Phosphoregulation of Cardiac Inotropy via Myosin Binding Protein-C During Increased Pacing Frequency or β₁-Adrenergic Stimulation

Carl W. Tong, MD, PhD; Xin Wu, MD; Yang Liu, MD, PhD; Paola C. Rosas, MD, PhD, RPh; Sakthivel Sadayappan, PhD; Andy Hudmon, PhD; Mariappan Muthuchamy, PhD; Patricia A. Powers, PhD; Héctor H. Valdivia, MD, PhD; Richard L. Moss, PhD

Background—Mammalian hearts exhibit positive inotropic responses to β-adrenergic stimulation as a consequence of protein kinase A–mediated phosphorylation or as a result of increased beat frequency (the Bowditch effect). Several membrane and myofibrillar proteins are phosphorylated under these conditions, but the relative contributions of these to increased contractility are not known. Phosphorylation of cardiac myosin-binding protein-C (cMyBP-C) by protein kinase A accelerates the kinetics of force development in permeabilized heart muscle, but its role in vivo is unknown. Such understanding is important because adrenergic responsiveness of the heart and the Bowditch effect are both depressed in heart failure.

Methods and Results—The roles of cMyBP-C phosphorylation were studied using mice in which either WT or nonphosphorylatable forms of cMyBP-C [ser273ala, ser282ala, ser302ala: cMyBP-C(t3SA)] were expressed at similar levels on a cMyBP-C null background. Force and [Ca²⁺]ₙ measurements in isolated papillary muscles showed that the increased force and twitch kinetics because increased pacing or β₁-adrenergic stimulation were nearly absent in cMyBP-C(t3SA) myocardium, even though [Ca²⁺]ₙ transients under each condition were similar to WT. Biochemical measurements confirmed that protein kinase A phosphorylated ser273, ser282, and ser302 in WT cMyBP-C. In contrast, CaMKIIβ, which is activated by increased pacing, phosphorylated ser302 principally, ser282 to a lesser degree, and ser273 not at all.

Conclusions—Phosphorylation of cMyBP-C increases the force and kinetics of twitches in living cardiac muscle. Further, cMyBP-C is a principal mediator of increased contractility observed with β₁-adrenergic stimulation or increased pacing because of protein kinase A and CaMKIIβ phosphorylations of cMyB-C. (Circ Heart Fail. 2015;8:595-604. DOI: 10.1161/CIRCHEARTFAILURE.114.001585.)

Key Words: contractility ■ myosin-binding protein-C ■ phosphorylation

The force and kinetics of the cardiac twitch exhibit remarkable plasticity on a beat-to-beat basis as a means for matching cardiac output to circulatory demand. Such demand-driven inotropy is depressed in heart failure, causing the majority of symptoms in patients; therefore, better understanding of the underlying mechanisms should lead to new approaches for treating this disease. Cardiac inotropy has usually been understood to result from alterations in Ca²⁺ delivery to thin filament regulatory proteins during the myocardial twitch or alterations in thin filament responsiveness to myoplasmic Ca²⁺ as a consequence of post-translational modifications of thick or thin filament accessory proteins. However, the relative contributions of these mechanisms to cardiac function under resting conditions or under stress, such as β₁-adrenergic stimulation, are not known. The present study was undertaken to determine the basis for cardiac inotropy in both to better understand this phenomenon and to suggest mechanisms of reduced function in heart failure.

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Here, measurements of force and intracellular Ca²⁺ transients were done in intact ex vivo myocardial preparations from either wild-type mice or mutant mice expressing a phosphorylation-deficient form of the thick filament regulatory protein, cardiac myosin-binding protein-C (cMyBP-C). cMyBP-C

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From the Department of Cell and Regenerative Biology, University of Wisconsin School of Medicine and Public Health, Madison (C.W.T., P.A.P., R.L.M.); Department of Medical Physiology (C.W.T., Y.L., P.C.R., M.M.) and Neuroscience and Experimental Therapeutics (X.W.), Texas A&M University Health Science Center College of Medicine, Temple; Baylor Scott & White Health, Temple, TX (C.W.T.); Department of Physiology, Loyola University Chicago Stritch School of Medicine, IL (S.S.); Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis (A.H.); and Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor (H.H.V.).


Correspondence to Richard L. Moss, PhD, Department of Cell and Regenerative Biology, UW School of Medicine and Public Health, 750 Highland Ave, Madison, WI 53705. E-mail rlmoss@wisc.edu

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binds to the thick filament and represses myosin–actin interactions and thereby slow cross-bridge cycling when an individual is at rest. Thus, cMyBP-C may be a major modulator of cardiac inotropy. Previous studies of hypo-phosphorylated cMyBP-C used skinned (ie, removed cellular membrane) myocardium at fixed concentrations of added calcium. The current study was undertaken to determine the roles of cMyBP-C in regulating cardiac contractility in living myocardium in the context of the time-varying Ca²⁺ transient during the twitch. Measurements were done as a function of increased stimulus frequency and in the presence and absence of β₁-adrenergic stimulation. The results, together with measurements of phosphorylation of other myofilament proteins under these conditions, show that phosphorylation of cMyBP-C is the predominant proximate mediator of both pacing-dependent and β₁-adrenergic-dependent potentiation of force and contraction kinetics. Remarkably, replacement of phosphorylatable serines in cMyBP-C with alanines blunted positive inotropic responses, even though the expected phosphorylations of other myofilament proteins and the expected increases in the amplitude and rates of the myoplasmic Ca²⁺ transients were observed to occur in both WT and mutant myocardium.

Methods

The experiments described here used previously generated mouse lines in which non-protein kinase A (PKA)-phosphorylatable cMyBP-C (ser273ala, ser282ala, ser302ala; the cMyBPC-C(t3SA) mouse) or WT cMyBP-C (the cMyBP-C(tWT) mouse) were expressed on a cMyBP-C null background. Expression levels in the lines used were 74% for cMyBP-C(t3SA) mice and 72% for cMyBP-C(tWT) mice, referenced to cMyBP-C expression in nontransgenic WT mice. The protocols for animal care and use were approved by the Animal Care and Use Committees of the UW School of Medicine and Public Health and Texas A&M Health Science Center College of Medicine.

[Ca²⁺]₈ and force were measured simultaneously in ex vivo intact papillary muscles to assess cross-bridge interactions in the context of the [Ca²⁺] transient during a twitch. Pacing frequency was varied and 1 μmol/L dobutamine (β₁-adrenergic agonist) was added to the bath to mimic β₁-adrenergic stimulation. Fura-2 AM was used to assess [Ca²⁺]₈. Experiments were performed at room temperature to avoid rapid extrusion of Fura-2 AM from myocardial cells that occurs at higher temperatures. At room temperature, increasing the pacing frequency from 1 to 3 Hz produced a positive force–frequency relationship much like that observed when pacing frequency is increased from 1 Hz to 3 Hz; and a Wilcoxon test for comparison between basal and post-dobutamine treatment; and analysis of variance with post hoc Tukey-HSD for multiple comparisons between groups. Data sets that do not have normal histograms are analyzed by exact tests: a Mann–Whitney U test for 2 independent samples; a Friedman’s 2-way analysis by rank for repeated measurements for increasing pacing frequency from 1 Hz to 3 Hz; and a Wilcoxon test for comparison between basal and post-dobutamine treatment. For all analyses, the choice of either a parametric test or an exact test did not affect the conclusions. Whenever possible, data were shown with Box-Whisker plots with the following percentiles: box-bottom 25th, median line-in-box, box-top 75th, bottom-whisker 10th, and top-whisker 90th.

