Cardiac Myosin-Binding Protein-C Phosphorylation and Cardiac Function

Sakthivel Sadayappan, James Gulick, Hanna Osinska, Lisa A. Martin, Harvey S. Hahn, Gerald W. Dorn II, Raisa Klevitsky, Christine E. Seidman, Jonathan G. Seidman, Jeffrey Robbins

Abstract—The role of cardiac myosin binding protein-C (cMyBP-C) phosphorylation in cardiac physiology or pathophysiology is unclear. To investigate the status of cMyBP-C phosphorylation in vivo, we determined its phosphorylation state in stressed and unstressed mouse hearts. cMyBP-C phosphorylation is significantly decreased during the development of heart failure or pathologic hypertrophy. We then generated transgenic (TG) mice in which the phosphorylation sites of cMyBP-C were changed to nonphosphorylatable alanines (MyBP-CAllP). A TG line showing ~40% replacement with MyBP-CAllP showed no changes in morbidity or mortality but displayed depressed cardiac contractility, altered sarcomeric structure and upregulation of transcripts associated with a hypertrophic response. To explore the effect of complete replacement of endogenous cMyBP-C with MyBP-CAllP, the mice were bred into the MyBP-C(t/t) background, in which less than 10% of normal levels of a truncated MyBP-C are present. Although MyBP-CAllP was incorporated into the sarcomere and expressed at normal levels, the mutant protein could not rescue the MyBP-C(t/t) phenotype. The mice developed significant cardiac hypertrophy with myofibrillar disarray and fibrosis, similar to what was observed in the MyBP-C(t/t) animals. In contrast, when the MyBP-C(t/t) mice were bred to a TG line expressing normal MyBP-C (MyBP-CWT), the MyBP-C(t/t) phenotype was rescued. These data suggest that cMyBP-C phosphorylation is essential for normal cardiac function. (Circ Res. 2005;97:1156-1163.)

Key Words: mouse ■ mouse mutants ■ muscle ■ muscle contraction ■ myocardial contractility

Understanding the structure/function relations for cardiac myosin binding protein-C (cMyBP-C) is clinically relevant, as cMyBP-C mutations are a widely recognized cause of familial hypertrophic cardiomyopathy. Various cMyBP-C transgenic (TG) and gene-targeted mouse models have demonstrated the importance of the protein for long-term integrity of sarcomeric structure and for maintaining normal cardiac contractility. Functional insight can be gained from appreciating the crucial structural differences between cMyBP-C and the skeletal isoform. Only the cardiac isoform contains an extra immunoglobulin domain at the N terminus (C0), an insertion of 28 residues within the C5 domain, and three phosphorylation sites (Ser273, -282, -302) that are substrates for cAMP-dependent protein kinase A (PKA), Ca2+-calmodulin-activated kinase and protein kinase C.

In vivo, PKA-mediated phosphorylation of cMyBP-C is linked to modulation of cardiac contraction. On adrenergic stimulation, PKA phosphorylates Ser273, -282, and -302, whereas protein kinase C phosphorylates only Ser273 and -302. These residues, located near the N terminus of the protein, are of particular interest, as this region binds to the S2 segment of the myosin heavy chain (MHC), which is close to the lever arm domain of myosin. It has been hypothesized that cMyBP-C/MHC interactions are dynamically regulated by the phosphorylation/dephosphorylation of cMyBP-C. In vitro experiments showed that after phosphorylation of cMyBP-C by PKA, the thick filaments exhibited a relative loose structure, preventing binding to myosin and, thus, changing the maximum Ca2+-activated force (Fmax). Electron microscopy of isolated thick filaments confirmed that phosphorylation of cMyBP-C initiates crossbridge movement away from the thick-filament backbone, although this interaction may also be determined by the MHC isoform that is present. In theory, cMyBP-C phosphorylation could change both filament orientation and contractile mechanics, although cMyBP-C phosphorylation did not alter the Ca2+ sensitivity of Mg2+-ATPase activity in reconstituted contractile protein systems. Additionally, phosphorylation of cMyBP-C did not affect the Ca2+ sensitivity of Mg2+-ATPase activity, force-Ca2+ relationship, or sarcomere length dependency of contraction in intact skinned fiber experiments, suggesting that MyBP-C phosphorylation plays a relatively minor role in regulating contraction. Given the above body of data, the role that cMyBP-C phosphorylation plays in either normal or pathological cardiac function remains unclear.

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We wished to determine whether cMyBP-C phosphorylation played an essential role in the maintenance of cardiac function. We first defined the phosphorylation status of cMyBP-C in the normal and diseased heart and showed that significant changes occurred during development of cardiac pathology. We then generated a TG mouse model in which the known phosphorylation sites in cMyBP-C were converted to alanine and a sequence encoding the myc epitope incorporated as described previously.2 The cDNA for mouse cMyBP-C (MyBP-C WT) was obtained by reverse transcription–PCR using RNA isolated from the mouse cardiac ventricle. The cDNA was subcloned into the mouse germline transmission of the transgene, termed MyBP-CAllP, in the absence of endogenous phosphorylatable cMyBP-C, mice showed significant changes during development of cardiac pathology compared to nonphosphorylatable alanines (MyBP-CAllP). The quantitative data are shown in tabular form in supplemental Table I.

Materials and Methods

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Transgenic and Targeted Lines

The cDNA for mouse cMyBP-C (MyBP-C WT) was obtained by RT-PCR using RNA isolated from the mouse cardiac ventricle. The cMyBP-C phosphorylation sites (Ser273, Ser282, and Ser302), along with 2 adjacent sites that could be potentially phosphorylated (Thr272, Thr281), were converted to alanine and a sequence encoding the myc epitope incorporated as described previously.2 The cDNA was subcloned into the mouse α-MHC promoter and used to generate multiple lines of TG mice for each construct. To ensure the absence of endogenous phosphorylatable cMyBP-C, mice showing germline transmission of the transgene, termed MyBP-C AllP−, were bred with mice that expressed less than 10% of normal cMyBP-C levels (MyBP-C t/t). The small amount of protein that is present in the nontransgenic (NTG) adults, animals that had undergone transverse aortic constriction (TAC), 24 hours (lane 1); 24 hours post-TAC (lane 2); 18 days sham (lane 3); 18 days post-TAC (lane 4); a NTG control (lane 5); 8-week-old calcineurin TG mouse (lane 6); 7- to 8-month-old mlp-deficient mouse (lane 7); a α-MHC TG mouse (lane 8); ischemic reperfusion (IR), 24 hours sham (lane 9); 24 hours post-IR (lane 10); 5 days post-IR sham (lane 11); and 5 days post-IR (lane 12). The 4 forms of cMyBP-C based on its phosphorylation status are shown as P0, P1, P2, and P3, which correspond to the de-, mono-, bi-, and triphosphorylated forms, respectively, with isoelectric points of 6.1, 5.9, 5.7, and 5.5. B, MHC isofrom shifts. Using the samples from A, the α- and β-MHC isoforms were separated in a 5% gelatin gel. The data show the expected isoform shifts characteristic of activation of the fetal gene program in the disease models (lanes 4, 6, 7, and 12). C, cMyBP-C phosphorylation in cardiac disease. Samples are as in A, and average values are shown as a percentage of total cMyBP-C (n=3). The quantitative data are shown in tabular form in supplemental Table I.

