PKA-dependent phosphorylation of cardiac myosin binding protein C in transgenic mice

Qinglin Yang, Timothy E. Hewett, Raisa Klevitsky, Atsushi Sanbe, Xuejun Wang, Jeffrey Robbins*

Department of Pediatrics, Division of Molecular Cardiovascular Biology, The Children's Hospital Research Foundation, 333 Burnet Avenue, Cincinnati, OH 45229-3039, USA

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Abstract

Objective: To investigate the physiological role of cAMP-dependent protein kinase A (PKA)-mediated, cardiac myosin binding protein C (MyBP-C) phosphorylation. Methods: A cardiac MyBP-C cDNA lacking nine amino acids, which contained a phosphorylation site, was made, and subsequently used to generate multiple lines of transgenic mice. Upon confirming that a partial replacement of endogenous protein with transgenic protein occurred, the biochemical and physiological consequences were studied. PKA-dependent phosphorylation assays were used to estimate the phosphorylation states of major cardiac PKA substrates. Myofibril Mg-ATPase activities were also measured. Isolated working heart and whole animal exercise studies were used to measure the physiological changes. Results: Transgenic mice displayed a compensatory response, with PKA-mediated phosphorylation of both troponin I and phospholamban showing significant increases. The remaining endogenous cardiac MyBP-C also showed increased phosphorylation levels. Maximal Mg$^{2+}$-ATPase activity was increased. Significant functional changes at both the whole organ and whole animal levels also occurred. Parameters reflecting cardiac contractility and relaxation increased about 22 and 25%, respectively, in the mutant relative to wild type mice (n = 5, P < 0.001). In young adults the capacity for stress exercise, quantitated using an exercise treadmill regimen, was substantially enhanced (n = 6, P < 0.01). Conclusions: Cardiac MyBP-C phosphorylation plays an important physiological role and that the protein’s degree of phosphorylation is coordinated with the phosphorylation levels of other proteins within the contractile apparatus. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Contractile apparatus; Contractile function; Histo(patholo)logy; Gene expression; Signal transduction; Protein phosphorylation

1. Introduction

It is well-established that generation of force and power in striated muscle in general, and cardiac muscle in particular, depends upon the thick and thin filaments interacting within a well organized sarcomere. Relaxation and contraction in cardiac muscle depend upon the controlled transition between resting and force-generating states. This control is mediated by a Ca$^{2+}$-dependent switch, which is made up of the thin filament protein troponymosin and the three subunits of troponin. Upon binding Ca$^{2+}$, the accessing state of the molecular motor, myosin, which is located in the thick filament, is modified such that it productively binds to actin, which is located in the thin filament [1].

It is now well established that β-adrenergic stimulation, either in intact hearts or in isolated myocardial cells, results in phosphorylation of MyBP-C [2] and the inhibitory subunit of troponin (TnI) [3], which are located in the thick and thin filaments, respectively. These post-translational modifications can have significant chronotropic, inotropic and lusitropic effects. However, the role or roles

*Corresponding author. Tel.: +1-513-636-8098; fax: +1-513-636-3852.
E-mail address: jeff.robbins@chmcc.org (J. Robbins).

Present address: Cardiovascular Research Institute, Morehouse School of Medicine, Westview Dr. SW, Atlanta, GA 30310, USA.

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phosphorylation of these proteins plays in the Ca\(^{2+}\)-independent regulation of cardiac contractility in vivo is not well understood.

MyBP-C, a major component of the thick filament, binds to both the myosin (thick) and titin filament systems. Discovered over 27 years ago [4], interest in the protein’s role(s) intensified after multiple mutations in the polypeptide were linked to familial hypertrophic cardiomyopathy [5]. MyBP-C is localized in the C region of the A band, and has a unique organization with seven to nine axial bands in each half-sarcomere. Like other myosin binding proteins and titin, MyBP-C belongs to the intracellular immunoglobulin (Ig) superfamily and is composed of repeated Ig and fibronectin domains [6]. In vitro modeling and reconstitution experiments, as well as experiments carried out using cell transfections, indicate that the protein probably plays an important role in assembling and maintaining the overall architecture of the sarcomere [7,8]. However, the cardiac-specific isoform may also play an important role in the heart’s response to β-adrenergic stimulation. While the skeletal muscle isoform has a single phosphorylation domain (MyBP-C), these sites, whose phosphorylation can change both filament stiffness and orientation and contractile mechanics [2,7,9,10]. One site, located at the cardiac isoform-specific insertion that forms a surface loop [2], is particularly interesting. When deleted or mutated, cardiac MyBP-C phosphorylation is markedly decreased. Thus, phosphorylation at this site may function as a conformational switch, rendering other sites on the protein more accessible to the relevant kinase [2].