Results

Initial experiments investigated the contributions of myofibrillar protein phosphorylations and variations in [Ca²⁺]₈ to the positive inotropy associated with increased pacing frequency or β₁-adrenergic stimulation. For these measurements, experimental recordings of force and [Ca²⁺]₈ during cardiac twitches are reported as force versus [Ca²⁺]₈ (Figure 1) to allow direct comparisons of force or [Ca²⁺]₈ for each experimental condition. During a pacing-triggered twitch, the [Ca²⁺]₈ transient rises to its maximum before the force peak (Figure 1A), so that plotting force versus [Ca²⁺]₈ yields a hysteresis loop because of the continuing rise in force during the initial decline in [Ca²⁺]₈ from its peak (Figure 1B). The hysteresis loop exhibits consistent phases: A to B, concordant rise, both force

Isolated cMyBP-C(tWT) hearts were first subjected to Langendorf retrograde perfusion to remove endogenous circulating catecholamines and establish baseline phosphorylation of thick and thin filament proteins. Pacing frequency was increased and dobutamine was infused to determine patterns of myofibrillar protein phosphorylation under these conditions. Western-blotting with site-specific antibodies for cMyBP-C phosphorylations was done on myofibers from the perfused hearts to determine which phosphorylatable serines were associated with the increased contractility caused by increased pacing or β₁-adrenergic stimulation.

Recombinant full length WT cMyBP-C, recombinant CaMK2δ, and CaMK2δ kinase inhibitors were used to test whether CaMK2δ mediates rate-dependent phosphorylation of cMyBP-C.

Phosphoprotein staining (Pro-Q Diamond) and total protein staining (Sypro-Ruby) were used to survey possible changes in phosphorylation of other myofilament proteins in cMyBP-C(t3SA) and cMyBP-C(tWT) papillary muscles and hearts under each experimental condition.

SPSS and R software were used to perform statistical analyses. The normality of a data set is determined by both a normal appearing histogram and nonsignificant Shapiro test for normality. Normal data sets are analyzed by an independent t test when comparing 2 lines; a repeated measure analysis of variance for increasing pacing frequency from 1 Hz to 3 Hz; a paired t test for comparison between basal and post-dobutamine treatment; and analysis of variance with post hoc Tukey-HSD for multiple comparisons between groups. Data sets that do not have normal histograms are analyzed by exact tests: a Mann–Whitney U test for 2 independent samples; a Friedman’s 2-way analysis by rank for repeated measurements for increasing pacing frequency from 1 Hz to 3 Hz; and a Wilcoxon test for comparison between basal and post-dobutamine treatment. For all analyses, the choice of either a parametric test or an exact test did not affect the conclusions. Whenever possible, data were shown with Box-Whisker plots with the following percentiles: box-bottom 25th, median line-in-box, box-top 75th, bottom-whisker 10th, and top-whisker 90th.

Figure 1. Hysteresis in plots of force vs [Ca²⁺]₈ during the cardiac twitch. A. On stimulation, [Ca²⁺]₈ rises to a peak well before twitch force. B. Plotting twitch force vs [Ca²⁺]₈ during the time progression of a twitch forms a hysteresis loop. Labels on the loop indicate diastole, A; peak of [Ca²⁺]₈, B; and peak of twitch force C. The segments formed between these points are described in the text.
and \([\text{Ca}^{2+}]_\text{in}\) increase; B to C, discordant rise, force increases whereas \([\text{Ca}^{2+}]_\text{in}\) decreases; C to A, concordant relaxation, both force and \([\text{Ca}^{2+}]_\text{in}\) decrease.9

Increasing the pacing frequency from 1 to 3 Hz substantially increased twitch force, that is, the Bowditch effect,11 in papillary muscles from cMyBP-C(tWT) hearts (Figure 2B, 2C, and 2E). However, the Bowditch effect was severely blunted in cMyBP-C(t3SA) muscles expressing non-PKA-phosphorylatable cMyBP-C. Recordings of \([\text{Ca}^{2+}]_\text{in}\) (Figure 2A and 2D) showed that there were similar progressive increases in the amplitudes of the \([\text{Ca}^{2+}]_\text{in}\) transients in the 2 types of muscle as frequency was increased over this same range. Thus, the dramatically reduced effect of increasing stimulus frequency on twitch force in myocardium expressing phosphorylation-deficient cMyBP-C suggests that such phosphorylation is required for full expression of the positive inotropy that characterizes the Bowditch effect. Conversely, any direct activating effect on thin filaments caused by the increased \([\text{Ca}^{2+}]_\text{in}\) transients at higher frequencies only partially increases peak twitch force in phosphorylation-deficient papillary muscles.

Next, the \(\beta_1\)-adrenergic agonist dobutamine was infused into the solution bathing the papillary muscles. At a frequency of 3 Hz, dobutamine substantially further increased twitch force in WT myocardium (Figure 2B, 2C, and 2E), an effect that was nearly absent in cMyBP-C(t3SA). In both instances, the \([\text{Ca}^{2+}]_\text{in}\) transients were increased to a similar degree (Figure 2A and 2D). Thus, the fullest potentiation of twitch force as a result of a \(\beta_1\)-agonist requires phosphorylation of cMyBP-C and seems to include a much smaller component that is a direct effect of the concomitant increase in the myoplasmic \([\text{Ca}^{2+}]_\text{in}\) transient. Thus, the increase in the myoplasmic \([\text{Ca}^{2+}]_\text{in}\) transient because of dobutamine induced, on average, a small increase in twitch force in cMyBP-C(t3SA) myocardium. This increase is likely because of a direct activating effect of Ca\(^{2+}\) binding to the thin filament.

For the Bowditch effect and \(\beta_1\)-adrenergic stimulation, the mechanism of twitch potentiation is thought to be increased activation of the myofibrils caused by the increased amplitude of the \([\text{Ca}^{2+}]_\text{in}\) transient,12,13 but our results above do not support this idea, implying that the mechanism is much more complex. From our data, positive inotropy in WT myocardium occurred primarily as a result of increases in the amplitude of the discordant rise segment (Figure 2C and 2F), which has previously been suggested to involve a cooperative activation process.9,14–16 In contrast, cMyBP-C(t3SA) myocardium exhibited much smaller increases in force with dobutamine or increased stimulus frequency, despite increases in \([\text{Ca}^{2+}]_\text{in}\) that were similar to cMyBP-C(WT) myocardium. The depressed force responses in cMyBP-C(t3SA) myocardium were associated with no significant increases in the discordant rise segment when pacing was increased (Figure 2C and 2F). Overall, the increases in amplitude of \([\text{Ca}^{2+}]_\text{in}\) transients accounted for a small component of the positive inotropic responses...
to increased pacing or dobutamine. Thus, phosphorylation of cMyBP-C seems to be the predominant mediator of the force–frequency relationship and the inotropy caused by a β1-adrenergic agonist, doubtless because of increased numbers of force-generating cross-bridges.

cMyBP-C(tWT) muscles also exhibited pacing-dependent acceleration of relaxation (Figure 3A) that was virtually absent in cMyBP-C(t3SA) (Figure 3B). Dobutamine increased the peak rates of force development (+dF/dt)$_{\text{max}}$ and relaxation (−dF/dt)$_{\text{min}}$ (Figure 3C) in both WT and cMyBP-C(t3SA) myocardium, but these effects were much greater in WT. A change in (−dF/dt)$_{\text{min}}$ might account for a change in (+dF/dt)$_{\text{max}}$ simply as a result of the corresponding change in peak force. Therefore, we used the ratio |(−dF/dt)$_{\text{min}}$|/(+dF/dt)$_{\text{max}}$ to assess the effects of inotropic stimuli on the kinetics of relaxation. Increased pacing frequency accelerated relaxation in cMyBP-C(tWT) but not in cMyBP-C(t3SA) muscles (Figure 3A, 3B, and 3D). Dobutamine did accelerate relaxation in cMyBP-C(t3SA) but to a lesser degree than in WT (Figure 3D). The differing magnitudes of effects in the 2 mouse lines were not caused by differences in the intracellular Ca2+ transients because myocardium from both lines exhibited similar increases in peak [Ca2+]$_{\text{in}}$ decay similarly in both mouse models. F, Increased pacing causes similar decreases in the time from the [Ca2+]$_{\text{in}}$ peak to 50% decay in both mouse models.