Molecular and Protein Analyses

For assessment of cMyBP-C transcript size, expression levels, and expression of hypertrophic marker genes, RNA transcript and blot analyses were performed as described.2,16 Enriched myofibrillar proteins were isolated using F60 buffer and solubilized in urea buffer.17 MyBP-C AllP− was identified via SDS-PAGE followed by Western blots using anti-myc monoclonal antibodies and anti-cMyBP-C rabbit polyclonal antibodies raised against the CO−C1 domains.18

Phosphorylation and Isoelectric Focusing

Myofibrillar proteins were treated with phosphatase and PKA as described.17 One-dimensional isoelectric focusing (IEF) was performed to identify the phosphorylated forms.19

Cardiac Function

In vivo cardiac function was assessed both by noninvasive echocardiography in nonsedated or sedated animals and by invasive catheterization.19

Results

Phosphorylation States of cMyBP-C in Normal and Heart Failure Mouse Models

As is the case for a number of other contractile proteins, cMyBP-C can be reversibly phosphorylated at multiple sites in response to altered physiological conditions.20 However, the in vivo phosphorylation levels of the protein under basal and stressed conditions are not well defined. To understand the level of phosphorylation in the nontransgenic (NTG) animals before we perturbed the cMyBP-C protein complement, cMyBP-C phosphorylation under normal and stressed conditions was determined using IEF followed by Westerns using a cMyBP-C CO−C1 motif–specific antibody.18 Total enriched myofibrillar proteins were extracted from unstressed, NTG adults, animals that had undergone transverse...
aortic constriction (TAC) to produce pressure overload hypertension, animals in failure as a result of cardiac specific expression of calcineurin, 21 mlp-deficient mice that develop dilated heart failure, 22 and animals that had been subjected to ischemia reperfusion. Because the degree to which cMyBP-C and its phosphorylated forms regulate cardiac function may depend, in part, on the MHC isoform that is present, 23 we also determined cMyBP-C phosphorylation levels in a mouse model in which we had replaced approximately 70% of the normal /MHC with /MHC. 24 Under basal conditions, the mono-, bi-, and triphosphorylated species of cMyBP-C made up >90% of the cMyBP-C population (Figure 1A, lane 5). A β-MHC shift, in of itself, had no effect on the normal phosphorylation pattern (Figure 1B, lane 8). In all of the models in which cardiac function was significantly compromised by either surgical or genetic manipulation, total cMyBP-C phosphorylation, particularly the triphosphorylated species, was decreased (Figure 1A and 1C; supplemental Table I). Invariably, in the animals that displayed overt cardiac failure as determined by labored breathing, anasarca, and failure to groom, the triphosphorylated state was reduced or absent (Figure 1, lanes 4, 6, 7, and 12). These data show that increased dephosphorylation of cMyBP-C is associated with contractile dysfunction and heart failure. 

**Figure 2.** MyBP-C AllP transgene expression. A, Schematic diagram of cMyBP-C. The 8 IgI-like domains are shown as ovals and the 3 F3 domains as octagons. Interaction sites are shown above the diagram. The cardiac-specific phosphorylation motif, which is highly conserved between species, is located between domains C1 and C2 and the sequence shown. The 3 known phosphorylation sites (Ser273, -282, and -302) and 2 potential alternative phosphorylation sites (Thr272 and -281) were each altered to a nonphosphorylatable alanine. B, Northern blot analyses of RNAs from left ventricles derived from 8- to 12-week-old MyBP-C WT, NTG, and 3 lines of MyBP-C AllP mice. The RNAs were hybridized with cMyBP-C and GAPDH probes to confirm expression of the intact and correctly sized transcript. C, Dot-blot analyses using the same RNAs and probes as in B were used to quantitate the levels of TG expression. D, Western blot analyses of myofibrillar proteins from NTG, MyBP-C WT (WT), and 3 MyBP-C AllP (lines 45, 262, and 272) hearts. Note the conservation of normal levels of total cMyBP-C expression. E, Total cMyBP-C levels as well as the presence of myc-tagged cMyBP-C protein in the same mice was confirmed by Western blot analysis. α-Sarcomeric actin was used as a loading control. F, Phosphorylation state of NTG, MyBP-C WT (WT), and MyBP-C AllP (AllP) cMyBP-C in myofibrillar extracts assessed by 1D IEF. Normal cMyBP-C in these extracts could be further phosphorylated by PKA, but MyBP-C AllP was not a PKA substrate. Phosphatase treatment (Phosp) of myofibrillar extracts dephosphorylated normal cMyBP-C.

**Figure 3.** Phenotypic analyses of MyBP-C AllP (AllP) mouse hearts. A, Longitudinal sections derived from 3-month-old left ventricle stained with hematoxylin/eosin (top) or Masson trichrome (bottom) demonstrate the absence of obvious pathology (x10). B, Transmission electron micrographs of the sarcomeres. Areas of the MyBP-C AllP ventricles occasionally show altered sarcomeric organization (NTG, left; AllP, right), with high magnification (lower panels) showing altered M-line definition (M). Magnification: upper panels, x10 000; lower panels, x30 000. C, Transmission electron micrographs of sarcomeres from 12-week-old NTG and MyBP-C AllP hearts. The abnormal H-lines and I-zones (right bottom panel) and perturbation of the normal sarcomere–mitochondria architecture (left bottom panel) in MyBP-C AllP are indicated (arrow). D, Immunofluorescent staining of cMyBP-C with either anti-cMyBP-C (top) or anti-myc antibodies (bottom) show normal incorporation. E, RNA dot-blot analyses of hypertrophic markers. Four micrograms of total RNA were loaded in each dot. The quantitative data are summarized in F. *P<0.001, **P<0.01: MyBP-C AllP (AllP) vs NTG.
Genetic Modulation of cMyBP-C
The phosphorylation motif of cMyBP-C is conserved among the human, mouse, chicken, and frog (Figure 2A). To investigate the role of cMyBP-C phosphorylation in relation to cardiac function, we generated a construct (MyBP-CAllP) in which the 3 phosphorylation sites (Ser273, -282, and -302) were altered to alanine (Figure 2A). Previously, we prepared a TG mouse that expressed myc-tagged, normal cMyBP-C (line 21.2) and mice in which cMyBP-C expression was ablated.3 To confirm the presence of myc-tagged TG cMyBP-C in the homozygous MyBP-C(t/t) hearts, samples derived from line 21.2 were used along with NTG littermates for comparison of transcript and protein levels for the MyBP-CAllP–null background. Samples treated with PKA or phosphatase (Phosp). PKA-treated myofilaments show levels of MyBP-CAllP–null background that are essentially identical to NTG levels. Lane 2, which corresponds to the protein derived from MyBP-C(t/t) hearts, showed no detectable signal and was omitted. E, Myosin isoform shifts in the samples shown in D. MyBP-CWT expression completely rescued the cardiac phenotype observed in MyBP-Cnull hearts in terms of the shift to the fetal hypertrophic pattern, as evidenced by β-MHC expression. In contrast, MyBP-CWT failed to prevent reversion to the fetal MHC isoform program. M indicates size markers.

