Multiple Forms of Cardiac Myosin-binding Protein C Exist and Can Regulate Thick Filament Stability

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Although absence or abnormality of cardiac myosin binding protein C (cMyBP-C) produces serious structural and functional abnormalities of the heart, function of the protein itself is not clearly understood, and the cause of the abnormalities, unidentified. Here we report that a major function of cMyBP-C may be regulating the stability of the myosin-containing contractile filaments through phosphorylation of cMyBP-C. Antibodies were raised against three different regions of cMyBP-C to detect changes in structure within the molecule, and loss of myosin heavy chain was used to monitor degradation of the thick filament. Results from Western blotting and polyacrylamide gel electrophoresis indicate that cMyBP-C can exist in two different forms that produce, respectively, stable and unstable thick filaments. The stable form has well-ordered myosin heads and requires phosphorylation of the cMyBP-C. The unstable form has disordered myosin heads. In tissue with intact cardiac cells, the unstable unphosphorylated cMyBP-C is more easily proteolyzed, causing thick filaments first to release cMyBP-C and/or its proteolytic peptides and then myosin. Filaments deficient in cMyBP-C are fragmented by shear force well tolerated by the stable form. We hypothesize that modulation of filament stability can be coupled at the molecular level with the strength of contraction by the sensitivity of each to the concentration of calcium ions.

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INTRODUCTION

Although absence or abnormality of myosin binding protein C (cMyBP-C) from the hearts of rodents and humans produces structural abnormalities (cardiac hypertrophy and dilatation, patchy myofibrillar disarray, and fibrosis) and serious dysfunction (reduced cardiac ejection, impaired diastolic filling, and reduced capacity to function with increased load) (Bonne et al., 1995; Rottbauer et al., 1997; Charron et al., 1998; Niimura et al., 1998; Yang et al., 1998, 2001; McConnell et al., 2001; Harris et al., 2002; Sadayappan et al., 2005, 2006), function of cMyBP-C is not clearly understood, and the cause of the abnormalities, unidentified (Watkins et al. 1995). One cMyBP-C consists of 11 domains or modules labeled C0 to C10 from N to C terminus (Fig. 1). Near its C terminus, cMyBP-C has binding sites for titin and the myosin rod, and at the N-terminal region, binding sites for actin (Kulikovskaya et al., 2003a; Squire et al., 2003; Herron et al., 2006) and the S2 fragment of myosin (Kunst et al., 2000). Three molecules of cMyBP-C are localized every 43 nm in the C zone of the sarcomere (Craig and Offer, 1976). Modules C5–C10 from the three molecules appear to interact, possibly resulting in a transverse "collar" around the thick filament that stabilizes the filament's structure (Moolman-Smook et al., 2002; McClellan et al., 2004). Three phosphorylation sites exist between modules C1 and C2, and the degree of phosphorylation determines whether the N-terminal region binds to myosin or actin (Hartzell and Glass, 1984; Schlender and Bean, 1991; Gautel et al., 1995; Kulikovskaya et al., 2003a). Dephosphorylation of cMyBP-C changes the structure of cardiac thick filament, resulting in a shift of myosin heads from well-ordered positions along the backbone of the filament to a disordered array extending from the backbone (Weisberg and Winegrad, 1996, 1998; Levine et al., 2001). Either Ca-calmodulin–activated kinase (CAMK) or PKA can phosphorylate isolated cMyBP-C (Schlender and Bean, 1991; McClellan et al., 2001; Gautel et al., 1995), but in intact cardiac muscle, isoproterenol can produce phosphorylation only if the first phosphate is already present (McClellan et al., 2001).

Cessation of contraction of intact cardiac trabeculae reversibly reduces maximum Ca-activated force (Fmax) and actomyosin ATPase activity while maximum unloaded shortening velocity remains unchanged (Lin et al., 1991). This suggests that the number of cycling force generators can be modified by contractile activity. The reduction can be reversed by any of several methods each of which increases intracellular Ca concentration and may also phosphorylate myofibrillar proteins. The only myofibrillar phosphorylation that consistently followed the change in force and ATPase activity in direction and amplitude was that of cMyBP-C (McClellan et al., 1994).

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Abbreviations used in this paper: CAMK, Ca-calmodulin–activated kinase; cMyBP-C, cardiac myosin binding protein; TNT, tropomyosin binding subunit of troponin.

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The rate of degradation of myosin in rhythmically contracting, cultured neonatal ventricular cells is closely related to the concentration of Ca in the extracellular space (Byron et al., 1996). The rate of degradation moves inversely with the concentration of Ca. The site of action of the Ca is intracellular because Ca channel blockade increases the rate of degradation. Ca does not appear to exert its effect on degradation of myosin by altering tension development. The addition of 2,3-butanedione monoxime, which inhibits development of Ca-activated tension in striated muscle (Zhao et al., 1995), does not prevent the effect of Ca on the rate of degradation. Stimulated by the results of Byron et al. (1996), we have tested the hypothesis that cMyBP-C and its phosphorylation modulate the stability of the myosin-containing thick filaments. These studies have been conducted on wild-type (WT) rat hearts in order to investigate the function of cMyBP-C in the normal heart.

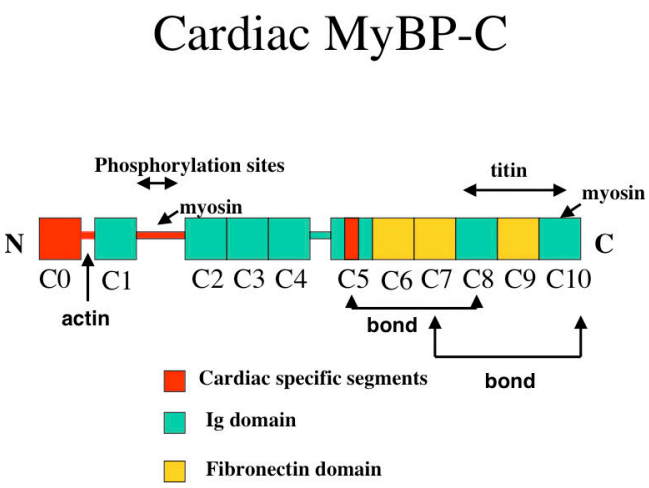

Figure 1. Diagram of cMyBP-C indicating modules, interactions with actin, myosin, and titin and interactions between modules. Location of phosphorylation sites is shown.