Figure 3. Cardiac myosin binding protein-C (cMyBP-C) phosphorylation accelerates relaxation. A, cMyBP-C(tWT) myocardium exhibits acceleration of relaxation in response to increased pacing or dobutamine, which is seen as increased (−dF/dt)$_{\text{min}}$/(+dF/dt)$_{\text{max}}$ in normalized $dF/dt$ traces. B, cMyBP-C(t3SA) myocardium exhibits no change in (−dF/dt)$_{\text{min}}$/(+dF/dt)$_{\text{max}}$ in response to increased pacing (upper arrow), but still exhibited a diminished increase in (−dF/dt)$_{\text{min}}$/(+dF/dt)$_{\text{max}}$ in response to dobutamine. C, Increased pacing increased absolute (+dF/dt)$_{\text{max}}$ for both lines. Increased pacing increased absolute (−dF/dt)$_{\text{min}}$ for both lines, although the effect was much greater in cMyBP-C(tWT) myocardium. cMyBP-C(t3SA) muscles also exhibited much smaller values of absolute (+dF/dt)$_{\text{max}}$ and (−dF/dt)$_{\text{min}}$ at 3 Hz with or without dobutamine. D, Increased pacing causes acceleration of relaxation, seen as increased |(−dF/dt)$_{\text{min}}$/(+dF/dt)$_{\text{max}}$| only in cMyBP-C(tWT). Dobutamine treatment causes increased |(−dF/dt)$_{\text{min}}$/(+dF/dt)$_{\text{max}}$| in both mouse models, although the effect was greater in cMyBP-C(tWT) myocardium. E, Normalized [Ca2+]$_{\text{in}}$ transients show that increased pacing or dobutamine accelerates the time course of [Ca2+]$_{\text{in}}$ decay similarly in both mouse models. F, Increased pacing causes similar decreases in the time from the [Ca2+]$_{\text{in}}$ peak to 50% decay in both mouse models.

**tWT**: n=9; **t3SA**: n=11

* p<0.05 vs. tWT; & p<0.05 (+) 1-to-3Hz frequency response within same mouse line; 
# p<0.05 Dob vs. basal within same mouse line at 3Hz
acceleration of transient decay times from peak to 50% maximal \([\mathrm{Ca}^{2+}]_o\) with either increased pacing frequency or dobutamine (Figures 2A, 2D, 3E–3F). These results strongly suggest that phosphorylation of cMyBP-C mediates the acceleration of relaxation by increasing the rate of cross-bridge cycling. This complex observation is addressed in detail in a companion paper and is not considered further here.

In vivo tissue Doppler echocardiography showed that cMyBP-C(t3SA) hearts exhibit slower relaxation velocity, and diastolic dysfunction, Sa is peak myocardial contraction velocity by tissue Doppler; Ea is peak myocardial relaxation velocity during early diastole. Both lines exhibit similar basal heart rates (cMyBP-C(tWT) 506±18 BPM, n=8; cMyBP-C(t3SA) 453±30 BPM, n=7). Dobutamine treatment increased heart rates on both lines in similar fashion (cMyBP-C(tWT) 635±7 BPM, n=8; cMyBP-C(t3SA) 660±12 BPM, n=7). A, In cMyBP-C(tWT) hearts, Ea>Sa. B, In cMyBP-C(t3SA) hearts, Ea<Sa. C–E, cMyBP-C(t3SA) hearts exhibit slower Ea resulting in depressed Ea/Sa (analogous to \(|-(dF/dt)_\text{min}|/(dF/dt)_\text{max}|\)). F, Left ventricular inner diameters during diastole (LVIDd) are similar between MyBP-C(tWT) and MyBP-C(t3SA) hearts. G, cMyBP-C(t3SA) hearts exhibit increased LV posterior wall thickness, indicating hypertrophy. H, Both models show similar ejection fraction and fractional shortening. J, cMyBP-C(t3SA) hearts exhibit prolonged iso-volumetric relaxation time (IVRT) in the basal state. IVRT was similar in both models when dobutamine was applied, likely because of an increase in heart rate and a probable increase in left atrial pressure. K, cMyBP-C(t3SA) hearts exhibited greater E/E (Doppler blood flow velocity across mitral valve at early filling/tissue Doppler velocity at medial mitral valve annulus during early filling) for all conditions, indicating persistent diastolic dysfunction. cMyBP-C indicates cardiac myosin binding protein-C.
Western blotting with site-specific phosphoantibodies revealed that Ser302 is the predominant site of phosphorylation when pacing is increased, with no increase in phosphorylation of Ser273, that is, Ser302-P>Ser282-P>Ser273-P (Figure 5A–5D). In contrast, dobutamine resulted in robust phosphorylation of all 3 residues (Figure 5A–5D), indicating that the kinases mediating these phosphorylations differ for the 2 interventions. Earlier studies showed that increasing the frequency of calcium pulses from 1 to 2.5 Hz activates CaMKδ50 and that inhibition of CaMKδ suppresses the phosphorylation of cMyBP-C.5 Thus, our results suggest that pacing-induced inotropy is mediated by CaMKδ phosphorylation of cMyBP-C principally at Ser302. Consistent with this, pharmacological inhibition of CaMKδ depressed the pacing-induced increase in Ser302 phosphorylation (Figure 5E).

Further experiments confirmed that CaMKδ preferentially phosphorylates cMyBP-C at Ser302. A bacterial expression system was used to produce unphosphorylated recombinant cMyBP-C, which was then treated with recombinant CaMKδ. This in vitro method for assessing site specificity of phosphorylation precludes potentially confounding effects on phosphorylation caused by other endogenous kinases or basally phosphorylated cMyBP-C in intact muscle. CaMKδ phosphorylated cMyBP-C at Ser302 within 15 to 30 seconds at [Ca2+] comparable to in vivo diastolic [Ca2+]i (Figure 5G). The results from Langendorff perfused hearts, and in vitro studies indicate that CaMKδ phosphorylates cMyBP-C at Ser302 when pacing frequency is increased, Ser282 to a much lesser degree, and Ser273 not at all, whereas PKA phosphorylates cMyBP-C at all 3 serines during adrenergic stimulation.

Experiments were conducted in solution on full-length recombinant WT cMyBP-C to explore the potential synergistic effects of CaMKδ and PKA when both are present. CaMKδ and PKA were added both singly and in combination for a reaction time of 5 minutes. CaMKδ caused a greater amount of phosphorylation than PKA at Ser302, and together, the combined kinases caused greater phosphorylation than either kinase alone. Autocamtide inhibitory peptide, a specific inhibitor of CaMKδ, was added to the kinase mixture, which reduced the effect of the combined kinases to the effect of PKA alone. These results suggest that CaMKδ and PKA act independently: CaMKδ specifically targets Ser302, whereas PKA targets all 3 but has a lesser effect on Ser302 than does CaMKδ.