Histological and Ultrastructural Consequences of MyBP-CAllP–null Expression
The gross histology of 3-month MyBP-CAllP–null hearts was unremarkable, with no obvious abnormalities, fibrosis, calcification, or disarray compared with NTG controls (Figure 3A). However, transmission electron microscopy revealed subtle ultrastructural changes. Whereas NTG hearts showed typical, well-organized and aligned sarcomeres with regularly distributed mitochondria, sarcoplasmic reticulum, and T-tubules (Figure 3B and 3C), MyBP-CAllP–null hearts occasionally displayed regions that lacked the regular sarcomere–mitochondria distribution, and some sarcomeres showed altered H-zones and M-lines that were relatively ill defined (Figure 3A). However, transmission electron microscopy revealed subtle ultrastructural changes. Whereas NTG hearts showed typical, well-organized and aligned sarcomeres with regularly distributed mitochondria, sarcoplasmic reticulum, and T-tubules (Figure 3B and 3C), MyBP-CAllP–null hearts occasionally displayed regions that lacked the regular sarcomere–mitochondria distribution, and some sarcomeres showed altered H-zones and M-lines that were relatively ill defined (Figure 3B, right panel; Figure 3C, lower panels).

The lack of definition at the central part of the sarcomere is reminiscent of the altered sarcomeres that were observed in mice in which cMyBP-C expression was ablated.4 To confirm that MyBP-CAllP–null was being incorporated normally into the sarcomere, we performed immunohistochemical analyses with both anti-myc and polyclonal anti-cMyBP-C antibodies. Both MyBP-CAllP–null and MyBP-CWT proteins were incorporated normally into the sarcomere (Figure 3D).

Despite the subtle ultrastructural changes, no cardiac hypertrophy and/or dilation in the MyBP-CAllP–null could be de-
tected. The heart/body weight ratios did not significantly differ between NTG (0.53±0.05, n=8) and MyBP-C\textsuperscript{AllP−} (0.60±0.05, n=8; P<0.08) littermates at 3 months. However, we reasoned that the ultrastructural changes reflected a structural deficit that might result in subtle alterations in the transcriptional patterns and, indeed, found that β-MHC and atrial natriuretic factor (ANF) transcript levels, which can serve as sensitive molecular markers for cardiac stress, were significantly increased in MyBP-C\textsuperscript{AllP−} hearts when compared with either NTG or MyBP-C\textsuperscript{WT} samples (Figure 3E and 3F).

**Depressed Cardiac Function in MyBP-C\textsuperscript{AllP−} Mice**

On the basis of the altered ultrastructure, we considered that partial replacement of cMyBP-C with a nonphosphorylatable form might affect whole-organ function. Cardiac function was evaluated both noninvasively and by catheterization in the intact animals at 3 months. M-mode echocardiography in nonsedated animals showed that the MyBP-C\textsuperscript{AllP−} animals had normal cardiac dimensions and function under baseline conditions (supplemental Table II). As cMyBP-C phosphorylation occurs in response to alterations in intracellular calcium levels (activating calcium/calmodulin-dependent kinase) and β-adrenergic stress (activating PKA), we reasoned that adrenergic stimulation might reveal a deficit in the ability of the MyBP-C\textsuperscript{AllP−} animals to respond to dobutamine. Hemodynamic load can be significantly altered via β-adrenergic stimulation, activation of PKA, and the subsequent phosphorylation of phospholamban, cTnI, and MyBP-C. Phosphorylation of cTnI and phospholamban leads to increases in cross-bridge cycling and enhanced relaxation, but the role that cMyBP-C plays in these processes, if any, is unclear. Cardiac function was assessed by in vivo catheterization and dobutamine stimulation. The complete data are shown in supplemental Table III. At baseline in this model, we were able to detect significant differences in the basal left ventricular (LV) end-systolic pressure (83.6±1.0), dP/dt\textsubscript{max} (6051.1±241.5), and dP/dt\textsubscript{min} (−5541.1±151.2) in the MyBP-C\textsuperscript{AllP−} hearts, compared with the NTG and MyBP-C\textsuperscript{WT} groups. The MyBP-C\textsuperscript{AllP−} hearts had decreased contractile performance (peak dP/dt\textsubscript{max}) at maximum dobutamine stimulation, suggesting that a lack of cMyBP-C phosphorylation inhibits maximum contractility. To confirm that these changes were not attributable to compensatory phosphorylation of the other contractile proteins, such as the myosin light chains, the phosphorylation status of these proteins was examined using 2D electrophoresis and found to be unchanged in the MyBP-C\textsuperscript{AllP−} mouse hearts (supplemental Figure I).

**MyBP-C\textsuperscript{AllP−} Fails to Rescue the MyBP-C\textsuperscript{t/t} Phenotype**

The data indicated that partial replacement with a nonphosphorylatable cMyBP-C led to both structural and functional...
deficits. If phosphorylatable cMyBP-C serves an essential function in the heart, complete replacement of endogenous cMyBP-C with MyBP-C\textsuperscript{CMIP−} should lead to significant functional deficits or even death. To test this hypothesis, line 262 was bred with MyBP-C\textsuperscript{C(t/t)}, which should result in a homogenous complement of MyBP-C\textsuperscript{CMIP−}, as MyBP-C\textsuperscript{C(t/t)} produces only a small amount of protein that is truncated and nonfunctional. The MyBP-C\textsuperscript{WT} line was also bred to MyBP-C\textsuperscript{C(t/t)} to confirm that a transgenic strategy could, in fact, rescue the MyBP-C\textsuperscript{C(t/t)} phenotype. Northern and dot-blot analysis confirmed robust expression of both MyBP-C\textsuperscript{WT} and MyBP-C\textsuperscript{CMIP−} in the MyBP-C\textsuperscript{C(t/t)} background (Figure 4A). SDS-PAGE (Figure 4B) and Western blot analysis (Figure 4C) using both anti-myc and anti–MyBP-C antibodies demonstrated the absence of cMyBP-C in MyBP-C\textsuperscript{C(t/t)} hearts and the presence of MyBP-C\textsuperscript{WT} and MyBP-C\textsuperscript{CMIP−} in the MyBP-C\textsuperscript{C(t/t)} background, at approximately the levels observed for the endogenous protein in NTG hearts. IEF was subsequently performed on untreated, PKA-treated, and phosphatase-treated samples to define the phosphorylation states of the total cMyBP-C complement in the mice and confirmed that MyBP-C\textsuperscript{CMIP−} “replacement” in the MyBP-C\textsuperscript{C(t/t)} background was complete (Figure 4D).