MATERIAL AND METHODS

Antibodies

Polyclonal antibodies raised against recombinant C0C1, C0C2, C5, and C8C9, (Fig. 1 and Fig. 2 A) produced from human cDNA encoding cardiac MyBP-C (cMyBP-C). The antibodies were tested for specificity against lysed rat cardiac ventricle that had been quickly frozen immediately after the chest had been opened. This tissue was chosen because it most closely resembled the in vivo state of the heart. The tissue was lysed and electrophoresed on polyacrylamide gels, and three Western blots were prepared using each of the different antibodies against cMyBP-C (McClellan et al., 2004). A single band was present at 145 kD in each blot of the quickly frozen muscle (Fig. 2 A).

Phosphorylation of cMyBP-C

PKA-mediated phosphorylation of MyBP-C was measured using an antibody against phosphorylated PKA substrate sites (Cell Signaling Technology phospho-serine/threonine substrate antibody #2851; referred in text as anti-phosphate). The antibody recognizes PKA substrate that has already been phosphorylated. Appropriate controls established that cMyBP-C was specifically stained by the antibody. Although CAMK normally adds the first phosphate to cMyBP-C in the intact filament lattice (Hartzell and Glass, 1984; Schlender and Bean, 1991; McClellan et al., 2001), PKA can add the first phosphate to isolated cMyBP-C and to the C1C2 fragment that contains the phosphorylation sites (Gautel et al., 1995). On denaturing gels, the antibody against PKA-phosphorylated sites recognizes mono-, di-, and triphosphorylated cMyBP-C (not depicted). Therefore it recognizes the CAMK-mediated phosphorylated site under these conditions. The absence of staining with the antibody on Western blots means that none of the three phosphorylateable sites was actually phosphorylated.

Skinned Trabeculae

Treatment of animals in all studies was in accordance with the guidelines of the University of Pennsylvania Animal Care Committee. All experiments were conducted at room temperature, 20–22°C. Trabeculae were chemically skinned with 1% Triton X-100 in a relaxing solution using solutions and protocols already described (Lin et al., 1991). The skinning solution was washed out with several changes of relaxing solution over 30 min. All solutions were collected and analyzed by SDS-PAGE followed by Western blotting with antibodies against cMyBP-C and MHC. Neither protein was found in either the skinning or the washout solutions with this sensitive technique.

Resistance to Shear Force

A solution of isolated thick filaments was passed through a capillary tube of measured diameter and length under a measured pressure. The shear force was calculated from the dimensions of the tube and the pressure. As the aim of this part of the study was to determine if loss of cMyBP-C altered the resistance of thick filaments to shear force, the study was conducted at only two levels of shear force covering a range of 2–3-fold.

Isolated Thick Filaments, Western Blots, IEF Gels

The methods for isolating thick filaments and preparing IEF gels and Western blots have been described in detail (Weisberg and Winegrad, 1996; Levine et al., 2001).

Data Analysis

The density of individual bands in gels and Western blots was determined by scanning densitometry and the NIH Image program (Fig. 2, A–C). Calibration curves for the density produced by cMyBP-C stained with each of the three antibodies were generated using different amounts of purified cMyBP-C and different amounts of the lysate of quickly frozen cardiac muscle (Fig. 2). The curves were the same nonlinear power functions with the two methods.

Tropomyosin binding subunit of troponin (TNT) was present in every sample of cardiac muscle and was not altered by the protocols employed in this study (see Fig. 8). When the same amount of protein from lysates of heart muscle was loaded on gels, the density of the TNT band was highly reproducible. These properties made TNT an excellent candidate protein for normalization of the cMyBP-C bands and quantitating changes in the amount of cMyBP-C.

Equal amounts of protein from experimental and control hearts were loaded on the lanes of gels. The ratio of the densities of the cMyBP-C to the TNT band from experimental tissue was compared with the ratio under control conditions (quickly frozen cardiac muscle). Deviations from the control ratio indicated an effect of the experimental protocol on the amount of cMyBP-C. The effect was quantified by determining the correct position for the control and experimental densities on the calibration curve.

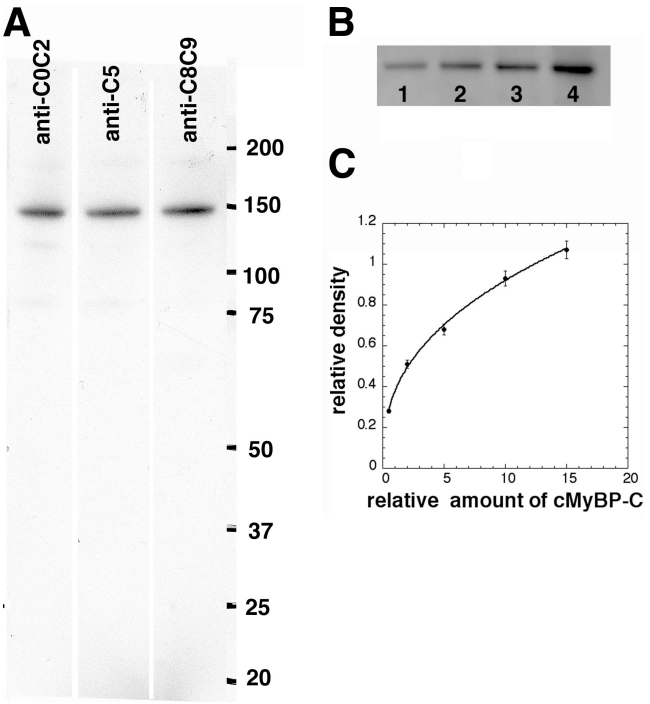

Figure 2. (A) Western blots showing the specificity of antibodies against of cMyBP-C fragments. The antibodies were tested against the lysate of a piece of ventricle rapidly frozen as soon as the chest had been opened. (B) Blots using anti-C5 with different amounts of lysate of quickly frozen heart. Lanes 1–4 contain 1, 4, 10, and 20 relative amounts of protein. (C) Calibration curve of cMyBP-C. Each point represents the mean of at least five muscles. Dose–response plots using anti-C0C2 and anti-C8C9 (not depicted) were very similar to the relation using anti-C5.