Phosphoprotein staining showed no differences in the phosphorylation of other myofilament proteins in WT and tSA myocardium under each experimental condition. Myofibrils from both mouse lines exhibited similar basal levels of phosphorylation of cardiac troponin-I, cardiac troponin-T, and regulatory light chain (Figure 6). Dobutamine treatment increased cardiac troponin-I phosphorylation to similar levels in both cMyBP-C(tWT) and cMyBP-C(tSA) myocardium (Figure 6). Therefore, the observed differences in functional responses to dobutamine are not caused by differential phosphorylation of myofibrillar regulatory proteins other than cMyBP-C.

A cMyBP-C phosphorylation mimetic mouse was also generated to determine whether replacement of the 3 PKA phosphorylatable serines with aspartic acid to mimic constitutive failure with preserved ejection fraction (HfPfE),51 that is, a combination of preserved ejection fraction and diastolic dysfunction (Figure 4F–4K).
phosphorylation would alter the responsiveness to increased pacing. cMyBP-C with Ser273D, Ser282D, and Ser302D was expressed on the cMyBP-C null background, creating the cMyBP-C(t3SD) mouse. Increasing pacing from 1 to 2.5 Hz in 0.5 Hz increments yielded the unexpected finding of a positive force–frequency response in cMyBP-C(t3SD) myocardium (Figure I in the Data Supplement). The existence of additional phosphorylatable sites could provide an explanation for a positive force/frequency relationship in cMyBP-C(t3SD) myocardium.21 However, such a site or sites must not be accessible in cMyBP-C(t3SA) myocardium because t3SA exhibits a severely depressed force–frequency relationship, as reported here. Thus, it may be that the introduction of glutamines induces conformational changes in cMyBP-C that are not observed in t3SA or tWT myocardium. Other mechanistic interpretations involving cMyBP-C are possible and are the subject of planned detailed investigations.

**Discussion**

The study was undertaken to determine the contributions of cMyBP-C phosphorylation in relation to increased [Ca^{2+}]_{i} or post-translational modifications of other myofibrillar proteins in mediating positive inotropy caused by increased pacing or β_{1}-adrenergic stimulation. Both heart failure with reduced ejection fraction and heart failure with preserved ejection fraction exhibit reduced inotropic reserve, which is evidenced by depressed inotropic responses to increased heart rate and β_{1}-adrenergic stimulation. Thus, elucidating the mechanisms of positive inotropy in response to these physiological interventions may provide insights into the mechanisms of dysfunction in heart failure and potential targets for therapeutic interventions.

The approaches used to study mechanisms of inotropy used intact myocardium expressing only non-PKA-phosphorylatable mutant cMyBP-C(t3SA) or phosphorylatable WT cMyBP-C(tWT), both to similar levels on a cMyBP-C null background. The key findings are (1) cMyBP-C(tWT) myocardium exhibited the expected steep force–frequency relationship (the Bowditch effect),11 whereas cMyBP-C(t3SA) myocardium exhibited dramatically smaller increases in twitch force when pacing frequency was increased; (2) dobutamine increased twitch force in cMyBP-C(tWT) but not cMyBP-C(t3SA) hearts. cMyBP-C is the only protein that showed significant interaction between ± dobutamine treatment and t3SA mutation. C, cTnT phosphorylation levels were similar in both mouse models. D, Basal cTnI phosphorylation levels were similar under basal conditions, and dobutamine induced similar increases in cTnI phosphorylation in both mouse models. E, RLC phosphorylation levels were similar in both mouse models.
Together, these results indicate that positive inotropy caused by the physiological interventions studied here is primarily mediated by phosphorylation of cMyBP-C with a smaller component because of increased binding of Ca\(^{2+}\) to thin filament proteins secondary to increased \([\text{Ca}^{2+}]_m\) under these conditions.

Based on different patterns of serine phosphorylation, the kinases mediating cMyBP-C phosphorylation seem to differ for pacing-induced and \(\beta\)-agonist-induced inotropy. Here we show that CaMK2\(\delta\) phosphorylation of cMyBP-C at Ser302 seems to be the primary mechanism for pacing-induced inotropy, although Ser282 is also phosphorylated to a much lesser degree. Activation of CaMK2\(\delta\) has previously been shown to contribute to the Bowditch effect by increasing delivery of Ca\(^{2+}\) to the myoplasm as a result of phosphorylation of L-type Ca\(^{2+}\) channels\(^{22,23}\) and ryanodine receptors RyR2.\(^{24}\) An increased frequency of calcium pulses, similar to the transients in successive twitches, has been shown to activate CaMK2\(\delta\),\(^{20}\) and once activated, CaMK2\(\delta\) can remain active despite a low average \([\text{Ca}^{2+}]_m\).\(^{20}\) These properties make CaMK2\(\delta\) an ideal sensor for increasing heart rate to affect inotropy. In our experiments, pacing induced cMyBP-C phosphorylation at Ser302, whereas inhibition of CaMK2\(\delta\) decreased Ser302 phosphorylation. Activated recombinant CaMK2\(\delta\) phosphorylated recombinant full length cMyBP-C at Ser302 at low calcium (<100 nmol/L) similar to \([\text{Ca}^{2+}]_m\) during diastole in <30 seconds. Thus, our data suggest that cMyBP-C contributes to pacing-induced inotropy through activation of CaMK2\(\delta\).

Although the present study has identified CaMK2\(\delta\) as the dominant mediator of cMyBP-C phosphorylation at Ser302 as the proximate basis for the positive force/frequency response, there are likely to be modulators of this mechanism in the heart in vivo. For example, reactive oxygen species when present can activate CaMK2\(\delta\)\(^{25}\) and angiotensin-II\(^26\) or aldosterone.\(^25\) In addition, protein kinase D can phosphorylate cMyBP-C at Ser302.\(^8\) Regardless of the identities of upstream signals as additional modulators of Ser302 phosphorylation, the present results show that pacing or adrenergic agonist–induced phosphorylation of Ser302 increases contractility.

Our conclusion that the force–frequency relationship in healthy myocardium is mediated by CaMK2\(\delta\) phosphorylation of cMyBP-C is consistent with earlier results,\(^{24}\) which showed depressed force–frequency relationships in mice expressing mutant ryanodine receptors (S2814A) that could not be phosphorylated by CaMK2\(\delta\). However, the mutant hearts in their study showed a marked residual positive force–frequency relationship,\(^{24}\) unlike our results showing near-elimination of the force–frequency relationship when nonphosphorylatable cMyBP-C is expressed. The residual force–frequency relationship in their experiments on S2814A myocardium was associated with a significant residual increase in myoplasmic Ca\(^{2+}\) when pacing frequency was increased,\(^{24}\) which presumably increased CaMK2\(\delta\) activity above basal levels and partially phosphorylated cMyBP-C. Conversely, they showed that inhibition of CaMK2\(\delta\) in WT mice with KN93 almost completely eliminated the positive force–frequency relationship,\(^{24}\) which is consistent with our finding that cMyBP-C(t3SA) papillary muscles exhibited minimal positive inotropy when pacing frequency was increased. In a different study, autocalcimide inhibitory peptide inhibition of CaMK2\(\delta\) depressed the force–frequency response, decreased cMyBP-C phosphorylation, and did not significantly change Ca\(^{2+}\) transients in intact papillary muscles.\(^5\) These results lend further support to the idea that CaMK2\(\delta\) phosphorylation of cMyBP-C is a principal mediator of the Bowditch effect. Our finding that the Ca\(^{2+}\) transient increased similarly in cMyBP-C(tWT) and cMyBP-C(t3SA) myocardium shows that the increased twitch force at higher pacing frequencies in cMyBP-C(tWT) myocardium is due mainly to the phosphorylation of cMyBP-C and to a much lesser degree the increased activation of the thin filaments as a consequence of increased Ca\(^{2+}\) binding to troponin.

