}}$, Ca$^{2+}$-independent force at pCa 9.0; $n_H$, Hill coefficient for Ca$^{2+}$-activated force; pCa$_{50}$, pCa required for half maximal activation.

Significantly different from - PKA control, $p < 0.05$. 
### Table 3S. Echocardiography summary data from WT and mutant mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cTnI_{ala2}</th>
<th>cMyBP-C^{-/-}</th>
<th>cMyBP-C^{-/-}/ cTnI_{ala2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>LV/BW (mg/g)</td>
<td>3.47 ± 0.24</td>
<td>3.45 ± 0.29</td>
<td>6.39 ± 0.31*</td>
<td>5.87 ± 0.35*</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>450 ± 18</td>
<td>443 ± 37</td>
<td>430 ± 35</td>
<td>467 ± 24</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>0.88 ± 0.06</td>
<td>0.88 ± 0.03</td>
<td>1.29 ± 0.06*</td>
<td>1.17 ± 0.06*</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.31 ± 0.03</td>
<td>1.38 ± 0.05</td>
<td>1.63 ± 0.08*</td>
<td>1.58 ± 0.06*</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.42 ± 0.11</td>
<td>3.55 ± 0.11</td>
<td>4.24 ± 0.12*</td>
<td>4.02 ± 0.13*</td>
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<tr>
<td>LVIDs (mm)</td>
<td>2.03 ± 0.21</td>
<td>2.08 ± 0.12</td>
<td>3.34 ± 0.16*</td>
<td>2.96 ± 0.13*</td>
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<tr>
<td>EF (%)</td>
<td>72.50 ± 4.17</td>
<td>72.97 ± 2.14</td>
<td>46.47 ± 4.24*</td>
<td>50.40 ± 2.17*</td>
</tr>
<tr>
<td>EnFS (%)</td>
<td>41.60 ± 3.52</td>
<td>41.54 ± 1.84</td>
<td>23.53 ± 2.70*</td>
<td>26.42 ± 1.43*</td>
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<tr>
<td>IVRT (ms)</td>
<td>17.72 ± 0.76</td>
<td>17.29 ± 1.84</td>
<td>33.66 ± 2.96*</td>
<td>30.01 ± 1.97*</td>
</tr>
</tbody>
</table>

All values are expressed as means ± SEM. LV/BW, left ventricle mass/body weight ratio; HR, heart rate; PWd, posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; LVDd, left ventricular dimension in diastole; LVIDs, left ventricular end-systolic internal dimension; EF, ejection fraction; EnFS, endocardial fractional shortening; IVRT, isovolumic relaxation time.

*Significantly different from WT, *p* < 0.05.
Figure 1S
Figure 2S

MyBP-C^−/−

Slope (proteins, OD/Volume)

PKA  -  +  -  +  -  +

MyBP-C  TnT  TnI

Slope (phosphoproteins, OD/Volume)

PKA  -  +  -  +  -  +

MyBP-C  TnT  TnI
Figure 4S

cMyBP-C<sup>−/−</sup>/cTnI<sub>ala2</sub>

Slope (proteins, OD/Volume)

PKA - + - + - + +
MyBP-C TnT TnI

Slope (phosphoproteins, OD/Volume)

PKA - + - + - + +
MyBP-C TnT TnI