With the exception of gain-of function approaches to study the potential dominant negative effects of some FHC mutations [11–13], studies concerning the structure–function relationships of this modular protein have been carried out in vitro using either transfection of cell culture with cDNAs [8] or biochemically defined filament reconstitutions with fragments of the protein [9]. X-ray diffraction analysis on thick filaments showed that the phosphorylation state of MyBP-C affects cross bridge extension and increases the overall order of the thick filament [14]. The authors suggested that phosphorylation of residues within the regulatory motif of MyBP-C changes the end-to-end interactions of adjacent molecules within the thick filament. This, in turn, may change the orientation or flexibility of the crossbridges, altering the rate constants of attachment/detachment [10,14]. Using a variety of biochemical and in vitro approaches, Gautel and co-workers demonstrated the potential importance of phosphorylation of this domain in regulating filament mechanics, noting that five of the six parameters measured were modified when exchanging the phosphorylated and non-phosphorylated fragments [9].

The above studies were performed in skeletal muscle fibers and involved infusing high doses of a soluble MyBP-C fragment into the skinned fibers. In contrast, we are interested in understanding the structure–function relationships of these post-translational modifications, and how other components of the contractile apparatus might react and compensate for any changes in the phosphorylation of the cardiac-specific domain of MyBP-C in vivo. In this study, we generated multiple lines of TG mice that express a MyBP-C that lacks the cardiac-specific phosphorylation domain (MyBP-C\(^{\Delta}\)). These mice were compared against both non-transgenic (NTG) and TG cohorts that expressed normal, wild-type MyBP-C [12,13]. The data show the in vivo functionality of this domain, and the interactions of the molecule’s phosphorylation state with respect to phosphorylation of other sarcomeric proteins, TnI and phospholamban, which function in controlling cardiac contractility. Long-term insufficiency of MyBP-C phosphorylation was quite benign, with no detectable hypertrophy at the cardiomyocyte level; in fact, improvement of performance, as measured by a stress exercise regimen, occurred. It appears that cardiac MyBP-C phosphorylation can play a role in regulating overall organ function and is coordinated closely with the phosphorylation states of the other cardiac contractile regulatory proteins.

2. Methods

2.1. Transgene construction

The full-length, c-myc-tagged murine cardiac MyBP-C cDNA has been described [12]. The sequence found in the human MyBP-C, LAGGGRIS reads LAGAGRRTS in the mouse. The cardiac MyBP-C with the deletion of this nine-amino acid domain was generated using the overlapping PCR method with flanking primers. All PCR products were sequenced completely and the fragments linked to the mouse α-myosin heavy chain promoter (α-MyHC) (Fig. 1A). The final constructs were digested free of vector sequence with NorI, purified from agarose gels and used to generate transgenic mice. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of health (NIH Publication No. 85-23, revised 1996).

2.2. Phosphorylation assays

Detergent-extracted cardiac myofibrils were prepared from frozen hearts. A protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, IN) was included in all isolation buffers. Only freshly prepared myofibrils samples were used. Ten μg of myofibril protein were used in the phosphorylation assays as described [15] with minor modifications. For the back-phosphorylation assays, myofibril proteins were incubated after adding [γ-\(^{32}\)P]ATP (15 μCi/reaction, reaction volume 15 μl), and the catalytic
Fig. 1. Transgenic constructs. (A) Two MyBP-C transgenes, encoding either the mouse cardiac wild type (MyBP-C.wt) or mutant (MyBP-C\A installation) proteins were linked to the α-MyHC promoter [29] and used to generate multiple lines of transgenic mice. The PKA phosphorylation domain residues within the MyBP-C motif near the N-terminus, which are lacking in the MyBP-C\A installation protein, are indicated. The two residues in the mouse sequence that differ from the human sequence are italicized. Both constructs contained a c-myc epitope sequence after the initiator methionine residue. The growth hormone polyA site (hGH; stippled boxes) was placed downstream of the cDNAs. (B) Levels of transcript over-expression relative to NTG littermates. RNA levels were quantitated as described previously [12]. (C) Western analysis of MyBP-C.wt and MyBP-C\A installation expression. Myofilament proteins were extracted [30], 5 μg loaded onto a 7% SDS-PAGE gel and transferred to nitrocellulose membranes for Western blot analyses using antic-myc monoclonal antibody to detect the TG proteins.