Our results also show that the greatest part of cardiac inotropy caused by \(\beta\)-adrenergic stimulation is because of phosphorylation of cMyBP-C, presumably by PKA, at all 3 phosphorylatable serines in the cMyBP-C motif, with only a small fraction of the inotropic response associated with the increased delivery of Ca\(^{2+}\) alone. This observation provides insight into the long-standing conundrum presented by the observation that \(\beta\)-adrenergic stimulation increases twitch force, despite a decrease in Ca\(^{2+}\) binding affinity of troponin when PKA phosphorylates cardiac troponin-I,\(^27\) as well as an additive decrease in Ca\(^{2+}\) sensitivity of force because of phosphorylation of cMyBP-C.\(^28\) The net increase in force is caused by phosphorylation of cMyBP-C and the resulting increases in the rate of cross-bridge binding. The observation that adrenergic inotropy is greater than pacing-induced inotropy can be related to the observation that PKA phosphorylation involves more serines, that is, positive inotropy seems to scale with the degree of phosphorylation of cMyBP-C.

Our results show that each cMyBP-C phosphorylation site can be phosphorylated independently. Sadayappan et al have suggested that ser282 phosphorylation is in some way permissive for ser302 phosphorylation,\(^3\) which they determined by generating a ser282ala mutant cMyBP-C. In contrast, our data show that ser302 is the residue predominantly phosphorylated during increased pacing, suggesting that phosphorylation of ser282 is not necessary for phosphorylation of ser302 in vivo, at least by CaMK2\(\delta\). Copeland et al found that ser302 can be phosphorylated at levels \(\approx 3\)-fold higher than ser282,\(^{29}\) and Gresham et al showed that PKA can phosphorylate both Ser273 and Ser302 in the presence of a Ser282Ala mutation.\(^6\) Both studies provide further support of our conclusion that phosphorylation of Ser282 is not permissive to phosphorylation at the other 2 sites.

The relevance of these observations in isolated muscle to cardiac function was confirmed with in vivo Doppler measurements showing that cMyBP-C(tWT) myocardium has a faster contraction velocity (Sa), a faster relaxation velocity (Ea), and an increased Ea/Sa ratio compared with cMyBP-C(t3SA) myocardium under basal conditions and during either increased pacing or \(\beta\)-agonist stimulation. The combination of results showing that paced ex vivo hearts exhibit predominantly Ser302 phosphorylation, that inhibition of CaMK2\(\delta\) decreased Ser302 phosphorylation, and recombinant CaMK2\(\delta\) robustly phosphorylated recombinant full length cMyBP-C mainly at ser302 strongly supports our hypothesis that activation of CaMK2\(\delta\) leading to phosphorylation of cMyBP-C mediates the positive force–frequency response.
cMyBP-C (tWT) myocardium exhibited much greater increases in (+\(dF/dr\))_{\text{act}} and (−\(dF/dr\))_{\text{num}} than cMyBP-C (t3SA) in response to increased pacing frequency or dobutamine, even though peak calcium and calcium decay kinetics were similar in both muscle types in each instance. Because of the absence of differences in the intracellular calcium transients, we conclude that the differences in maximum rates of contraction and relaxation are caused by CaMK2δ or PKA phosphorylation of cMyBP-C to cause faster cross-bridge cycling kinetics. This conclusion is consistent with our prior study demonstrating that PKA treatment of skinned myocardium from cMyBP-C (t3SA) mice does not accelerate cross-bridge cycling kinetics.\

The more robust force–frequency and \(\beta_1\)-adrenergic responses observed in cMyBP-C (tWT) myocardium can be accounted for by effects of cMyBP-C phosphorylation to increase the rate of myosin binding to actin and thus the number of strongly bound cross-bridges. Consistent with this idea, phosphorylation of cMyBP-C induces displacement of myosin toward actin,\(^{33,35}\) which would increase the probability of myosin binding to actin. cMyBP-C binding to actin also seems to restrict structural torsion dynamics of thin filaments, an effect that is relieved by phosphorylation of cMyBP-C.\(^{32}\) Either or both of these structural changes have the potential to promote cross-bridge interactions and to increase the rate of myosin binding to actin. Force can also increase because of enhancement of positive cooperativity in the binding of myosin to actin. Initial Ca\(^{2+}\) binding to tropomyosin (Tm) from a Tm_{\text{blocked}} to a Tm_{\text{at}} state in which cross-bridges bind weakly to actin.\(^{33}\) The subsequent transition of cross-bridges from weakly to strongly bound states switches Tm_{\text{at}} to a Tm_{\text{off}} state, a process that is accompanied by force development and promotion of cooperative binding of cross-bridges to actin.\(^{33,33}\) The activation dependence of the rate of force development has been proposed by some to manifest the longer time taken at low levels of activation for the cooperative spread of cross-bridge binding along the thin filament from the initial site of cross-bridge binding.\(^{36}\) Such a model supposes that the rate of cross-bridge interaction is constant and maximal, so that force development at low activation is slowed only by the relatively slow spread of cooperative cross-bridge binding to the thin filament. In the present context, the dramatically reduced inotropy in cMyBP-C (t3SA) myocardium was related to much lower forces generated in the decremental rise segment of the force-[Ca\(^{2+}\)]_{\text{i}} loop, that is, the cooperative phase of the twitch in which force continues to increase despite declining [Ca\(^{2+}\)]_{\text{i}}.

Although the present study shows clearly that phosphorylation of the phosphorylationatable serines within the M-domain of cMyBP-C has profound physiological effects, some care in extrapolating these results to other systems or conditions is warranted. However, published data showing positive force/frequency responses in mouse hearts under a wide range of conditions lend confidence in the conclusions drawn here. The living papillary muscle has intact membranes, calcium handling proteins, preserved myofilament lattice structure, and plausible time courses of force and calcium during the twitch. Also, our earlier studies showed that pacing papillary muscle at room temperature at 1 to 3 Hz yields the same overall force frequency effects as pacing at 34°C and 1 to 7 Hz.\(^{6}\) Rottman et al have shown that mouse hearts in vivo exhibit robust, positive force/frequency relationships from 150 to 700 beats per minute in both conscious and anesthetized mice.\(^{37}\) Because pacing in our study overlaps the lower range, it seems reasonable to conclude that our findings at room temperature are broadly applicable to normal physiology.

Physiological differences can potentially limit the applicability of the present results from mice to humans. Mice have much higher resting heart rates (350–600) than humans (60–70),\(^{38}\) much smaller hearts with higher overall metabolism, and faster contractile protein isoforms. An overarching consideration in the selection of mice for this study is the ability to perform gene deletion and mutant gene expression in murine hearts, but studies in higher animal models will be necessary to confirm the degree to which the mechanisms reported here are also operable in the human heart.