The MyBP-C\textsuperscript{C(t/t)} mice have been characterized previously.\textsuperscript{3} The homozygous animals are viable but soon after birth display a progressive dilated cardiomyopathy. Myocyte hypertrophy, disarray, fibrosis, and calcification are observed, and these progress as the animals mature. The effectiveness of MyBP-C\textsuperscript{CWT} expression in preventing reactivation of a fetal transcription program was underscored by the lack of β-MHC expression, which serves as a sensitive marker for a nascent hypertrophic response (Figure 4E).

TG expression of MyBP-C\textsuperscript{WT} also effectively rescued the overt hypertrophy displayed by the MyBP-C\textsuperscript{C(t/t)} hearts. In contrast, equal levels of MyBP-C\textsuperscript{CMIP−} expression did not rescue the MyBP-C\textsuperscript{CMIP−}-induced hypertrophy (Figure 5A). The MyBP-C\textsuperscript{CMIP−} and MyBP-C\textsuperscript{C(t/t)} mouse hearts showed markedly enlarged chambers, with significant cardiac hypertrophy and myocyte disarray, as compared with the NTG and MyBP-C\textsuperscript{WT}\textsuperscript{(t/t)} mice (Figure 5B). Light microscopic analyses showed pathology typical of cardiac hypertrophy in both the MyBP-C\textsuperscript{C(t/t)} and MyBP-C\textsuperscript{CMIP−}(t/t) ventricles, whereas the MyBP-C\textsuperscript{CMIP−}(t/t)-derived sections appeared normal (Figure 5C and 5D). Heart/body weights in the MyBP-C\textsuperscript{C(t/t)} and MyBP-C\textsuperscript{CMIP−}(t/t) mice were significantly elevated, whereas, in contrast, the values derived from the MyBP-C\textsuperscript{WT}(t/t) mice were essentially normal, confirming the absence of physiological hypertrophy (Figure 5E).

Ultrastuctural analysis showed the expected lack of M-band definition in the MyBP-C\textsuperscript{C(t/t)} sarcomeres, as described previously in this model.\textsuperscript{3} In contrast, regular A- and I-bands and M-lines in both the MyBP-C\textsuperscript{CMIP−}(t/t) and MyBP-C\textsuperscript{WT}(t/t) compared with MyBP-C\textsuperscript{C(t/t)} sarcomeres were apparent (Figure 6A). To confirm correct incorporation of MyBP-C\textsuperscript{CMIP−} and MyBP-C\textsuperscript{WT} in the MyBP-C\textsuperscript{C(t/t)} background, we performed immunohistochemistry with both cMyBP-C and myc antibody. As expected, cMyBP-C was absent in the MyBP-C\textsuperscript{C(t/t)} hearts, but each transgenically encoded species showed the expected pattern of incorporation at approximately equal levels (Figure 6B). The inability of MyBP-C\textsuperscript{CMIP−} to rescue the MyBP-C\textsuperscript{C(t/t)} phenotype was confirmed at the functional level. M-mode echocardiography showed the MyBP-C\textsuperscript{CMIP−}(t/t) and MyBP-C\textsuperscript{C(t/t)} mice had increased LV end-diastolic and end-systolic dimensions, as well as reduced fractional shortening, whereas normal shortening fractions were observed in the MyBP-C\textsuperscript{WT}(t/t) hearts (Figure 7A and the Table). Although both MyBP-C\textsuperscript{CMIP−} and MyBP-C\textsuperscript{WT} incorporate normally into the sarcomere, MyBP-C\textsuperscript{WT} expression appears to be able to rescue the MyBP-C\textsuperscript{C(t/t)} phenotype, whereas MyBP-C\textsuperscript{CMIP−} cannot. To define this more completely, we looked for activation of the fetal gene program in these mice, as upregulation of ANF, brain natriuretic peptide (BNP), β-MHC, and skeletal α-actin and downregulation of α-MHC, phospholamban (PLN), and the sarcoplasmic reticulum Ca\textsuperscript{2+} pump (SERCA) often serve as sensitive markers for hypertrophy or cardiac stress.\textsuperscript{25} The data illustrate the completeness of the rescue, as no differences, even at the molecular level, were detected between the NTG and MyBP-C\textsuperscript{WT}(t/t) groups (Figure 7B and 7C). In contrast, ANF, β-MHC, BNP, and skeletal α-actin were upregulated, whereas α-MHC, PLN, and SERCA were significantly downregulated in both the MyBP-C\textsuperscript{C(t/t)} and MyBP-C\textsuperscript{CMIP−}(t/t) groups (Figure 7B and 7C), a pattern consistent with compromised cardiac function.

Discussion

Heart failure is associated with diminished β-adrenergic responsiveness, loss of cardiac contractility, abnormalities in Ca\textsuperscript{2+} handling,\textsuperscript{26,27} and altered contractile protein phosphorylation.\textsuperscript{18,28} Although cMyBP-C is extensively phosphorylat-
phosphorylation. Basal levels of cMyBP-C phosphorylation may be necessary for maintaining thick-filament orientation, dynamic regulation, and contractile mechanics, and compromised phosphorylation patterns almost certainly reflect alterations in these cardiac parameters. Protein phosphatase activities appear to be increased in heart failure, and this increased activity, along with decreased β-adrenergic responsiveness during heart failure, may be responsible for the decrease in cMyBP-C phosphorylation.

We next addressed whether altered cMyBP-C phosphorylation patterns could actually cause cardiac disease. Mice in which approximately 40% of the endogenous cMyBP-C was replaced with MyBP-C WT appeared overtly normal. However, ultrastructural analysis did show subtle alterations at multiple foci, consistent with the hypothesis that normal cMyBP-C phosphorylation levels are needed to maintain normal myofibrillar structure. These mice also showed up-regulation of transcripts usually associated with a nascent hypertrophic response, and invasive catheterization showed that contraction and relaxation were significantly decreased. Rapid and reversible changes in thick-filament structure and ordering of myosin heads can be produced in cardiac muscle by changes in the degree of cMyBP-C phosphorylation, and these changes in structure are accompanied by changes in force production. Cardiac MyBP-C and its phosphorylation state may play an important role in determining thick/thin-filament interaction, with the force of contraction and time to half-relaxation dependent on the phosphorylated state of the C1–C2 domains. Although it is not clear that dephosphorylated cMyBP-C directly causes cardiac disease, our data suggest that cMyBP-C phosphorylation patterns can have a significant effect on whole-heart function and compromise cardiac hemodynamics.