With control tissue, the density on the curve for cMyBP-C equaled the actual measured density of the cMyBP-C band when the standard amount of protein was loaded on the gel. The amount of protein was chosen to place the density on the relatively linear portion of the calibration curve. Provided the measured densities of TNT from the control and the experimental tissues were the same (as they normally were), the correct position on the calibration curve for the density of cMyBP-C from the experimental tissue was the actual measured density on the gel. In this way the difference between the control and experimental amounts of cMyBP-C was determined, and possible confounding effects of the nonlinearity of the calibration curve were eliminated. All quantitative values given in the Results have been normalized in this way.

TNT was not present in the washout of skinned trabeculae and therefore could not be used for normalization in measurements of cMyBP-C and myosin heavy chain (MHC) in these solutions. The amount of the washouts applied to the lanes of the gel was set so that the amount of cMyBP-C or MHC produced a density similar to those of our standard control on the same gel. The relative amounts of cMyBP-C and MHC were determined from the calibration curves.

Staining and conditions for exposure of the film to the gel were kept constant.

Statistics

Statistical significance was determined by the Student's *t* test and ANOVA, with $P < 0.05$ considered to be significant. Where appropriate, the Bonferroni correction was made.

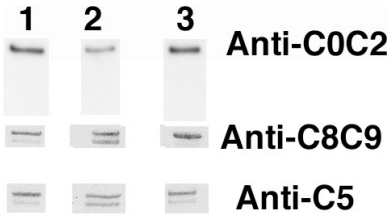

Figure 3. Western blots of rat heart using three antibodies. Lane 1, quickly frozen tissue from a heart of a control animal (had not received propranolol); lane 2, quickly frozen tissue from a heart that had received propranolol before euthanasia; and lane 3, quickly frozen tissue from heart of an animal that had received propranolol before euthanasia, and after removal from the heart had been soaked in propranolol-free medium for 15 min followed by medium with 0.1 μM isoproterenol for 15 min.

RESULTS

Multiple forms of cMyBP-C in the whole animal

Western blots of lysed rat ventricle quickly frozen immediately after euthanasia were produced using antibodies against C0C2, C5 and C8C9 fragments of cMyBP-C. Each blot had a dense band with an apparent molecular weight of 145 kD (Fig. 2 A) and, in some cases, a faint suggestion of a band at 130 kD in the anti-C5 and anti-C8C9 blots (Fig. 3). During euthanasia of a rat there is a large release of catecholamines that increases phosphorylation of cMyBP-C. This release of catecholamines can be blocked by intraperitoneal injection of the β adrenergic blocker, propranolol. When 10 μg/kg of propranolol was injected into the peritoneal cavity 20 min before euthanasia to block the action of the catecholamine, Western blots with anti-C5 and anti-C8C9 produced two clear, separate bands, one at 145 kD and a second at 130 kD (Fig. 3). There was no staining of a 130-kD band with anti-C0C2. The density of the single 145-kD band stained with anti-C0C2 was significantly reduced by the propranolol injection, suggesting that some cMyBP-C existed in a form not stained by that antibody. In every one of 17 experiments the second band disappeared after washing out the propranolol and incubating the intact trabecula in 0.1 μM isoproterenol for 15 min (Fig. 3). These results indicate that under normal physiological conditions two different forms of cMyBP-C under adrenergic control can exist in vivo and the conversion of one to the other can occur within minutes.

Beta adrenergic agonists cannot cause phosphorylation of cMyBP-C in intact cardiac cells unless one phosphate is already present (McClellan et al., 2001). A similar restriction exists on the conversion of the 130- to the 145-kD form by β adrenergic agonists. 0.1 μM isoproterenol was added to the bathing solutions of trabeculae that had been soaked in 1.0 or 2.5 mM Ca for 2 h to produce, respectively, low and moderately high levels of phosphorylated cMyBP-C (McClellan et al., 2001). The drug increased the density of the 145-kD band where a major

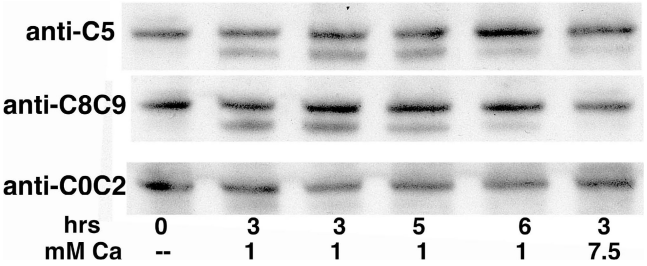

Figure 4. Western blots with three antibodies against different regions of cMyBP-C. The protocols (duration of soak and Ca concentration) for the various muscles are shown. Note the single bands with quickly frozen trabeculae, and the second band with antibodies against C8C9 and C5. The second band is not present when the bathing solution contained 7.5 mM Ca. The density of the second band increases over the first 3 h in 1.0 mM Krebs' solution and then gradually becomes less dense. Note the reproducibility in lanes 2 and 3.

fraction (>60%) of cMyBP-C was phosphorylated before the addition of the drug and had much less effect where only a minor fraction of the cMyBP-C (<20%) was phosphorylated (unpublished data).