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Disclosures

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Our study shows that cardiac myosin-binding protein-C (cMyBP-C) phosphorylation is a critical mediator of positive inotropy in myocardium in response to increased heart rate or β₁-adrenergic stimulation. Measurements of twitch force and [Ca²⁺]ₙₙ strongly suggest that the inotropic effects as a result of phosphorylation of cMyBP-C result from modulation of cross-bridge interaction kinetics unrelated, at least directly, to calcium handling during the twitch. Although the phosphorylation of cMyBP-C in response to β₁-agonists is mediated by protein kinase A, phosphorylation in response to increased heart rate seems to be caused by activation of CaMKII as a consequence of an increased frequency of Ca²⁺ transients. The greater positive inotropic response to a β₁-agonist corresponds to protein kinase A phosphorylation of 3 sites on cMyBP-C (Ser273, Ser282, and Ser302), whereas CaMKII phosphorylates a single site robustly (Ser302) and another to a lesser degree (Ser282), suggesting that the positive inotropic effects scale with the extent of phosphorylation of cMyBP-C at these 3 residues. Because the failing heart exhibits diminished inotropic responsiveness, targeting of cMyBP-C phosphorylation holds excellent potential for development of novel therapeutics.
Phosphoregulation of Cardiac Inotropy via Myosin Binding Protein-C During Increased Pacing Frequency or β1-Adrenergic Stimulation
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SUPPLEMENTAL MATERIAL

Phosphoregulation of cardiac inotropy via myosin binding protein-C during increased pacing frequency or β1-adrenergic stimulation

Carl W. Tong MD, PhD1,2,3, Xin Wu MD2, Yan Liu MD, PhD2, Paola C. Rosas MD, PhD, RPh2, Sakthivel Saddayappan PhD4, Andy Hudmon PhD5, Mariappan Muthuchamy PhD5, Patricia A. Powers PhD1, Hector H. Valdivia MD, PhD6, Richard L. Moss PhD1

1University of Wisconsin School of Medicine and Public Health, Madison WI
2Texas A&M University Health Science Center College of Medicine, Temple TX
3Baylor Scott & White Health, Temple TX
4Loyola University Chicago Stritch School of Medicine, Chicago IL
5Indiana University School of Medicine, Indianapolis IN
6University of Michigan Medical School, Ann Arbor MI

CORRESPONDING AUTHOR:

Richard L. Moss, Ph.D.
Department of Cell and Regenerative Biology
UW School of Medicine and Public Health
750 Highland Avenue
Madison, WI 53705-2221
Email: rlmoss@wisc.edu
Phone: (608) 265-0523
FAX: (608) 265-0522
**Supplemental Methods**

**Simultaneous Force and Intracellular Calcium Measurement on Intact Papillary Muscle**

We chose to perform simultaneous force and intracellular calcium concentration $[Ca^{2+}]_{in}$ measurements on intact papillary muscle to better elucidate the effects of cardiac myosin binding protein-C phosphorylation regulation of cross-bridge cycling on contractile function. The intact papillary muscle provides advantage over the previous skinned myocardium experiments by: (1) intact cardiac myocytes with working kinases, phosphatases, and organelles, (2) intact 3-dimensional myofilament lattice, and (3) triggered calcium release and re-uptake by endogenous calcium handling proteins to reflect in vivo environment of a beating heart. We expect the force/calcium relationships within the intact papillary muscle to be different from the skinned myocardium experiments due to both changing $[Ca^{2+}]_{in}$ and intact multi-cellular structure. Particularly, the measuring force as response to increasing and decreasing transients $[Ca^{2+}]_{in}$ will be able to detect differences caused by faster cross-cycling kinetics that cannot be detected by steady state measurements of skinned myocardium experiments. Bulk of the protocol was developed by a previous study.\(^1\)

The force measurement equipment and fluorescence optics were purchased from Scientific Instruments (Heidelberg, Germany). We used our own implementation of a data acquisition suite (National Instruments A/D and Labview\(^{TM}\)) and software to acquire and analyze the data. Papillary muscles were dissected free at 4°C in Krebs Henseleit (KH) solution with 30 mmol/L of 2,3-butanedione monoxime (BDM), taking care that surgical instruments did not touch surface of papillary muscle and avoiding over-stretch. Damaging the papillary muscle during dissection causes low twitch force and negative force/frequency response. The papillary muscle was attached to the force transducer and motor by the chordae tendenae at its tip and a large mass of surrounding ventricular tissue around the base. KH solution (in mmol/L: NaCl 119, Glucose 11, KCl 4.6, CaCl\(_2\) 1.8, MgCl\(_2\) 1.2, NaHCO\(_3\) 25, and K\(_2\)HPO\(_4\) 1.2) was continuously bubbled with 95% O\(_2\) and 5% CO\(_2\) gas mixture for 30 minutes to reach a stable pH of 7.4. KH solution is continuously bubbled throughout the experiment. The KH buffer is pumped through the superfusion chamber at sufficient rate to support strongest possible contraction at 3 Hz stimulation. This corresponds to > 4ml/minute in the current system.

Maximum twitch force was achieved by carefully stretching the relaxed muscle to the point beyond which a further increase in length as reflected in resting tension failed to elicit a further increase in twitch force. All measurements were obtained at a length corresponding to maximum twitch force. Stimulation voltage and pulse duration were adjusted to ensure that stimulus conditions did not limit peak force; standard conditions consisted of 7 volts with a 5 millisecond pulse duration. A Tektronix oscilloscope with Wavestar\(^{TM}\) computer interface continuously measured, logged, and displayed trends in twitch force and resting tension to guide adjustments. Pacing at 0.5 Hz for at least 90 minutes was required for extracted papillary muscle to become stable. Papillary muscles with stable diastolic force baselines were used for experimental measurements.

FURA-2AM loading buffer consisted of KH with the additions of FURA-2AM 10 µmol/L, cremophor 5 g/L (facilitates FURA-2AM diffusion into cells), and N,N,N',N'-Tetakis(2-pyridylmethyl) ethylenediamine (TPEN) 4.3 mg/L (chelates manganese to avoid manganese quenching of Fura2 fluorescence\(^2\)). The KH to dimethyl sulfoxide (DMSO) volume ratio was 399. A KH to DMSO volume ratio < 200 will cause significant cellular damage. Loading FURA-2AM for 2 hours at 0.5 Hz pacing yielded a consistent fluorescence signal between twitches and allowed muscle to achieve stable twitch forces. De-esterification duration after FURA-2AM loading was 15 minutes at 1 Hz pacing. Fura2 fluorescence was captured from the entire papillary muscle. The $[Ca^{2+}]_{in}$ was estimated by:\(^2\):

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Calcium-bound Fura2 fluoresces with 340 nm excitation ($F_{Ca}$). Calcium-free Fura2 fluoresces with 380 nm excitation ($F_{\text{free}}$). $R$ is calculated by $F_{Ca}/F_{\text{free}}$. $R_{\text{min}}$ is measured when Fura2 is completely free of calcium. $R_{\text{max}}$ is measured when Fura2 is saturated with calcium. $K_{\text{apparent}}$ is the apparent calcium association constant of Fura2 for the entire system. System calibrations for all configurations provided the corresponding $R_{\text{min}}$, $R_{\text{max}}$, and $K_{\text{apparent}}$ for each experiment. We used estimated $[Ca^{2+}]$ instead of $R$ because estimating $[Ca^{2+}]$ by equation DS-1 adjusts for changes in $R$ due to normal equipment maintenance such as replacing the mercury light bulb (Different mercury light bulbs will give different intensities at 340 nm and 380 nm to vary Fura2 fluorescence on the same $[Ca^{2+}]$ resulting in different $R$ values).

System calibration uses no calcium with 10 mmol/L of ethylene glycol tetra acetic acid (EGTA) for $R_{\text{min}}$, 10 mmol/L of CaCl$_2$ for $R_{\text{max}}$, and a range of $[Ca^{2+}]$ standards with magnesium that were purchased from Molecular Probes for $K_{\text{apparent}}$. Auto-fluorescence of papillary muscles were measured before Fura-2 loading and subsequently subtracted from recorded data before calculating $R$.