Subunit of cAMP-dependent protein kinase (PKA catalytic subunit, 30 U) for 50 min at 30°C. A series of preliminary experiments were done to ensure these conditions resulted in maximal 32P incorporation. Basal phosphorylation was minimal in the presence of calyculin A (0.1 mM) and Na3VO4 (1 mM). Boiling for 5 min in gel-loading buffer terminated phosphorylation. Control samples were incubated without the PKA catalytic subunit or with the PKA inhibitor, PKI15-24 (Sigma, St. Louis, MO). Proteins were then separated by 12% SDS-PAGE. Gels were fixed, stained with Colloidal Brilliant Blue and then subjected to autoradiography. Protein loading was normalized to actin, as quantified by scanning densitometry. Radioactive signal was quantitated using a StormImager860 (Molecular Dynamics, Sunnyvale, CA). Maximal PKA-dependent phosphorylation was determined by pretreatment with alkaline phosphatase (AP) followed by treatment with the PKA catalytic subunit and [γ-32P]ATP to rephosphorylate only the PKA sites. Samples were preincubated in F60 buffer (60 mM KCl, 30 mM imidazole, 7.2 mM MgCl2, pH 7.0), AP added (1:100 enzyme:protein) and the reaction carried out for 30 min at 37°C. Calyculin A and Na3VO4 were
added after dephosphorylation. Samples were heated at 65°C for 15 min to ensure the termination of the dephosphorylation reaction. Negligible amounts of 32P incorporation occurred in AP-treated samples that were not heated. The phosphorylation levels of phospholamban were also measured by PKA back phosphorylation using 20 μg of isolated sarcoplasmic reticulum [15].

2.3. Actomyosin Mg2+ ATPase assays

Myofibrillar actomyosin Mg2+ ATPase activity was determined by measuring inorganic phosphate release as described by Dobrowolski et al. [16] and Wattanapermpool [17] with slight modifications. Assays were carried out in 96-well microtiter plates at pCa2+ concentrations of 8.000–4.875 at room temperature during the linear P, release phase of the reaction.

3. Results

3.1. Generation of mice carrying MyBP-C transgenes

Two constructs were used in these studies (Fig. 1A). The full-length, wild-type MyBP-C cDNA was used previously to generate TG mice [12,13] and a cohort of these animals served as a control to rule out any epiphenomena arising merely as a result of TG expression of MyBP-C. Both lines were tagged with c-Myc such that the TG-encoded protein could be distinguished from endogenous MyBP-C. In comparing the mouse and human sequences for the protein’s cardiac isoform, we noted that the nine-amino acid residues comprising a serine phosphorylation site in the cardiac-specific sequences present in the amino termini were almost completely conserved. Subsequently, we generated a MyBP-C cDNA (MyBP-C9) in which these nine amino acids were deleted. The cDNA was then linked to the α-MyHC promoter [18] and used to generate TG mice. Three MyBP-C9 TG lines expressing the transgene at different levels were generated and the degree of expression relative to the endogenous MyBP-C transcripts was established (Fig. 1B). Modest differences were observed between the three lines, with transcript levels varying from 1.5- to 3.5-fold with respect to the endogenous mRNA. These expression levels were significantly below the level (5-fold) observed for the wild-type over-expressor. Western blot analyses of myofibril protein extracted from the TG lines (8–12 weeks) were carried out using anti-C-Myc monoclonal antibody in order to detect the TG proteins (Fig. 1C). The data confirm the correct size of the transgenically encoded polypeptides. The mutant MyBP-C protein appears to be stable and the protein levels from the three lines correspond roughly to their transcript levels (Fig. 1C). Based on standard curves performed with MyBP-C.wt, we estimate that the highest expressing lines (lines 156/157) resulted in approximately 30–40% substitution of endogenous MyBP-C with MyBP-C9.