The inability of MyBP-C WT to rescue the MyBP-C WT phenotype is consistent with the hypothesis that cMyBP-C phosphorylation is essential for normal cardiac function. Strikingly, equivalent TG expression of MyBP-C WT effectively rescued the MyBP-C WT mice, resulting in restoration of normal morphology, preventing activation of the fetal gene program, and resulting in normal cardiac hemodynamics (Table). Both MyBP-C WT and MyBP-C WT appear to incorporate normally into the sarcomere, yet only the phosphorylatable form is effective in suppressing the MyBP-C WT phenotype. In addition to the structural roles of cMyBP-C, and ability to bind to titin, phosphorylation of cMyBP-C extends the crossbridges from the backbone of the thick filament, with the crossbridges from the backbone of the thick filament, with the crossbridges from the backbone of the thick filament, with the crossbridges from the backbone of the thick filament, with the crossbridges from the backbone of the thick filament, with the crossbridges from the backbone of the thick filament, with the crossbridges from the backbone of the thick filament, with the crossbridges from the backbone of the thick filament.
filament, changes their orientation, increases the degree of order of the crossbridges, and decreases crossbridge flexibility.33,34,35 Although our data do not address crossbridge mechanics directly, it is clear that the mutant cMyBP-C is present in the sarcomere in a pattern that is indistinguishable from normal protein but appears to lack some critical function necessary for maintaining the overall sarcomere architecture, as manifested by the alterations observed in the sarcomere–mitochondrial spatial relationships. Further studies using these mouse models should provide valuable insight into the mechanical consequences of cMyBP-C phosphorylation.

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References


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Cardiac Myosin Binding Protein-C Phosphorylation and Cardiac Function

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Running title: Cardiac Myosin Binding Protein-C

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Materials and Methods

Transgene Construction

The cDNA encoding the endogenous form of mouse cardiac MyBP-C (MyBP-C^{WT}) was obtained by RT-PCR using total RNA isolated from the mouse cardiac ventricle. Full length MyBP-C^{WT} (~3.8kb) was subcloned, completely sequenced in both directions and compared with Genebank cDNA database (Accession number: NM_008653). The known phosphorylation sites (Ser-273, -282 and -302\textsuperscript{1}) and two neighboring potentially alternative phosphorylation sites (Thr-272 and -281) were converted to alanines using standard PCR-based methods and subcloned into the ApoI and SphI restriction sites. This construct was named MyBP-C^{AllP}. Sequence encoding the human c-myc peptide (EQKLISEEDL) was inserted after the initiation methionine codon to differentiate the transgenic (TG) species from endogenous cMyBP-C.\textsuperscript{2,3} Earlier studies confirmed that introduction of the c-myc epitope was benign.\textsuperscript{3,4} The cDNAs were subcloned into the mouse α-myosin heavy chain promoter and the construct purified from the plasmid backbone after NotI digestion. The DNAs were used to generate multiple lines of FVB/N TG mice.\textsuperscript{5} Six founders were obtained for this construct, the one chosen for further experimentation, line 262, contained 11 copies of the MyBP-C^{AllP} transgene and expressed the protein at a level such that approximately 40% of endogenous cMyBP-C was replaced with the TG species.

MyBP-C\textsuperscript{(\textit{t/t})} Background.

MyBP-C\textsuperscript{(\textit{t/t})} mice\textsuperscript{6} were back-crossed for more than 6 generations into the FVB/N background. MyBP-C^{AllP} mice (line 262) were crossed with homozygous MyBP-C\textsuperscript{(\textit{t/t})} mice to ensure the absence of endogenous phosphorylatable cMyBP-C. An animal that expresses the normal cMyBP-C isoform,\textsuperscript{3} MyBP-C^{WT}, was also bred into the MyBP-C\textsuperscript{(\textit{t/t})} background to serve as a control. Except where noted, 5-6 mice, 12-15 weeks old of
mixed gender were used in each experiment after preliminary experiments showed no gender differences.

**Genotyping**

DNA samples were extracted from ear clips at 10 days and PCR was employed in two different reaction sets for either the cMyBP-C transgene or MyBP-C$^{(t/t)}$. The forward (5’- TGTCAGCCTCAACAAAGCCAAGC-3’) and reverse (5’- CTTCAAGACTTGAGACATTCTAAC-3’) oligonucleotide sequences were used for genotyping cMyBP-C transgene. Oligonucleotide sequences for MyBP-C$^{(t/t)}$ genotyping have been described.\(^6\)

**Mouse Models of Heart Failure**

Four different mouse models of heart failure were used to determine if cMyBP-C phosphorylation changed during the development of cardiac pathology:

(1) **TG model.** This mouse (supplied by Dr. Jeffery Molkentin, Cincinnati) over-expresses the active form of calcineurin in the heart, developing cardiac hypertrophy and heart failure that mimics human heart disease.\(^7\) It shows a dramatic increase in heart size with the heart weight/body weight averaging 5-fold greater as compared to control littermates at 8 weeks post-birth.

(2) **Gene targeted model.** The striated muscle-specific LIM-only protein MLP (muscle LIM protein) homozygous knockout mice (supplied by Dr. Pico Caroni, Basal, Switzerland) show dilated cardiomyopathy and heart failure.\(^8\)

(3) **Pressure overload hypertrophy.** Acute pressure overload is typically used as a means of inducing a pathological profile of cardiac hypertrophic growth and ventricular remodeling. Initiation of pressure overload induced hypertrophy in the first 2 days postsurgery is associated with immediate early gene activation although less is known of the signaling factors or genes that sustain the long-term hypertrophic growth of the
myocardium and its transition to dilated failure. Pressure overload hypertrophy was induced by constriction of the transverse aortic arch (TAC). The arch was visualized through a median sternotomy and 7-0 silk ligature was tied around the aorta (27-gauge constriction) between the right brachiocephalic and left common carotid arteries. Heart weight and body weight ratios were calculated to confirm the presence of cardiac hypertrophy. Total myofibrillar proteins were obtained from both ventricles at either 1 day or 18 days after TAC surgery.

(4) Ischemia-reperfusion. In order to induce myocardial infarction, cardiac ischemia-reperfusion injury was performed in eight-week old FVB/N mice as described previously. The thoracotomy was closed and the mice were revived for a 1 hour ischemic period, after which the knot was released and the heart reperfused for 24 hours or 5 days. Upon completion of the reperfusion period, mice were sacrificed by CO₂ asphyxiation and the hearts quickly removed for analysis of infarction injury with 2% triphenyl tetrazolium chloride. Infarcts were ~40-45% of the left ventricle after 5 days of reperfusion. Total myofibrillar proteins were obtained from both ventricles, and included the area not at risk, area at risk, and infarcted area.

**Molecular Analyses**

For evaluating cMyBP-C transcript levels, hearts were homogenized in Tri-Reagent (Molecular Research Center) and total RNA extracted according to the manufacturer’s protocol. For assessment of cMyBP-C transcript size, northern blot analyses were performed using 7.5 µg of total RNA, as described. Expression levels were determined by RNA dot blot analysis with γ³²P-labeled cMyBP-C and human growth hormone (hGH) probes using transcript-specific oligonucleotides. GAPDH was used for the loading control. Expression levels of atrial natriuretic factor (ANF), brain natriuretic protein (BNP), α-skeletal actin, α- and β-myosin, phospholamban and SERC2a were
used to detect activation of transcription reflecting cardiac hypertrophic and/or heart failure.

