

Effect of MyBP-C Binding to Actin on Contractility in Heart Muscle

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ABSTRACT In contrast to skeletal muscle isoforms of myosin binding protein C (MyBP-C), the cardiac isoform has 11 rather than 10 fibronectin or Ig modules (modules are identified as C0 to C10, NH₂ to COOH terminus), 3 phosphorylation sites between modules C1 and C2, and 28 additional amino acids rich in proline in C5. Phosphorylation between C1 and C2 increases maximum Ca-activated force (F_{max}), alters thick filament structure, and increases the probability of myosin heads on the thick filament binding to actin on the thin filament. Unphosphorylated C1C2 fragment binds to myosin, but phosphorylation inhibits the binding. MyBP-C also binds to actin. Using two types of immunoprecipitation and cosedimentation, we show that fragments of MyBP-C containing C0 bind to actin. In low concentrations C0-containing fragments bind to skinned fibers when the NH₂ terminus of endogenous MyBP-C is bound to myosin, but not when MyBP-C is bound to actin. C1C2 fragments bind to skinned fibers when endogenous MyBP-C is bound to actin but not to myosin. Disruption of interactions of endogenous C0 with a high concentration of added C0C2 fragments produces the same effect on contractility as extraction of MyBP-C, namely decrease in F_{max} and increase in Ca sensitivity. These results suggest that cardiac contractility can be regulated by shifting the binding of the NH₂ terminus of MyBP-C between actin and myosin. This mechanism may have an effect on diastolic filling of the heart.

KEY WORDS: C1C2 • C0 • cardiomyopathy • relaxation • phosphorylation

INTRODUCTION

Myosin binding protein C (MyBP-C) was first discovered in skeletal muscle by Offer et al., (1973), and subsequent work from several laboratories (Craig and Offer, 1976; Starr and Offer, 1978; Koretz, 1979; Bennett et al., 1986; Davis, 1988; Okagaki et al., 1993; Seiler et al., 1996; Sebillon et al., 2001) has shown that it can play an important role in the formation of normal thick filaments in striated muscle. In its absence, isolated myosin does not form thick filaments with uniform thickness, uniform length or helically ordered myosin heads (Koretz, 1979; Lin et al., 1994; Rhee et al., 1994). Under conditions where it may exist as an impurity, more normally appearing thick filaments can be made (Maw and Rowe, 1986). Two to four (most likely three) molecules are distributed every 43 nm within the C zone of the sarcomere (Craig and Offer, 1976). Three isoforms of MyBP-C, two skeletal and one cardiac, exist. The cardiac isoform is found only in cardiac muscle, and the two skeletal isoforms are not normally expressed in the heart. The skeletal isoform has 10 modules (labeled C1-C10) that resemble either fibronectin or immunoglobulin domains (Gautel et al., 1995; Schwartz et al., 1995; Gilbert et al., 1996).

Cardiac MyBP-C has two myosin-binding sites. One site in C10 at the COOH terminus binds to the rod portion of myosin (LMM, Okagaki et al., 1993) and the other near the NH₂ terminus binds to the S2 region of myosin (Gautel et al., 1995; Gruen and Gautel, 1999; Gruen et al., 1999). The cardiac isoform differs in three ways from the two skeletal isoforms (Fig. 1). It has an extra module at the NH₂-terminal (C0), 28 additional residues in C5, and 3 phosphorylation sites in a sequence connecting C1 to C2 (the MyBP-C motif). Bound to the thick filament in heart muscle is a Ca-calmodulin-regulated kinase that is specific for these sites (Hartzell and Glass, 1984; Schlender and Bean, 1991). Binding of the motif to the S2 region of myosin reduces maximum Ca-activated force (F_{max}) by ~50%, but its binding to myosin is markedly decreased by phosphorylation of these sites. The phosphorylated C1C2 fragment does not bind to skinned fibers and does not alter F_{max} (Gruen and Gautel, 1999; Kunst et al., 2000).

The recognition that as much as 40% of familial hypertrophic cardiomyopathy may be due to mutations in MyBP-C has fostered considerable interest in its function and the changes in structure and function produced by its phosphorylation (Bonne et al., 1995; Watkins et al., 1995; Yang et al., 1998; Flavigny et al., 1999).

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Abbreviation used in this paper: MyBP-C, myosin binding protein C.