Previous studies in which FHC mutations in MyBP-C were expressed in cardiomyocytes showed significant alterations in the pattern of protein incorporation in the sarcomere, as revealed by immunofluorescent staining using anti-C-Myc antibody, coupled with confocal microscopy [12,13]. In contrast with those results, MyBP-C9 was capable of incorporating into the A-band of the sarcomere in a pattern that was essentially indistinguishable from that of the endogenous protein (Fig. 2). There was no apparent disruption of sarcomeric structure, as detected by immunofluorescent staining (Fig. 2) and by standard light and electron microscopy (data not shown). Consistent with these data, when we determined the cell volume, profile area and length, the sarcomere length, as well as the transverse sectional area and cardiomyocyte minor and major diameter using Coulter™ Sizer analyses and computer-aided image analysis, no statistically significant differences presented (data not shown), indicating that, if any hypertrophic response occurs, it is subtle, not fully penetrant and certainly does not lead to any overt pathology.

3.2. Partial substitution with MyBP-C9 alters endogenous phosphorylation levels

To determine the in vivo consequences of the phosphorylation domain’s deletion in cardiac MyBP-C, we performed phosphorylation assays using MyBP-C9 MyBP-C.wt and NTG mice. As expected, both back and maximal PKA-dependent phosphorylation of myofibril samples from the MyBP-C9 mice were decreased (Fig. 3). Maximal phosphorylation (Fig. 3A), a measure of the total PKA-dependent phosphorylation sites in the protein, was decreased by 35%, which corresponds well to the estimated degree of replacement of the endogenous protein with the TG species. In the back phosphorylation assay, which gives one an estimate of the phosphorylated sites in the isolated MyBP-C protein pools, values obtained with the MyBP-C9 myofibril samples were decreased by approximately 65%, relative to the NTG and MyBP-C.wt samples (Fig. 3B). Thus, as expected, there are fewer sites on the total MyBP-C population available for PKA-dependent phosphorylation due to the partial substitution of endogenous MyBP-C with MyBP-C9. Additionally, the significant decrease in the back-phosphorylation studies (65%), relative to the decreases in the maximal phosphorylation values (35%), implies that the remaining, endogenous protein is highly phosphorylated, certainly more so than in the NTG and MyBP-C cohorts.

The phosphorylation states of other contractile proteins that function as potential regulators of contractility were also examined. Maximal phosphorylation of TnI was not decreased and maximal phosphorylation of phospholamban (PLB), derived from sarcoplasmic reticulum, was unaffect-
back phosphorylation studies showed that in the MyBP-C<sup>−/−</sup> mice significant decreases in both TnI and PLB presented (Fig. 3C). Since the back-phosphorylation assay reflects the degree of phosphorylation of the isolated proteins, the data indicate that these endogenous proteins also appear to be more highly phosphorylated relative to the MyBP-C.wt and NTG control cohorts. We next examined the ATPase activities present in the NTG, MyBP-C.wt and MyBP-C<sup>−/−</sup> experimental cohorts. While the MyBP-C.wt mice were statistically indistinguishable for the NTG controls, the MyBP-C<sup>−/−</sup> line showed a significant increase in maximal ATPase activity (Fig. 4). These results are consistent with previous data indicating that phosphorylation of MyBP-C decreased actin-activated myosin ATPase activity [19].

### 3.3. Cardiac function

In an attempt to detect functional changes that might occur in response to the partial inability of MyBP-C to respond to β-adrenergic stimulation, both Langendorff and working heart [20] preparations were carried out on the three experimental cohorts. In the mature adults (15–25 weeks), differences in contractility and relaxation (±dP/dt) presented (Table 1). The increases in contractility in the MyBP-C<sup>−/−</sup> mice are consistent with the increased ATPase values. However, when similar experiments were carried out in an intact, closed chest model, no differences in cardiac responses to β-agonist stimulation could be observed (data not shown). It should be noted, however, that the neural-humoral axis would be intact in those animals, and therefore it is likely that the system can compensate, at least during the short period (30–180 min) during which the experiment takes place. Maintenance of function may therefore reflect alterations in the phosphorylation levels of the other proteins able to participate in Ca<sup>2+</sup>-independent regulation of contractile function.