**Protein Analyses**

To identify modifications at the protein level, enriched myofibrillar proteins were isolated using F60 buffer (60 mmol/L KCl, 30 mmol/L imidazole, 7.2 mmol/L MgCl₂, pH 7.0) with protease/phosphatase inhibitors (Cocktail I and II, Sigma) as described,¹¹ and solubilized in urea buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 20 mmol/L spermine, 1 mmol/L PMSF and 20 mmol/L DTT). The presence of MyBPC⁹⁻ protein was confirmed in SDS-PAGE (4-15% criterion gradient Tris-glycine pre-cast gels, Bio-Rad) followed by western blots using an anti-c-myc monoclonal antibody (clone 9E10, Roche) and an anti-cMyBP-C rabbit polyclonal antibody raised against the C0-C1 domains.¹²,¹³ Myc-tagged MyBP-C⁹⁻ was used as a positive control and for comparing protein levels.

**In Vitro PKA Phosphorylation and Phosphatase Treatment**

Total enriched myofibrils were isolated as described above in the presence of 1% Triton X-100 (Fisher Scientific) and protease/phosphatase inhibitors (Cocktail I and II, Sigma). The PKA phosphorylation assays contained 100 µg of freshly isolated total skinned myofibrils as described previously.¹⁴ Myofibrils were incubated 90 minutes at 37°C in 100 µl of 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 20 mmol/L MgCl₂, 10 µM ATP and 30 U of the PKA catalytic subunit (PKAce kit, Novagen). A PKA inhibitor, PKI 6-22 Amide (Calbiochem), was used for control studies to confirm the specificity of PKA activity. Similarly, 100 µg of freshly isolated total myofibrils were treated with 10 U of alkaline phosphatase (Roche) in 1× dephosphorylation buffer (0.5 mol/L Tris-HCl and 1mmol/L EDTA, pH 8.5). Myofibrils were washed 3 times in F60 and solubilized in 7 mol/L urea buffer for IEF. Protein was determined by the Bradford method (Bio-Rad).
Measurement of MyBP-C Phosphorylation

One dimensional isoelectric focusing (IEF) was used to detect the multiple phosphorylated species of a subset of the contractile proteins (de-, mono- bi- and tri-phosphorylated) as described in detail previously\textsuperscript{12,13} with some modifications. IEF slab gels were prepared using empty criterion cassettes (Bio-Rad) consisting of 5% Duracryl (Genomic Solutions), 5% glycerol, 1× of both pH 5-7 and pH 3-10 ampholites (Bio-Rad) were degassed for 1 hour and the three catalysts (riboflavin-5’-phosphate (FMN), ammonium persulfate, and N,N,N,N-,tetramethylethylenediamine (TEMED) added separately. Total myofibrillar protein was loaded in buffer containing 10 mol/L urea, 40% glycerol, 1× of both pH 5-7 and pH 3-10 ampholites, and 2% Triton X-100 with 0.025% bromophenol blue. The cathode buffer consisted of 30 mmol/L NaOH (pH 10.1), and the anode buffer consisted of 15 mmol/L phosphoric acid (pH 2.4) (Bio-Rad). Standard IEF markers (Bio-Rad) were used for calculating isoelectric points (pI). The running conditions were 1 W for 2 hours, 2 W for 30 minutes and 500 V for 2 hours per gel. The transfer took place in 0.7% acetic acid (pH 3), placing the nitrocellulose membrane towards the anode at 200 mA for 10-12 hours at 4°C. After the transfer, an immunoblotting procedure was performed using a polyclonal anti-cMyBP-C antibody as described.\textsuperscript{2,3} Percentage phosphorylation was expressed as cMyBP-C (phosphorylated species)/(P0+P1+P2+P3) x 100%.

Two-Dimensional Electrophoresis

Myofilament proteins suspended in 8 mol/L urea buffer were analyzed via two-dimensional analysis to assess the phosphorylation status of selected contractile proteins. Total myofilament proteins, 50-75 µg, were diluted in 185 µl IPG rehydration buffer (Bio-Rad) containing 1× ampholytes (Bio-Rad) and subjected to IEF on IPG strips (11 cm, pH 4-7 or pH 7-10 linear gradient, Bio-Rad) according to the manufacture’s instructions. This was followed by 10-20% gradient SDS-PAGE (Criterion gradient Tris-
glycine pre-cast gels, Bio-Rad). The gels were stained with SYPRO-Ruby (Bio-Rad) for 8-12 hours and scanned on the Typhoon 9400 scanner (Amersham Biosciences) or transferred to nitrocellulose membranes (Bio-Rad) to quantitate the level of myofibrillar protein phosphorylation by western blotting.

**Echocardiography Measurements**

For two-dimensional M-mode echocardiography, mice were anesthetized with 2% isoflurane (Table 1). In some cases, conscious unsedated mice were used (Table 2S, Online Supplement), and hearts visualized with a Hewlett Packard Sonos 5500 instrument and a 15 MHZ transducer as described previously. Measurements were taken three times per animal from different areas and then averaged for left ventricular (LV) diastolic and systolic dimensions (LVEDD and LVESD) and septal and posterior wall thickness (SWT and PWT), from which fractional shortening (FS) and LV mass was derived. Pulsed wave Doppler was used to measure aortic ejection time (E-TIME) and calculate the velocity of circumferential shortening, $V_{cf}$ (FS/E-TIME). Diastolic transmitral inflow Doppler indices included the following: peak E wave velocity, peak A wave velocity, E wave deceleration time, and isovolumic relaxation time (IVRT).

**In Vivo Cardiac function and β-adrenergic Responsiveness**

Invasive hemodynamic studies was performed in the intact animal as previously described. Mice were anesthetized with ketamine-thiobutabarbital and following tracheotomy (PE-90), the right femoral artery and vein were cannulated with polyethylene tubing for measuring systemic arterial pressure and for the infusion of experimental drugs. To assess myocardial performance, closed chest animals were studied with a high fidelity 1.4F Millar micromanometer tipped catheter placed retrograde across the aortic valve into the LV (via right carotid cut-down) for pressure measurement and determining $dP/dt_{max}$ and $dP/dt_{min}$. Cardiovascular responses to
increasing doses of dobutamine were determined during 3 minute constant infusions (0.1 µl/min/gm body weight), and average values determined during the final 30 seconds of each infusion. Online heart rate, telemetry, LV developed pressure, +dP/dt, and -dP/dt were archived on a Macintosh G4 using Maclab software.

**Histochemistry**

Heart weight/body weight was measured to determine if hypertrophy had occurred. For histopathological examinations, the hearts were removed while still beating from deeply anesthetized mice, drained of blood, and fixed in 10% formalin. The hearts were bisected longitudinally, dehydrated through a graded series of alcohols, and laid open before being paraffin embedded. Step-serial sections (5 µm) were taken from 2-3 hearts per group. Sections were stained with hematoxylin-eosin or Masson’s trichrome and images evaluated with the SPOT software (Diagnostic Instruments, Inc) using an Olympus BX60 microscope. The presence of necrosis, fibrosis, myocyte disarray and calcification were evaluated by an expert who was blinded to genotype.