Cardiac MyBP-C

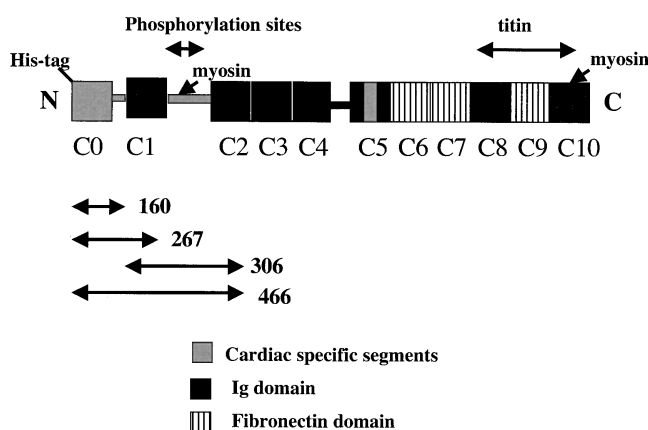


FIGURE 1. Diagram of cardiac MyBP-C showing 11 modules, binding sites, phosphorylation sites, and regions specific for the cardiac isoform. Also shown are the fragments used in this study, an estimate of their length in numbers of amino acid residues and the position of the His tag used in preparing the fragments. The length of a skeletal MyBP-C is ~ 35 nm (Offer et al., 1973).

Although phosphorylation of the protein in reconstituted contractile systems does not seem to have a major effect on actomyosin ATPase activity, the degree of phosphorylation of MyBP-C in intact heart muscle is changed in the same direction as force development by the 5 most common modulators of contractility (α and β adrenergic agonists, cholinergic transmitters, Ca^{2+} , endothelin; McClellan et al., 1994; Winegrad, 1999). In skinned fibers partial extraction of MyBP-C produces a change in Ca sensitivity, in the velocity of shortening (Hofmann et al., 1991a), and in Fmax (Kulikovskaya et al., 2003). Phosphorylation is associated with a change in structure of thick filament (Weisberg and Winegrad, 1996, 1998) and in the probability of interaction between isolated thick and thin filaments (Levine et al., 2001). These results have led to the hypothesis that the probability of forming weak interactions between myosin in thick filaments and actin in thin filaments can be regulated by the degree of phosphorylation of MyBP-C (Levine et al., 2001; McClellan et al., 2001).

Although it has not been possible to produce normal thick filaments *in vitro* without MyBP-C present, in cardiac MyBP-C knockout mice, sarcomere pattern in the heart has a normal appearance (Harris et al., 2002). Nevertheless the hearts of these mice still undergo hypertrophy and have impaired function. The reason for this apparent discrepancy is not clear, although it may be due to compensation such as up-regulation of some other myosin-binding protein during development of cardiac muscle.

Recently Moolman-Smoock et al. (2002) demonstrated that modules in the COOH-terminal half of the mole-

cule bind to each other, specifically C5 to C8 and C7 to C10. Based on these interactions and the data favoring three molecules of MyBP-C every 43 nm in the C zone, they proposed a model in which the three molecules form a collar around the thick filament. The C5-C10 portions of each of the three molecules interact with each other and the C0-C4 regions extend in some undefined manner from the thick filament.

MyBP-C also binds to actin (Moos et al., 1978), but neither the location of the binding site on MyBP-C nor the effect of this binding on the function of the contractile system has been clearly determined. There is some indication that MyBP-C binding to actin increases actomyosin ATPase activity in solution by producing links between actin and myosin to form aggregates of the two contractile proteins (Moos et al., 1978). Because the NH_2 terminus of MyBP-C contains three phosphorylation sites that can regulate force development and the COOH terminus may be involved in forming a collar around the thick filament, we considered the possibility that the NH_2 terminus also contains the actin binding site. Phosphorylation between C1 and C2 may regulate whether the NH_2 terminus binds to myosin or to actin. We examined the structure of MyBP-C for sequences in the NH_2 terminus that resemble known actin binding sequences and found such a sequence in the region of C0. The properties and functional consequences of C0 binding to actin are reported here.