The above assays are all performed under acute conditions. We reasoned that intermittent cardiac stress over a prolonged period might point out some of the physiologica consequences of an altered response to β-adrenergic stimulation and the ability of the animals to perform during a stress exercise regimen was tested. Quantitation of performance during repeated trials on a motorized treadmill apparatus has proven to be a useful measurement of a transgenic animal’s capability to exercise [13,21]. Young adult (16 week) mice were acclimated to the treadmill over a 2-week period. They were then exercised for 50 min twice per week over a 5-week period (Fig. 5). Implantable telemetry detected no significant differences in the heart rates of the three experimental cohorts, either before, during or after exercise. However, consistent with the augmented function detected in both the ATPase and isolated working heart assays, the exercise capacity of the MyBP-C<sup>−/−</sup> mice was significantly increased as compared to the MyBP-C.wt and NTG controls (Fig. 5).
4. Discussion

In this study, we utilized a transgenic approach in dissecting structure function relationships in cardiac MyBP-C. As in previous experiments where a contractile protein is expressed in a cardiac-specific manner at high levels [12,13,18,22], the cardiomyocyte regulates the protein’s stoichiometry such that TG expression results, not in ‘over-expression’ but rather in a dose-dependent (partial) replacement of the endogenous protein with the transgenically encoded species. Emphasizing the fact that any observable phenotype is not simply an epiphenomenon of TG expression are the data obtained from the TG control cohort, in which wild-type MyBP-C is expressed at levels higher than those observed for the construct under study: in this case, MyBP-C^Δ9. Long-term diminished cardiac MyBP-C phosphorylation in vivo results in increased endogenous phosphorylation in at least three major cardiac proteins (MyBP-C, Tnl and PLB) that are involved in adrenergic regulation of cardiac contraction. Within the context of the isolated working heart, as well as the whole animal, statistically significant increases in performance...
were noted. The modifications are, for the most part, fairly benign and no noticeable pathology resulted nor could a hypertrophic response be detected at the molecular or cellular levels, even in older animals. These data confirm the hypothesis that cardiac MyBP-C phosphorylation, either directly or by effectively being coordinated with the phosphorylation of other controlling proteins such as PLB and TnI, plays an important in vivo role in regulating cardiac contraction.

4.1. Altering the phosphorylation status of the contractile apparatus via genetics

To date, biochemical methodology in which different target proteins are partially or completely extracted from myofibrillar samples, followed by the subsequent reconstitution of the contractile apparatus and measurement of the functional outcomes have been used to dissect the role that phosphorylation can play in regulating cardiac contraction [23–25]. While these studies are informative, functional outcomes at the whole organ and whole animal levels have not been possible. The transgenic approach, when carefully controlled, allows one to examine the long-term consequences in the animal of diminished phosphorylation of cardiac MyBP-C. It is important to note that the mutated protein is incorporated normally into the sarcomere at the A-band in a manner indistinguishable from the endogenous protein. We focused on lines in which only partial replacement (35%) of MyBP-C with MyBP-C<sup>Δ9</sup> protein occurred, in order to mimic modulation of phosphorylation levels as they occur in vivo. Removal of a subset of phosphorylation sites in MyBP-C resulted in increased endogenous phosphorylation levels in the remaining MyBP-C as well as in TnI and PLB and, in fact, may potentiate the heart’s ability to respond to chronic stress. These compensatory changes may partially explain the rather benign phenotype that resulted and the increases in certain performance parameters. Not only was there an absence of morbidity, mortality, pathology, and a hypertrophic response, but the animals actually demonstrated increased Mg<sup>2+</sup>-ATPase activity, increased ±dP/dt in the Langendorff and working heart preparations, and an increased exercise capacity as measured on the motorized treadmill.