The 130-kD protein was not a proteolytic fragment of cMyBP-C. The N-terminal sequence of the 130-kD band (PEPGKRPVSA), identified by Edman degradation, was the same as the sequence in human, mouse, and dog cMyBP-C except for substitution of arginine for lysine at position 6, both of which carry a negative charge (xPASy database). It was not possible to sequence the N terminus of the 145-kD band because the protein appeared to be blocked to Edman degradation. Proteolysis of C10 and/or C9 at the C terminus was an unlikely cause of the more rapid migration on the gel because of the following: (a) anti-C8C9 still recognized the 130-kD form and anti-C0C2 did not; (b) rapid reversibility of the migration pattern; and (c) the rapid change in density of the 145- and 130-kD bands after exposure to isoproterenol. The increase in the density of the 145-kD bands and the decrease of the 130-kD bands occurred too rapidly for in vitro protein synthesis. Restoration of cMyBP-C after the proteolysis that occurs during transient ischemia requires ~10 d (Decker et al., 2005). Very small changes in a protein can produce changes in its rate of migration on SDS gels that normally would indicate a larger change in molecular weight (Bottinelli et al., 1998; Katori et al., 2004). This is particularly true of phosphorylation (Burden and Sullivan, 1994).

Effect of [Ca] on the two forms of cMyBP-C

The density of both bands in gels from resting trabeculae varied with the concentration of Ca in the superfusion solution and the duration of incubation (Figs. 4 and 5; $n = 16$; $P < 0.05$). Trabeculae frozen within 3 min of euthanasia produced the same single band as quickly frozen ventricles. After 2 or more hours at rest

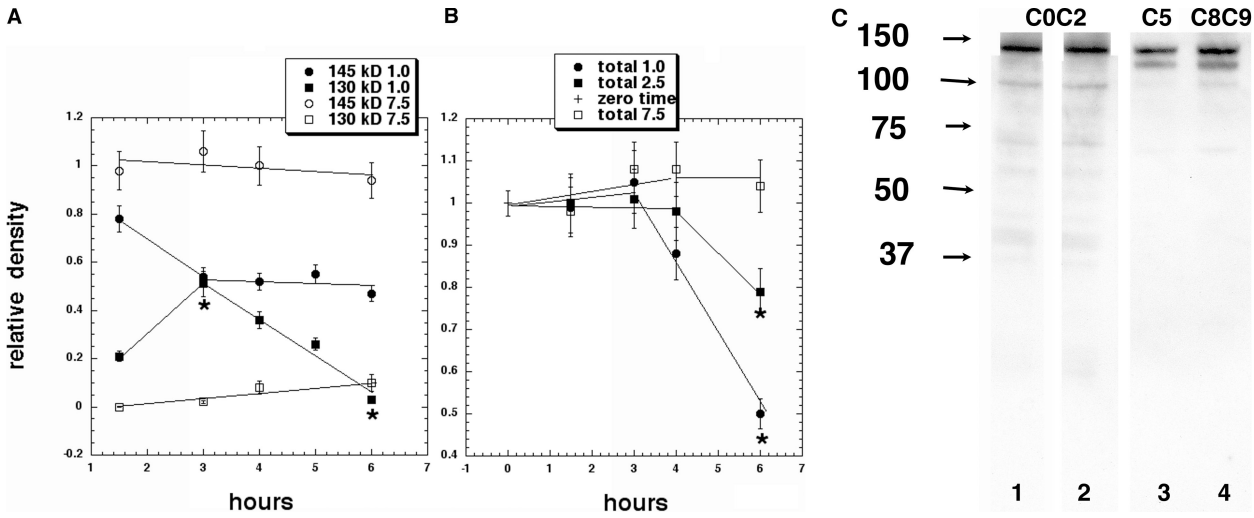

Figure 5. (A) Densities of 145- and 130-kD forms plotted as a function of the duration of incubation of the trabeculae in 1.0 or 7.5 mM Ca. Each point represents the mean ± SEM of at least five experiments. The antibody against C5 was used in the Western blots. Results with the antibody against C8C9 were not statistically different (not depicted). Lines between points are interpolations and do not represent a specific function. Open circles 145 kD in 7.5 mM Ca, open squares 130 kD in 7.5 mM Ca, filled circles 145 kD in 1.0 mM Ca, and filled squares 130 kD in 1.0 mM Ca. Asterisk indicates statistical significance from 1.5 h. (B) Plot of combined densities of 145- and 130-kD forms as a function of the duration of the incubation in electrolyte solution with 1.0, 2.5, or 7.5 mM Ca. Asterisks indicate significant difference from value at 3 h. Lines between points are interpolations and do not represent a specific function. Filled circles 145+130 kD in 1.0 mM Ca, filled squares 145+130 kD in 2.5 mM Ca, and open squares 145+130 kD in 7.5 mM Ca. (C) Comparison of peptides in lysates stained with each antibody after 4 h incubation in 1.0 mM Ca. Density of TNT bands after SDS-PAGE, stained with a specific antibody and used for normalization, was the same in all three lanes (not depicted). Note the larger number of peptides stained with anti-C0C2 than with either anti-C5 or anti-C8C9.