**Immunohistochemistry**

Localization and integration of cMyBP-C into the sarcomere was determined by confocal microscopy. Hearts were perfused in 4% paraformaldehyde/cardioplegic buffer (50 mmol/L KCl, 5% dextrose in phosphate buffered saline (PBS)), fixed overnight in 4% paraformaldehyde/PBS at 4°C, transferred to 10% sucrose for 3 hours, followed by 30% sucrose for at least overnight. After fixing, the hearts were embedded in OCT, frozen on dry ice and the blocks stored at -80°C until use. Five µm frozen sections were fixed on microscope slides (SuperfrostPlus, Fisher) and stored at -80°C. Before processing for immunostaining, the frozen sections were allowed to dry at room temperature for 15 minutes. The slides were placed in a slide jar and microwaved with antigen retrieval buffer (10 mmol/L citric acid and 0.05% Tween 20, pH 6.0) for 2, 5 and 25 minutes at
power levels 100, 20 and 10, respectively (Kenmore) and then cooled to room temperature. The slides were washed with PBS (3 times, 5 minutes each), incubated in blocking solution (1% BSA, 0.1% cold water fish skin gelatin, 0.05% NaN₃ and 0.1% Tween-20 in PBS) for 1 hour at room temperature and probed with 1:1300 diluted polyclonal antibody against cMyBP-C (C0-C1) in PBS for overnight at 4°C. The slides were washed with PBS 3 times for 5 minutes each, incubated with blocking solution for 20 minutes, and incubated with secondary goat anti-rabbit IgG antibody (1:100) conjugated with Alexa-488 (green fluorescence) in PBS. Finally, the slides were washed with PBS 3 times for 5 minutes each, and mounted with Vecta Shield. For long-term storage, immuno-labeled slides were placed in the dark at 4°C. The vector biotin-avidin blocking and M. O. M. kits (Vector Laboratories, Inc) were used for immunostaining with the monoclonal anti-myc antibody and with the Alexa-488 conjugated goat anti-mouse IgG antibody. Specimens were examined using confocal microscopy (Nikon PCM 2000) and software SimplePCI v.4 (C.Imaging Systems) by an investigator blinded to genotype.

**Transmission Electron Microscopy**

Structural analyses at the light and electron microscopy levels were performed as described.²,³ For electron microscopic ultrastructural analysis, mice were anesthetized with isofluorane and the hearts fixed by perfusion with 3.5% glutaraldehyde in cardioplegic buffer for 2 minutes, followed by 3.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.3) for 2 minutes. The fixatives were gravity fed (600 mm) into the hearts through the apex and right ventricle. Immediately after the hearts started to fill with the fixative, the aorta and pulmonary artery were cut to allow the output of liquids. The hearts were then excised and subsequently separated into six regions: left atrial flap, right atrial flap, left ventricular free wall, right ventricular free wall, septum, and apex. Each region was divided into small (1 mm) fragments and fixed in the glutaraldehyde-
cacodylate fixative overnight at 4°C. The tissue fragments were post-fixed on ice in 1% O\textsubscript{4}O\textsubscript{4} in cacodylate buffer, dehydrated in acetone, and embedded in a Poly/Bed 812 resin mixture. Thin sections were counterstained with uranyl acetate and lead citrate and examined on a Zeiss 912 transmission electron microscope at an accelerating voltage of 100 kV. Multiple sections were cut from 2-4 mice of mixed gender and >50 fields were observed by a blinded observer.

**Statistical Analysis**

Data are expressed as means±SE. The significance of differences among means was evaluated either using analysis of variance (ANOVA) or Student’s t test. Statistically significant differences were considered as $P<0.05$. 

![Diagram showing pH gradient and protein expression]
Figure 1S. Phosphorylation status of myosin essential light chain (ELC1v) and regulatory light chain (RLC2v) in MyBP-C\textsuperscript{WT} (WT), MyBP-C\textsuperscript{AllP\textsuperscript{-}} (AllP\textsuperscript{-}) and MyBP-C\textsuperscript{(t/t)} (t/t) mouse hearts. Two-dimensional electrophoresis (pH 4-7) was performed as described previously\textsuperscript{17} to determine if any compensatory changes in the phosphorylation status of ELC1v and RLC2v had occurred in response to the presence of MyBP-C\textsuperscript{AllP\textsuperscript{-}} protein. Changes in phosphorylation status of these proteins can sometimes be associated with heart failure.\textsuperscript{18} No significant differences could be detected between the NTG, MyBP-C\textsuperscript{WT} and MyBP-C\textsuperscript{AllP\textsuperscript{-}} samples.
Table 1S. Quantification of cMyBP-C phosphorylation species (n=3)

<table>
<thead>
<tr>
<th>Lanes</th>
<th>Models</th>
<th>%P3</th>
<th>%P2</th>
<th>%P1</th>
<th>%P0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24 hours Sham</td>
<td>23.1 ± 1.1</td>
<td>43.1 ± 5.4</td>
<td>27.1 ± 3.0</td>
<td>6.7 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>24 hours TAC</td>
<td>11.5 ± 1.0‡</td>
<td>45.2 ± 1.7</td>
<td>30.6 ± 2.4</td>
<td>12.6 ± 0.9‡</td>
</tr>
<tr>
<td>3</td>
<td>18 day Sham</td>
<td>21.7 ± 1.4</td>
<td>43.7 ± 3.3</td>
<td>26.7 ± 1.0</td>
<td>7.9 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>18 day TAC</td>
<td>2.2 ± 2.9†</td>
<td>13.0 ± 3.1†</td>
<td>16.6 ± 4.4††</td>
<td>68.1 ± 10.1†</td>
</tr>
<tr>
<td>5</td>
<td>NTG</td>
<td>24.3 ± 1.8</td>
<td>42.9 ± 3.6</td>
<td>25.3 ± 2.3</td>
<td>7.5 ± 1.1</td>
</tr>
<tr>
<td>6</td>
<td>Calcineurin TG</td>
<td>2.0 ± 1.1*</td>
<td>22.9 ± 1.3*</td>
<td>27.0 ± 4.6**</td>
<td>57.2 ± 3.9*</td>
</tr>
<tr>
<td>7</td>
<td>MLP-KO</td>
<td>1.5 ± 0.9 *</td>
<td>24.4 ± 1.8*</td>
<td>34.7 ± 3.8**</td>
<td>39.4 ± 4.8*</td>
</tr>
<tr>
<td>8</td>
<td>β-MHC TG</td>
<td>27.0 ± 0.8</td>
<td>47.7 ± 1.9</td>
<td>21.7 ± 3.8</td>
<td>3.6 ± 2.7</td>
</tr>
<tr>
<td>9</td>
<td>24 hours Sham</td>
<td>32.6 ± 2.9</td>
<td>45.9 ± 0.5</td>
<td>18.3 ± 5.5</td>
<td>3.2 ± 2.7</td>
</tr>
<tr>
<td>10</td>
<td>24 hours IR</td>
<td>34.4 ± 1.8</td>
<td>45.4 ± 0.5</td>
<td>15.9 ± 0.7</td>
<td>4.3 ± 2.3</td>
</tr>
<tr>
<td>11</td>
<td>5 day Sham</td>
<td>23.4 ± 16.1</td>
<td>52.9 ± 9.0</td>
<td>20.4 ± 3.8</td>
<td>3.3 ± 4.7</td>
</tr>
<tr>
<td>12</td>
<td>5 day IR</td>
<td>25.2 ± 3.2</td>
<td>24.9 ± 3.4##</td>
<td>14.3 ± 4.0</td>
<td>34.7 ± 4.3#</td>
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</table>