The pacing protocol consisted of (1) increasing the pacing frequency from 1 to 2 and then to 3 Hz every 30 seconds to verify the integrity of muscle (intact muscle will show a positive force frequency response; this occurred > 90% of time), (2) returning the pacing frequency to 1 Hz for 5 minutes to allow the papillary muscle to re-equilibrate, (3) increasing pacing frequency from 1 to 2 and then to 3 Hz every 2 minutes, (4) changing the solution to KH with 1 µmol/L dobutamine with continuation of 3 Hz pacing for 5 minutes, (5) recording at a minimum of 20 contractions at the end of each pacing frequency and condition period. A wash period of 10 minutes with normal KH returned the twitch force to pre-dobutamine treatment levels.

Room temperature was chosen because FURA-2AM exits the cells too quickly at 37°C. We have noticed that at room temperature of 20-21°C, the myocytes do not respond appropriately to pacing frequency above ~ 3.5 Hz without pre-treatment with a β-adrenergic agonist. Thus a maximum pacing frequency of 3 Hz was chosen. Our previous study have shown that increasing pacing frequency from 1 Hz to 3 Hz at room temperature causes similar positive force/frequency effects as increasing pacing frequency from 1 Hz to 7 Hz at 37°C.

Multiple techniques were used to ensure good signal quality. These involve optimizing the initial fluorescence to current transduction signal to noise ratio (SNR), taking advantage of the hardware, and signal averaging.

Optimization of Initial Fluorescence to Current Transduction SNR

The photomultiplier tube (PMT) converts incident fluorescence to electronically measurable current (i.e., light to current transduction). At a constant supply voltage, the PMT performs this signal transduction at a stable photon to electron gain. The amplitude of Fura2 fluorescence from the entire muscle is more than sufficient enough to be directly converted to a corresponding current by the PMT. Thus, the system operates the PMT in some literature noted as analog mode. One can estimate photomultiplier tube SNR by:

\[
[Ca^{2+}] = \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) \cdot K_{\text{apparent}}, \text{ where, } R = \frac{F_{Ca}}{F_{\text{free}}}
\]
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[Equation DS-2]\[4\]

\[
SNR = \frac{I_K}{(2eB \cdot (d/(d-1)) \cdot (I_K + 2I_d))^{(1/2)}}
\]

I<sub>K</sub>: cathode current (A), function of incident light power and anode radiant sensitivity
e: electron charge (C)
B: bandwidth (Hz)
d: secondary emission ratio, ~ 6, so (d/(d-1) ~ 1.2
I<sub>d</sub>: dark current, noise independent of light source (i.e. noise produced by the equipment in total darkness), increases with supply voltage

One can increase the photon to electron gain (e.g. radiant sensitivity) by increasing the supply voltage. This allows one to detect lower intensity fluorescence signal. However, increasing the supply voltage will increase the dark current noise resulting shot noise as given by the denominator of the above equation. Thus, the supply voltage to the PMT is optimized for each experiment. The optimization is done by first recording the auto-fluorescence before Fura2 loading at range of 600-800V. This region provides good linearity between supply voltage and photon-to-electron gain. After loading, fluorescence was measured again at 600-800V. Papillary muscle contraction induced fluorescence change is than analyzed to find the optimum supply voltage. Once found, the supply voltage is set to this optimum value before start of the actual experiment. The corresponding auto-fluorescence is subtracted from the collected experimental data before further processing. This method eliminated the dark current "shot" noise associated with high PMT gain as well as producing the best possible signal to noise ratio on the raw electrical output from the optical system.

**Taking Advantage of Hardware**

Unlike an inverted microscope, the entire objective to PMT suite is completely enclosed in one assembly to minimize path length. This significantly reduced the long path length and multiple intervening optical parts that would cause fluorescence signal loss in an inverted microscope type of configuration.

A filter wheel passes the alternating 340nm and 380nm excitation ultraviolet (UV) light to the tissue at 250 Hz with a non-continuous time window for each wavelength. PMT than converts the resultant 510 nm fluorescence signal to an electrical signal. A phase-lock-loop controlled sample/hold with integrator circuit times averages the output of PMT to give a smoothed continuous signal. We sample this continuous analog output. Using this integrator output provide the advantage of a smoothed continuous signal.

**Signal Averaging**

Averaging more than 30 contractions at each frequency produced the force and [Ca\(^{2+}\)]<sub>in</sub> curves that were used for the analyses. An electrical pacing pulse was used as the reference starting time for averaging of traces. Averaging greater than 30 contractions increased signal to noise ratio because noise occurs randomly in time where paced contraction signal occur in stationary manner with respect to the stimulation pulse. Emissions from separate 340 nm and 380 nm excitations were examined. Only experiments showing conjugate changes in emissions in response to both 340 nm and 380 nm excitations during a contraction were used because ineffective coupling of 340 nm excitation to tissue will artifactualy depress the estimated [Ca\(^{2+}\)]<sub>in</sub> by about 10-fold. Proper conjugate emission response consisted of simultaneous increasing emission by 340 nm excitation and decreasing emission by 380nm excitation with expected increase in [Ca\(^{2+}\)] after an electrical stimulation. Sampling frequency must be fast enough to allow good approximation of the actual signal after digital to analog conversion. The Nyquist criterion states that a minimum sampling frequency of 2-times the highest frequency component
is needed. One can estimate the highest frequency component by calculating Fourier transform of the expected signal and finding the frequency where intensity in the frequency-domain Fourier spectrum has decreased by 90%. In practice, one needs about 10-times the highest frequency component to reconstruct the actual signal after digitization. The sharp rising edge of the Weibull-shaped $[\text{Ca}^{2+}]_\text{in}$ transient most likely has the fastest frequency components in these experiments. For these reasons, sampling frequencies of 1 KHz or faster were used.

**Langendorff Perfusion**

Hearts were excised, aorta was cannulated, and perfused in retrograde fashion via the cannulated aorta with KH buffer at 37°C in a Langendorff apparatus (AD Instruments, Colorado) to remove blood and catecholamines from the coronary circulation. The perfusion was done in the constant pressure mode ~ 90 mmHg. The pacing protocol consisted of 5-10 minutes of spontaneous rhythm, followed by pacing at 7 Hz for 15 minutes, and then pacing at 10 Hz for 15 minutes. After the initial 15 minutes at 10 Hz, hearts were either continually paced with perfusion alone, or paced with addition of 1 µmol/L dobutamine, or paced with addition of 10 mmol/L of KN-93.

**Recombinant Wild-Type Cardiac Myosin Binding Protein-C**

Wild-type (WT) murine MyBPC3 cDNA was spliced into pET-28 vector with His-Tag. The resultant plasmids containing MyBPC3 cDNA were cloned and maintained in DH5α competent E coli. The plasmids were extracted from DH5α competent E coli and re-cloned into BL21(DE3)pLysS competent E coli for production of full length WT murine MyBPC3. The full length WT MyBPC3 was difficult to produce in bacteria due to its length and rare-codons to bacteria (ccc, gga, cgg) that can stop translation in bacteria. We followed the increases in 600 nm absorbance to ensure the bacteria culture remained in the log-phase growth before harvest. Furthermore, reducing temperature from 37°C to 25°C at the point of induction increased the efficiency of full length WT MyBPC3 protein production. Finally, completing all protein extraction tasks at 4°C helped to prevent protein from precipitating.