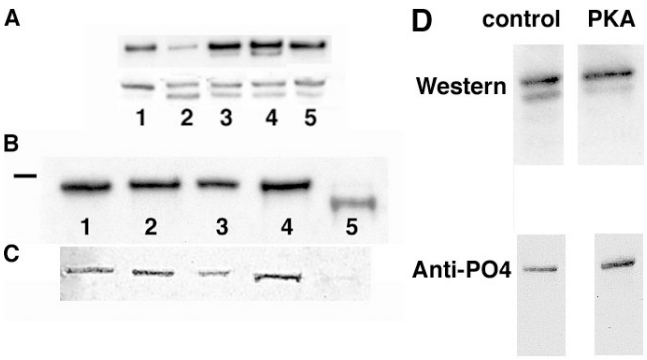

Figure 6. (A) Blots of antibody against C5 (bottom) and phosphorylated PKA substrate (top). The same gel is shown. Lane 1, quickly frozen; lane 2, 4 h in 1.0 mM Ca; lanes 3 and 4, stimulated at 12/min for 30 min in 2.5 mM Ca after 3 h at rest in 2.5 mM Ca; lane 5, soaked for 5 h in 7.5 mM Ca. The 130-kD band is absent from the anti-PKA substrate Western except where the trabecula was rhythmically contracting (lanes 3 and 4). (B) The effect of total dephosphorylation on the conversion of the 145- to the 130-kD form. Western blots with anti-C8C9 (lanes 1–5) of lysed cardiac muscle that had been quickly frozen. In lane 5, the same lysate has been incubated with 10 U alkaline phosphatase for 4 h. Lane 1, alkaline phosphatase buffer (Promega); lane 2, urea-thiourea-CHAPS (UTC) lysate; lane 3, pellet in lithium dodecyl sulfate; lane 4, UTC lysate; lane 5, UTC lysate (same as lane 1) incubated at 37°C for 4 h with calf intestinal phosphatase (Promega). Marker on left is 150 kD. (C) Shows the same proteins after incubation with anti-PKA substrate phosphate. (D) Phosphorylation converts 130- to 145-kD form. Controls are isolated thick filaments prepared from of trabecula soaked for 2 h in 1.0 mM Ca to dephosphorylate cMyBP-C. PKA are thick filaments from same preparation exposed to 30 U of the catalytic subunit of PKA for 90 min. Western blots were stained with anti-C5 and the anti-PO_4 with antiphosphate antibody.

in 1.0 mM Ca (during which bound catecholamine was washed out), Western blots of trabeculae produced the same bands as hearts from propranolol-injected animals. Trabeculae soaked at rest in 7.5 mM Ca for up to 6 h had little or no 130-kD form and a constant density of their 145-kD form (Fig. 4). Decreasing Ca concentration from 7.5 to 1.0 mM produced a 130-kD band and decreased the 145-kD band. Raising Ca from 1.0 to 7.5 inhibited further change in the two bands, but it did not significantly reverse the changes that had already occurred in 1.0 mM ($n = 4$, $P < 0.05$).

Density of both bands changed with the duration of the exposure to 1.0 mM Ca (Figs. 4 and 5). For 3 h the density of the 130- and 145-kD forms, respectively, increased and decreased, but the combined densities remained constant (Fig. 5 A). The intensity of the 130-kD band increased to a peak at 3 h, and then declined with further superfusion (Fig. 5). After 3 h, the combined densities decreased (Fig. 5 B). 2.5 mM Ca produced a similar course, but the magnitude of the changes was smaller.

Proteolysis of the 130-kD Form of cMyBP-C

The sum of densities of the 145- and 130-kD forms remained constant for 3 h regardless of the Ca concentration in the soak solution (Fig. 5 A). After >3 h in 1 mM Ca, the amount of the 130 kD and the sum of the two densities gradually decreased (Fig. 5 B). Initially the 145 kD was converted to the 130-kD form, but after 3 h, proteolysis of the 130-kD form became apparent. Lower molecular weight bands stained by the antibodies against cMyBP-C appeared (Fig. 5 C). Most of the visible smaller peptides were stained by anti-C0C2 and not by anti-C5 or -C8C9, indicating that the proteolysis was probably occurring in the C5 to C10 region, which forms a putative collar around the thick filament.

The ability of anti-C0C2 to stain proteolytic fragments of the 130-kD form of cMyBP-C supports the conclusion from sequencing that the N-terminal region has not been lost in the conversion of the 145- to the 130-kD form. The proteolysis in the C5C9 region has apparently had an effect on the epitope in the C0C2 region enhancing the recognition of the protein by the anti-C0C2, consistent with other data that events in one end of the molecule can influence the nature of the other end of the molecule (McClellan et al., 2004).

Differences in Phosphorylation in the Two Forms of cMyBP-C

Anti-phosphate antibody measures total phosphate and cannot distinguish among the three phosphorylation sites. The antibody always stained the 145-kD band and with one exception never stained the 130-kD band (Fig. 6). The extent of antiphosphate antibody staining varied considerably depending on prior treatment. Incubation with 7.5 mM Ca, which prevented the appearance of the 130-kD band, was associated with a high level of phosphorylation. A 2-h soak in 1 mM Ca reduced antiphosphate staining by >50%.

Incubation with 10 U of alkaline phosphatase for 4 h reduced phosphorylation to an undetectable level and converted all of the 145-kD form to the 130-kD form (Fig. 6, B and C). Incubation with 3 U decreased the phosphate content of cMyBP-C by 64 ± 7% ($n = 4$, $P < 0.05$), and converted 23 ± 4% ($n = 4$, $P < 0.05$) of the 145- to the 130-kD form. After incubation of a preparation having a low level of phosphorylation of cMyBP-C (as a result of incubation in a 1.0 mM Ca solution) with PKA, the level of phosphorylation and the relative amount of the 145-kD form were increased substantially (Fig. 6 D).

Effect of Contraction on the Two Forms of cMyBP-C

After 3 h of superfusion at rest in 2.5 mM Ca, several trabeculae were stimulated for 30 min at 12 Hz. The stimulation, which would be expected to raise time-averaged concentration of intracellular Ca, increased antiphosphate staining of the 145-kD band by 42 ± 6% ($P < 0.05$; $n = 5$), and produced the only detectable staining of the 130-kD band. A portion of the 130-kD band was converted to the 145-kD band. The relative intensity of the 145-kD band increased by 37 ± 5%

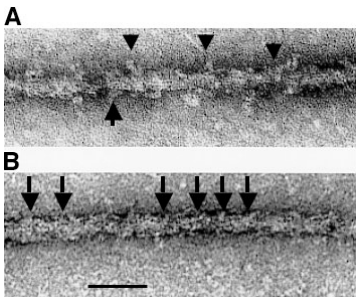

Figure 7. Electron micrographs of a part of two isolated thick filaments. (A) From a trabecula that had been soaked in 1.0 mM Ca to produce dephosphorylated cMyBP-C and a band at 130 kD with anti-C5 or anti-C8C9. Arrowheads point to irregular, extended myosin heads. (B) From a trabecula that had been soaked in 7.5 mM Ca to produce phosphorylated cMyBP-C and prevent a band at 130 kD with anti-C5 or anti-C8C9. Arrows point to regular myosin heads lying along filament backbone. Line indicates 70 nm.