One dimensional IEF focusing (pH 5-7) of total myofilament proteins followed by western blot analysis using cMyBP-C antibody as described in detail in the Online Supplemental Materials and Methods. A representative western blot is shown in Figure 1A. The percent of de-, mono, di- and tri-phosphorylated MyBP-C (P0, P1, P2, and P3, respectively) is shown for each model. Signal on the membrane was quantitated using a STORM 860 (GE Healthcare) and ImageQuant v.5.2. Percentage of phosphorylation is expressed as cMyBP-C (Phosphorylated species)/(P0+P1+P2+P3)X100. Values are expressed as means±SE.

‡‡P<0.05 and ‡P<0.001, significant difference vs 24 hours TAC and 24 hours Sham.
††P<0.05 and †P<0.001, significant difference vs 18 day TAC and 18 day Sham
**P<0.05 and *P<0.001, significant difference vs with NTG
##P<0.05 and #P<0.001, significant difference vs 5 day IR and 5 day Sham
Table 2S. Cardiac Function Assessed by M-mode Echocardiography

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVED (mm)</th>
<th>LVES (mm)</th>
<th>SW (mm)</th>
<th>PW (mm)</th>
<th>HR (bpm)</th>
<th>E-TIME</th>
<th>LVM (g/m2)</th>
<th>FS (%)</th>
<th>Vcf</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG</td>
<td>3.1 ± 0.08</td>
<td>1.7 ± 0.1</td>
<td>0.7 ± 0.01</td>
<td>0.7 ± 0.02</td>
<td>693.3 ± 27.2</td>
<td>38.2 ± 1.1</td>
<td>63.4 ± 3.7</td>
<td>47.2 ± 2.2</td>
<td>11.7 ± 0.8</td>
</tr>
<tr>
<td>WT</td>
<td>3.3 ± 0.15</td>
<td>1.8 ± 0.2</td>
<td>0.6 ± 0.02</td>
<td>0.6 ± 0.02</td>
<td>711.6 ± 14.2</td>
<td>40.8 ± 1.6</td>
<td>65.3 ± 5.7</td>
<td>46.0 ± 3.7</td>
<td>10.5 ± 1.2</td>
</tr>
<tr>
<td>AllP⁻</td>
<td>3.2 ± 0.19</td>
<td>1.7 ± 0.1</td>
<td>0.7 ± 0.02</td>
<td>0.7 ± 0.02</td>
<td>683.5 ± 13.7</td>
<td>40.9 ± 1.3</td>
<td>66.2 ± 8.1</td>
<td>47.0 ± 2.8</td>
<td>10.9 ± 1.1</td>
</tr>
</tbody>
</table>

Conscious unsedated mice at 3 months were used. Measurement values were averaged from at least 3 separate cardiac cycles: left ventricular (LV) end diastolic thickness (LVED), LV end systolic thickness (LVES), septal wall thickness (ESW), posterior wall thickness (PW), aortic ejection time (E-TIME), LV mass (LVM), fractional shortening (FS), heart rate (HR) and velocity of circumferential shortening, Vcf (FS/E-TIME). The MyBP-C⁻AllP⁻ TG hearts demonstrated normal cardiac function compared to NTG and MyBP-C⁻WT TG cohorts (n=6). Data are expressed as means±SE.
Table 3S. Cardiac Function Assessed by In Vivo Catheterization

<table>
<thead>
<tr>
<th>Groups</th>
<th>Heart rate (bpm)</th>
<th>LVP (mm Hg/s)</th>
<th>dP/dt\textsubscript{max} (mm Hg/s)</th>
<th>dP/dt\textsubscript{min} (mm Hg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Dobutamine</td>
<td>Basal</td>
<td>Dobutamine</td>
</tr>
<tr>
<td>NTG</td>
<td>400 ± 4.7</td>
<td>606 ± 11.3</td>
<td>108.6 ± 3.0</td>
<td>121.8 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>7895.7 ± 47.5</td>
<td>17594.5 ± 125.0</td>
<td>-7029.9 ± 87.8</td>
<td>-9542.7 ± 219.6</td>
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<tr>
<td>WT</td>
<td>425 ± 4.8</td>
<td>625 ± 6.4</td>
<td>92.1 ± 2.4</td>
<td>99.9 ± 1.0</td>
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<tr>
<td></td>
<td>8356.5 ± 260.1</td>
<td>16229.4 ± 182.5</td>
<td>-7019.9 ± 266.0</td>
<td>-8361.8 ± 107.0</td>
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<tr>
<td>AllP-*</td>
<td>383 ± 5.5#</td>
<td>548 ± 7.2</td>
<td>83.6 ± 1.0**</td>
<td>99.6 ± 1.5</td>
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<tr>
<td></td>
<td>6051.1 ± 241.5**#</td>
<td>13648.5 ± 132.6**##</td>
<td>-5541.1 ± 151.2*</td>
<td>-7630.8 ± 217.5**</td>
</tr>
</tbody>
</table>

Measurements of LV hemodynamic parameters, including heart rate, peak LV pressure (LVP), LV dP/dt\textsubscript{max} (an index of myocardial contractility) and LV dP/dt\textsubscript{min} (an index of myocardial relaxation), were determined. The LV dP/dt\textsubscript{max} and dP/dt\textsubscript{min} in the MyBP-C\textsuperscript{AllP-} mice was markedly decreased by 76\% and 78\%, respectively, compared with to the NTG and MyBP-C\textsuperscript{WT} mice. LV dP/dt\textsubscript{max} and dP/dt\textsubscript{min} in MyBP-C\textsuperscript{AllP-} TG mice remained substantially lower than in NTG and MyBP-C\textsuperscript{WT} TG mice. All data are presented as means±SE. **P<0.05 and *P<0.001 vs NTG. #P<0.05 and ##P<0.001 vs WT (n = 6) for all cohorts.
References


15. Hahn HS, Marreez Y, Odley A, Sterbling A, Yussman MG, Hilty KC, Bodi I, Liggett SB, Schwartz A, Dorn GW, 2nd. Protein kinase Ca negatively regulates