**In Vitro Calcium Calmodulin Kinase**

Dr. Andy Hudmon kindly provided the recombinant CaMK2d that was produced via baculovirus system. The recombinant CaMK2d was transferred from -80°C freezer onto ice for slow thawing. After thawing, we activated CaMK2d in buffer consisting in mmol/L of CaCl2 0.5, calmodulin 0.02, KCl 60, MgCl2 10, HEPES 50 at pH=7.4, and ATP 1 for 5 minutes. The target recombinant MyBPC3 was placed in reaction buffer with final concentrations consisting in mmol/L of EGTA 1, KCl 60, MgCl2 10, HEPES 50 at pH=7.4, and ATP 1. The reaction was started by adding pre-activated CaMK2d into reaction buffer containing MyBPC3. We used 2.5 microgram of CaMK2d and 11.7 microgram of MyBPC3 in final reaction volume of 50 microliters. Due to EGTA, the final reaction has estimated $[\text{Ca}^{2+}]$ of 12 nmol/L. The reactions were stopped by addition of SDS sample buffer and immediately placing at 95°C for 5 minutes. We chose stopping times of immediately after mixing, 15 seconds, 30 seconds, 60 seconds, 5 minutes, and 10 minutes. The negative controls consisted of adding activation buffer without CaMK2d to demonstrate there is no endogenous kinase from the recombinant protein production and inhibit CaMK2d with autocamtide-2 inhibitory peptide (which is specific inhibitor of CaMK2d, EMD-Calbiochem Catalog# 18940) at 10 µmol/L to further demonstrate it was CaMK2d that phosphorylated MyBPC3.

**Phospho-protein Staining and Analyses**

We treated WT mice with metoprolol (5 microgram/gram) via intra-peritoneal injection at 30 minutes prior to harvesting in order to produce hearts that were used for reference (WT+BB). Metoprolol, which
is a selective β₁-adrenergic receptor blocker, is used to reduce phosphorylation levels of myofilament proteins. We then extracted and concentrated myofibrils. The myofibrils were then solubilized and electrophoresed on 4-15% gradient gel. We loaded 3 different total protein amounts on 3 different lanes for each experiment. We used Pro-Q Diamond to stain gel for phosphorylated protein. After Pro-Q Diamond de-staining, we stained the same gel with Sypro-Ruby to quantify total protein. We then performed linear regression to find slopes of specific phosphorylated protein band vs. total protein loading (Pro-Q slope) and specific protein band vs. total protein loading (Sypro-Ruby slope). As described previously the ratios of 

\[
\frac{(\text{Pro-Q slope of experiment})/\text{(Pro-Q slope of WT+BB)}}{(\text{Sypro-Ruby slope of experiment})/\text{(Sypro-Ruby slope of WT+BB)}}
\]

were used to compare phosphorylation levels.

**Major Changes in the New Intact Papillary Muscle Simultaneous Force and \([Ca^{2+}]_s\), System**

Small intact muscle test system (Aurora Scientific, Model 1500A) was used. This system included a very sensitive and stable force transducer (Aurora Scientific, Model 405A). The muscle is now mounted using 7-0 silk suture instead of metal clips. This system brought improved long term force transducer stability. Furthermore, the North American location of the vendor allowed easy repairs and updates instead of very difficult trans-Atlantic maintenance.

An inverted Olympus microscope is now being used with long working distance objectives that allow 340 nm and 380 nm excitation. This allowed direct visualization of the portion of papillary muscle being excited. Previously, the excitation pathway occurred in free air at 90° angle from the upright microscope. This new configuration improved \([Ca^{2+}]_s\) imaging sensitivity and improved % success in obtaining quality Fura-2AM signals. However, one can no longer see the entire papillary muscle and measure the diameters at the experimental configuration.

An IonOptix Hyper-Switch system is now being used to (A) provide 340 nm and 380 nm excitation, (B) detect 510 nm fluorescence, and (C) record raw data, and (D) perform signal averaging. The Hyper-Switch system eliminated the 20-Hz motor wobble artifact that occurs with the older system. Furthermore, the Hyper-Switch system does not breakdown quickly like the spinning wheel of the older system. The hyper-switch system uses threshold-photon counting method of detection; therefore, we lost the ability to adjust the gain of the photomultiplier tube to optimize signal/noise ratio. Furthermore, the North American location of the vendor allowed easy repairs and updates instead of very difficult trans-Atlantic maintenance.

**Cytosolic and Myofibril Preparation**

Three months old mice from all three lines (cMyBP-C(tWT), cMyBP-C(t3SA), cMyBP-C(t3SD)) were selected. Mice were anesthetized with isoflurane. Hearts were extracted and quickly rinsed free of blood in phosphate buffered saline. Afterwards, hearts were quickly immersed in cooled 4°C solution containing KCl 60 mmol/L, MgCl2 2 mmol/L, EGTA 1 mmol/L, HEPES 20 at pH = 7.4 mmol/L, DTT 2 mmol/L, phosphatase inhibitor okadaic acid 1 micromol/L, and protease inhibitor cocktail (Sigma P8340). The hearts were homogenized for 30 seconds 3-times on ice. Triton-X 100 was then added to reach final concentration of 1%. The mixture set on ice for 30 minutes with vortexing the solution at least 3-times. Centrifuging at 1200g 4°C for 10 minutes pelleted down myofibrils without mitochondria (this is the myofibril fraction). The supernatant is then centrifuge again at 12,000g 4°C for 20 minutes to pellet down mitochondria. The supernatant was then used for western blotting for calcium calmodulin kinase 2 delta (CaMK2d) and protein kinase-A (PKA) BioRad DC assay was used to quantify protein concentration.

The myofibril fraction was then purified using a previously developed protocol.

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Western Blotting for Kinases

A collection of antibodies were tested against both cytosolic fraction and myofibril fraction. Both CaMK2d and PKA occur in the cytosolic fraction. 50 microgram of cytosolic proteins were loaded for each lane. The final set of antibodies that detected kinases and its phosphorylation status are: CaMK2d (Santa Cruz, SC-5392, polyclonal goat, 1:250 dilution), Phos-CaMK2d (Santa Cruz, SC-12886, polyclonal rabbit, 1:250 dilution), PKA catalytic subunit (Santa Cruz SC-903, polyclonal rabbit, 1:500 dilution), Phos-PKA catalytic subunit (Sigma, SAB4503969, 1:500 dilution), and GAPDH (Santa Cruz, SC-166504, monoclonal mouse, 1:250 dilution). A cooled CCD camera system (BioRad) was used to capture chemiluminescence intensity for quantification.
Figure S1: Force Frequency Relationships. A different set of intact papillary muscles from cMyBP-C(tWT), cMyBP-C(t3SA), and cMyBP-C(t3SD) mice were mounted on a completely new system (see material and methods supplement section). Peak twitch force was normalized to peak twitch force at 1 Hz to eliminate differences in muscle size. Both cMyBP-C(tWT) and cMyBP-C(t3SD) papillary muscles demonstrate robust positive force frequency response far exceeding cMyBP-C(t3SA) papillary muscles.

Supplemental Figures and Figure Legends

![Force Frequency Relationships](chart.png)

**tWT:** n=9; **t3SA:** n=9; **t3SD:** n=9

Error Bar=SEM; *p<0.05 vs tWT; #p<0.05 vs t3SD
Figure S2: Effect of CaMK2δ and PKA combination. All kinase reactions were conducted at room temperature for 5 minutes. Addition of 4X SDS sample buffer at the end of 5 minutes solubilized the enzyme protein mixture to stop the reaction. Recombinant protein alone without addition of kinases was used as negative control. CaMK2δ phosphorylated Ser302 to greater extent than PKA. CaMK2δ + PKA caused greater amount of phosphorylation than either kinases alone. Inhibition of CaMK2δ with autocomtide inhibitory peptide (AIP) returned the combined kinases phosphorylation level back down to PKA phosphorylation alone. These results suggest that CaMK2δ and PKA act independently on Ser302. The net effect is additive.
Supplemental References