($n = 5$, $P < 0.05$) and the intensity of the 130-kD band decreased (Fig. 6 A). Since it was not possible to stimulate isolated trabeculae at in vivo rates, we could not ascertain whether physiological rates of stimulation would produce greater phosphorylation and transition between the two forms.

Ultrastructure of Thick Filaments

Isolated, negatively stained thick filaments had different structures depending on which form of cMyBP-C was predominant (Fig. 7). Populations of predominately the 130- or the 145-kD form were produced by using, respectively, 1.0 or 7.5 mM Ca in the soak solutions. Thick filaments with the 130-kD form had disordered myosin heads that were often extended at different angles to the filament backbone (Fig. 7 A). With the 145-kD form, myosin heads were well ordered, lying uniformly along the backbone of the filament (Fig. 7 B).

Loss of MHC Follows Loss of cMyBP-C

Trabeculae were chemically skinned to permit control of the microenvironment of the myofibrils, to detect changes in the composition of the contractile filaments, and to determine whether the cMyBP-C or its proteolytic fragments were bound to myofibrils. Bound protein remained with the skinned cardiomyocytes while protein not bound in the cell interior appeared in the soak solution.

After dissection, intact resting trabeculae were soaked in electrolyte solution containing either 1.0 or 7.5 mM Ca for 2 h to produce cells with respectively low (<20%) and high (>80%) levels of phosphorylation of cMyBP-C. During and immediately after their skinning, there was no loss of myosin or cMyBP-C in the several bathing solutions, indicating that no cMyBP-C or MHC had been removed from the trabeculae. 4 h after skinning, the relaxing solution that had bathed trabeculae from the

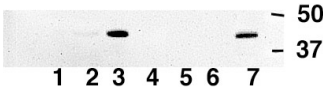

Figure 8. Western blot of soak solutions from skinned trabeculae using anti-TNT. Lane 1, relaxing solution after 4 h; lane 2, relaxing solution after 18 h; lane 3, lysate of relaxed trabecula after 4 h; lane 4, contraction solution used 3 times; lane 5, relaxing solution used for 3 h between contractions; lane 6, relaxing solution used for 15 h after three series of contraction solutions; lane 7, lysate of trabecula that had contracted. The entire incubation solution was concentrated for use in the blot, but only 1/3 of the lysate was used in the blot.

7.5 mM Ca group contained 3 ± 0.4% of the total cMyBP-C ($P < 0.05$) and 1% of myosin heavy chain (MHC; $P > 0.05$; the reference value for 100% protein was the total amount of each protein in trabeculae after their skinning). There was no statistically significant change in the cMyBP-C or MHC content of the trabeculae. Over the same period in relaxing solution, trabeculae from the 1.0 mM Ca group lost significantly more cMyBP-C and MHC, respectively, 18 ± 3% cMyBP-C and 6 ± 2% MHC ($n = 6$; $P < 0.05$). These washouts of cMyBP-C and MHC were accompanied by significant declines in the content of cMyBP-C and MHC in the trabeculae of, respectively, 23 ± 5 and 8 ± 4% ($P < 0.05$). The total cMyBP-C lost may have been underestimated by this method because washout solutions contained low molecular weight peptides stained by antibodies against regions of cMyBP-C. TNT was not present in any soak solutions, indicating that the instability was limited to the thick filament (Fig. 8).

After extraction of ~20% of the total cMyBP-C, release of MHC began (Fig. 9 A). Release of MHC always followed, never preceded or accompanied, the initial extraction of cMyBP-C (Fig. 9 B). Thick filament stability appeared to exist until the filaments had lost at least 20% of cMyBP-C. The rate at which cMyBP-C was extracted was faster when the trabeculae had been exposed to 1.25 mM than to 2.5 or 7.5 mM Ca, but the threshold of cMyBP-C loss at which the loss of MHC began was not significantly different.

Isolated thick filaments were much less resistant to shear forces after a large fraction of cMyBP-C has been extracted (Kulikovskaya et al., 2003b). Filaments with 35% of cMyBP-C extracted (using the protocol of Kulikovskaya et al., 2003b) began to fragment at ~17 dynes/cm^2. Thick filaments with a normal complement of cMyBP-C were able to withstand shear forces at least two to three times higher without fragmenting (Fig. 10). The length of the fragments varied from over 0.8 μm to the dimensions of individual double-headed myosin molecules.

Thick filaments were isolated from trabeculae that had been bathed at rest for 2 h in 1.25 mM Ca solutions to produce a low level of phosphorylation of cMyBP-C

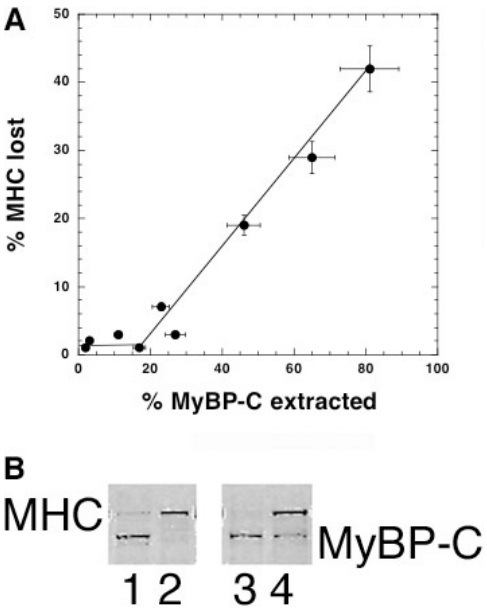

Figure 9. (A) Relation between cMyBP-C extracted and MHC lost. Each point represents the mean of at least four different experiments. Lines between points are interpolations and do not represent a specific function. (B) Western blots with anti C0C2 (bottom) and anti-MHC (top). Lane 1, extraction solution for 4 h from skinned trabeculae; lane 2, relaxing solution for the next 14 h from the same trabecula; lane 3, extraction solution for 4 h; lane 4, the extraction solution from the same trabecula for an additional 14 h.