At this point, we have no experimental data that directly address the compensatory processes that appear to operate in linking the phosphorylation status of the contractile proteins. Experiments that would illustrate directly the operative mechanisms are not currently feasible, as they will necessitate not only measuring the in vivo rates of both the kinases and phosphorylases but also their spatial locations. An alternative approach is to modify the potential phosphorylation status of those proteins that appear

### Table 1
<table>
<thead>
<tr>
<th>Isolated cardiac function</th>
<th>Control</th>
<th>MyBPC-P</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Working heart</strong></td>
<td>(n=5)</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats per min)</td>
<td>326±2</td>
<td>330±3</td>
<td>NS</td>
</tr>
<tr>
<td>+dP/dt (mmHg/ms)</td>
<td>5677±221</td>
<td>6952±137</td>
<td>+22***</td>
</tr>
<tr>
<td>−dP/dt (mmHg/ms)</td>
<td>4109±163</td>
<td>5148±205</td>
<td>+25***</td>
</tr>
<tr>
<td><strong>Langendorff heart</strong></td>
<td>(n=5)</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats per min)</td>
<td>312±5</td>
<td>336±5</td>
<td>+8**</td>
</tr>
<tr>
<td>+dP/dt (mmHg/ms)</td>
<td>2576±76</td>
<td>2852±38</td>
<td>+11**</td>
</tr>
<tr>
<td>−dP/dt (mmHg/ms)</td>
<td>1615±69</td>
<td>1813±38</td>
<td>+12**</td>
</tr>
</tbody>
</table>

S.E.M., standard error of the mean; NS, not significant.

**p=0.01; ***p=0.001, transgenic versus control, unpaired Student’s t-test.
to be affected in the MyBP-CΔ9 animals. Therefore, our current approach is to generate a series of animals in which the different phosphorylation sites are ablated, both in MyBP-C as well as in MLC2v, phospholamban, the L-type Ca²⁺ channel, TnI and TnT. As these animals are made the phosphorylation status of each of these mutations, both in isolation and as they are crossed to the other, transgenic animals whose contractile protein phosphorylation sites are modified, can be examined. In this manner, the tools needed to understand the mechanistic underpinnings of the compensatory process(es) can be prepared and used to determine the interplay between the different proteins and their relative degrees of PKA or PKC-mediated phosphorylation. Such an approach will rigorously test in vivo the role(s) of MyBP-C phosphorylation within the context of the post-translational modifications that the other contractile proteins can undergo. The structure–function relationships of these sites, both in the basal and hemo-

dynamically challenged states can subsequently be determined.

The results from this study demonstrate an important physiological role for cardiac MyBP-C phosphorylation and indicate that the phosphorylation states of three major cardiac proteins, which are adrenergically regulated, appear to be linked in some manner. Phosphorylation of cardiac MyBP-C appears to be operationally coordinated with other phosphorylation events in the cardiomyocyte’s contractile apparatus. Interactive regulation of the phosphorylation states of the three major cardiac proteins involved in adrenergic regulation could be achieved by a variety of mechanisms. These include circulating and/or local catecholamine levels, cAMP activity or PKA levels and/or activity, which, in turn, can be modulated by the phosphorylation status of the regulatory subunit [26]. Protein phosphatases 1 and 2A activity, which have both been implicated in dephosphorylation of cardiac myofibrillar proteins [27,28] may also play a potential regulatory role in determining the phosphorylation state of cardiac MyBP-C, TnI and PLB. In isolation, modulation of MyBP-C or TnI may not have that dramatic an effect. For example, previous studies showed that the time course of the positive inotropic response of isolated perfused hearts to β-adrenergic stimulation did not correlate with changes in TnI phosphorylation [25]. Undoubtedly, many different mechanisms operate together, both positively and negatively, to regulate the overall contractile state of the myocardium. Even though we are able to produce, either through gene targeting or transgenesis, a primary, unigenetic change, this invariably leads to a series of changes within the web of control points represented by the working heart. Because of this complexity, it will remain difficult to assess the relative contribution of any single mechanism to the overall cardiac response. A combination of inducible transgenesis and proteomic analysis approaches should prove useful in unraveling the combinatorial processes brought about as a result of the primary


genetic change that modifies the phosphorylation status of MyBP-C in particular, and myofibrillar proteins in general.

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