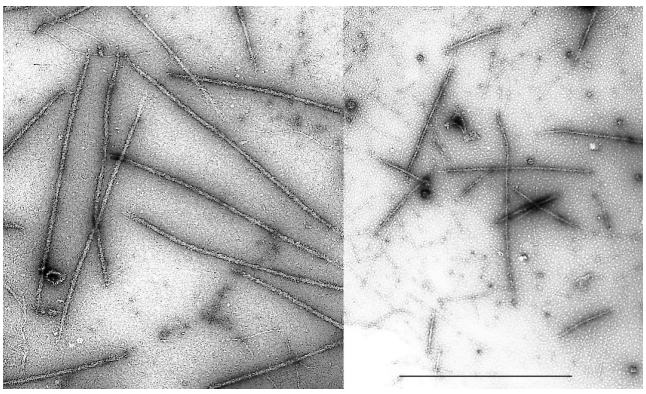

Figure 10. Left, electron micrographs of negatively stained, isolated thick filaments with a normal content of cMyBP-C; right, filaments from a trabecula that had had 36% of its cMyBP-C extracted. Both sets of filaments had been subjected to the same shear force of ~17dynes/cm^2. Note the intact thick filaments with central bare zones and tapered ends when cMyBP-C content is normal and the fragmented thick filaments where cMyBP-C had been extracted. Line indicates 1 μm. The magnification for the two micrographs is the same. The thinner images in the fragmented filaments indicate that degradation of the thick filaments deficient in cMyBP-C is not simply a fracture through the entire filament.

(McClellan et al., 2001; Kulikovskaya et al., 2003b) and then subjected to the same level of shear force as extracted filaments. These thick filaments fragmented into pieces that were similar to those in the preparation of extracted filaments. As only a limited range of shear force was used, it was not possible to determine whether dephosphorylation of cMyBP-C produces the same degree of filament instability as extraction. (This point is currently being studied using a more quantified level of shear force and a larger population of thick filaments.)

DISCUSSION

Multiple Forms of cMyBP-C

In the intact heart in vivo, cMyBP-C can exist in at least two different forms, identifiable from the different migration on SDS gels and the different binding patterns of antibodies. One is phosphorylated, relatively resistant to proteolysis, and associated with greater stability of thick filaments (145-kD form). The other is unphosphorylated, relatively sensitive to protease, and associated with lower stability of thick filaments (130-kD form). Different electron micrograph images of thick filaments with the two different forms of cMyBP-C were observed. In the presence of the 145-kD form, myosin heads lay along the thick filament backbone and were well ordered, but with the 130-kD form the myosin heads were extended and disordered.

In both intact and skinned cells, the 145-kD form can be converted completely to the 130-kD form by dephosphorylation of cMyBP-C, and the 130-kD form can be converted to the 145-kD form by phosphorylation with PKA. The 130-kD form in the in situ contracting heart and in vitro trabeculae can be converted to the 145-kD form by isoproterenol, which both raises intracellular Ca and phosphorylates cMyBP-C, and by rhythmic contractions. Even when contractile activity ceases, the 145-kD form persists as long as elevated extracellular Ca concentration maintains phosphorylation. In in vitro cardiac muscle, contractile activity alone could not produce complete phosphorylation of cMyBP-C, presumably because the slow rate of contraction did not raise intracellular Ca concentration sufficiently (Gautel et al., 1995; McClellan et al., 2001). The rate at which these transitions occur is sufficiently fast to modulate cardiac function but not on a beat-to-beat scale. The rate is consistent with the rate of phosphorylation and dephosphorylation of cMyBP-C (McClellan et al., 2001) and the rate of change in the structure of isolated thick filaments associated with phosphorylation (Weisberg and Winegrad, 1998).

Although the data show that a Ca-regulated phosphorylation is necessary for the transition, they do not indicate how much phosphorylation is required, whether it is one, two, or three phosphates per molecule of cMyBP-C. The data also do not distinguish between the consequence of multiple phosphates on a single molecule and monophosphorylation of several molecules per thick filament. These questions are currently being actively investigated

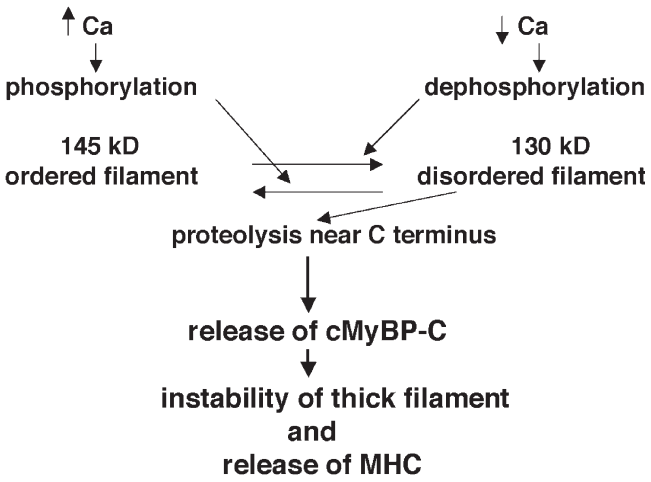

Figure 11. Diagram showing our working hypothesis for in vivo function of the phosphorylation-dependent modulation of thick filament stability. In vitro evidence for each of the steps has been presented in this study. Confirmation of a similar function in vivo will require further study.

in our laboratory. According to Moolman-Smook et al. (2002), three cMyBP-C molecules form a double-stranded collar around the thick filament every 43 nm within the C zone. Removal of one molecule of cMyBP-C should leave a single-stranded collar, but loss of two molecules at a given locus would interrupt the integrity of the collar at that location. Loss of myosin from the thick filament commences only after cMyBP-C content has been reduced by ~20% as would be expected if the integrity of at least one ring of the collar is required for filament stability.

The significance of the two forms of cMyBP-C and the transitions between them become clear when the mechanism of phosphorylation of cMyBP-C is considered. cMyBP-C has three phosphorylation sites, but a specific one appears to require CAMK and to be phosphorylated first before the other two can be phosphorylated (Schlender and Bean, 1991; Gautel et al., 1995; McClellan et al., 2001). The concentration of Ca for Ca-sensitive phosphorylation of cMyBP-C is less than for activation of contraction, but higher than exists in a resting cardiac cell bathed in normal extracellular concentration of Ca (McClellan et al., 2001). Therefore the resting cell should have a low level of phosphorylation of cMyBP-C, and respond weakly to PKA activation by β adrenergic agonists. Experimental results support both predictions (McClellan et al., 2001). Either elevated extracellular Ca or contraction at a rate in the physiological range produces phosphorylation and permits further phosphorylation from β adrenergic stimulation.

The effect of Ca concentration on thick filament degradation in these studies appears to be very similar to the role of Ca in regulating myosin degradation in cultured neonatal cardiomyocytes (Bryon et al., 1996). In both studies, by a mechanism that does not require activation of contraction, raising extracellular Ca reduces myosin degradation. Raising Ca concentration increases phosphorylation of cMyBP-C (McClellan et al., 2001), which inhibits its proteolysis. The peptides produced by proteolysis in this study are stained by anti-C0C2 but not with anti-C5 or -C8C9, indicating that the cleavage has occurred in the C5C10 region. Proteolysis in this part of the cMyBP-C molecule should weaken interactions among the three cMyBP-C molecules and the rod portion of myosin that are necessary for the formation of the putative cMyBP-C collar (Moolman-Smook et al., 2002). The result of proteolysis is dissociation of cMyBP-C, release of MHC, and disassembly of the thick filament (McClellan et al., 2004). The greater the amount of unphosphorylated cMyBP-C, the more rapidly MHC is lost and the thick filaments degraded under the conditions of our experiments. Apparently changes in phosphorylation at sites between C1 and C2 can modify the sensitivity of the C5 to C10 region to proteolysis, perhaps by changing the folding of cMyBP-C in this region. Module C5 has a dynamic structure (Idowu et al., 2003), a property that would facilitate the transfer of information between the C0C5 and the C5C10 collar. Since TNT remains bound to the myofibrils, there is not corresponding degradation of thin filaments.

Although thick filaments deficient in cMyBP-C will degrade over time even in the absence of forces exerted on the filament during contraction, loss of cMyBP-C makes the thick filament more susceptible to fragmentation when exposed to a shear force that intact filaments can withstand and that may exist in parts of the normally contracting heart.

In cMyBP-C knockout (KO) mice, sarcomere structure has a normal appearance at low magnification and maximum Ca-activated force is not reduced, indicating that cMyBP-C is probably not required for thick filament stability in this preparation (Harris et al., 2002). The KO, in which cMyBP-C is absent from the embryonic state, can apparently compensate for the instability of thick filament structure that appears when cMyBP-C is partially extracted from WT. To explain this difference or inconsistency in results with the different preparations and to elucidate the physiological and pathophysiological functions of cMyBP-C, studies with both types of preparations will be necessary.

Possible Physiological Functions

Several possible physiological functions for a mechanism of the proposed type are suggested by published observations (Fig. 11). The dependence of both phosphorylation of cMyBP-C and the force of contraction on Ca concentration could form the basis for a mechanism that could inversely couple the rate of degradation of thick filaments to the level of contractile force (Schlender and Bean, 1991; Gautel et al., 1995; Byron

et al., 1996; McClellan et al., 2001). The rate of degradation would decrease as the concentration of activating Ca rises and contractile force increases. The converse would also be true. Contractile activity, time-averaged intracellular Ca concentration, and phosphorylation of the second and third sites on cMyBP-C are all sensitive to adrenergic stimulation, making adrenergic activity a potentially important component in regulation of filament degradation. At this point the existence of this type of mechanism in intact hearts requires additional supporting evidence.

Although it is not clear whether turnover of the contractile system occurs at the individual molecule or the individual filament and whether damaged molecules or filaments are preferentially selected for turnover, dephosphorylation of cMyBP-C could be a marker for physiological turnover. The transition between the 145- and the 130-kD forms can take place within minutes, sufficiently rapidly to modulate turnover.

There is evidence that dephosphorylation of cMyBP-C may contribute to myocardial dysfunction during myocardial ischemia (Decker et al., 2005) and in heart failure (Paolocci, N., L. Tomiolo, I. Kulikovskaya, T. Katori, D.A. Kass, C. Reggiani, and S. Winegrad. 2006. *Biophys. J.* 90:274a). In ischemic hearts, dephosphorylation and proteolysis of cMyBP-C consistently and closely precede the decline in shortening of myocardial segments and the decrease in the number of myosin heads bound to thin filaments that occur during contraction. Dephosphorylation and proteolysis rise in parallel with the increase in end-diastolic pressure as hearts progress into failure from rapid pacing (Paolocci et al., 2006). Phosphorylation of cMyBP-C increases the ability of intact hearts to tolerate a chronically increased load (Sadayappan et al., 2006).

It is clear that the role of cMyBP-C is complex and may involve several functions that appear different depending on the nature of the preparation and the conditions of the cardiac myocytes. A rigorous description of the physiological role of the proposed dephosphorylation-proteolysis mechanism will require further experimentation with different preparations.

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