MATERIALS AND METHODS

Measurement of Fmax

Trabeculae were isolated from the endocardial surface of the right ventricle of rats that had been killed according to AAALAC guidelines. The trabeculae were suspended isometrically at sarcomere length of ~ 2.4 μm at room temperature. They were allowed to recover in a modified Krebs' solution (Lin et al., 1991) containing 2.5 mM Ca for 15 min and then bathed in solution of the same composition containing 2.5 or 1.25 mM Ca for 2 h to produce two populations of trabeculae with little dephosphorylated and little phosphorylated MyBP-C. The trabeculae were maintained quiescent. The trabeculae then were skinned with Triton X-100 as previously described (Lin et al., 1991), and the [Ca]-force relation was determined after the Triton X-100 had been washed out. Sarcomere length was not controlled during the contractions. The skinned trabeculae were then soaked in a relaxing solution (100 mM KCl, 3 mM EGTA, 7 mM MgCl_2 , 5 mM ATP, 15 mM creatine phosphate, and 25 mM imidazole, pH 7.0) containing one of four fragments of cardiac MyBP-C, either C0, C0C1, C0C2, or C1C2 at concentrations between 0.3 and 30 μM . After 30–60 min, a Ca-force curve was determined in solutions containing MyBP-C fragments. Trabeculae not exposed to a fragment but otherwise treated in the same way served as controls. After determination of the Ca-tension relation in solution with a fragment, the muscles were very briefly rinsed with relaxing solution containing no fragment. The solution was changed to a relaxing solution without fragment for different lengths of time from 5 min to 60 min and then a Ca-force curve was repeated.

Sarcomere length in the resting trabeculae was not significantly altered by the addition of any of the fragments. All solutions were kept for measurement of the concentration of each fragment by Western blotting. After determination of the Ca-force relation after the washout of fragment, the trabeculae were lysed, subjected to SDS-PAGE, and analyzed by Western blotting for the relative concentration of MyBP-C and fragment. Density of bands was quantified by scanning the gels into NIH Image and measuring the average density times the area for each band. The amount of protein added to each lane of the gel was carefully controlled to keep the densities of bands in the Western blots within the linear range.

Expression of Recombinant C0C2

The DNA sequence of C0C2 was amplified by polymerase chain reaction (PCR), using pMT21-myc-mybp3 as a template (the cDNA is human type; Flavigny et al., 1999). Primers were designed on C0C2 fragment's NH₂ and COOH termini and included BamHI and SalI restriction sites respectively. They did not include the myc tag. Primer 1 was 5'-AAAAGGATCCCCG-GAGCCGGTAAGAAGCCGGTC-3', and Primer 2 was 5'-TAT-AGTCGACTTAGGGCTCTTTCACAAAC-3'. In the first primer the codons encoding two prolines (CCT) and glycine (GGG) were modified to produce the synonymous codons preferred for usage in *E. coli* (CCG, GGT). PCR was performed in a 50 μ l mixture using the standard program of the Expand High Fidelity PCR System (Roche Molecular Biochemicals) and an Eppendorf Mastercycler. The reaction mixture contained 3 units of Expand High Fidelity (Expand HF) thermostable polymerase enzyme mix, 50 ng template plasmid DNA, 50 pmol of each primer, and 200 μ M each of dNTP in the supplied Expand HF buffer. Amplification parameters were an initial 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 1 min annealing at 56°C, and 1 min elongation at 72°C. The PCR product was purified with a QIAquick PCR Purification Kit (QIAGEN), cut with restriction endonucleases Bam HI and Sal I, run on agarose gel and finally purified from the gel using the QIAquick Gel extraction kit (QIAGEN).

For expression of proteins, Plasmid pQE-80L, containing 6 histidines on the NH₂ terminus (QIAGEN), was cut with restriction endonucleases BamHI and Sal I, cleaned with a QIAquick Nucleotide removal kit (QIAGEN), dephosphorylated with calf intestinal alkaline phosphatase (Promega, Madison, WI) and finally purified from an agarose gel using QIAquick Gel Extraction Kit (QIAGEN). Ligation of the plasmid and PCR product insert was performed using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals). 50 μ L of BL21-Star chemically competent cells (QIAGEN) were transformed with the ligation mixture following a standard protocol (20 min on ice, 40 s at 42°C, 3 min recovery on ice, dilution with 5,000 μ l of S.O.C. media [Invitrogen: 2% trypton, 0.5% yeast extract, 10 mM NaCl, 2 ml 1 N NaOH, 10 mM MgCl₂, 2.5 mM KCl, 10 mM MgSO₄, 20 mM glucose, pH 7.0], and a 60-min incubation in 37°C shaker). The transformed cells were plated onto a LB/ampicillin/kanamycin plate and incubated at 37°C overnight.

Several colonies were picked up and grown in LB/ampicillin/kanamycin media for DNA sequencing and analysis of protein expression. Automated cycle sequencing was performed by the University of Pennsylvania DNA sequencing facility. The sequence was analyzed to corroborate its accuracy. The sequencing primer was designed for the promoter area of pQE-80L plasmid. After selection of the appropriate clone, the cells were grown in 1 liter of medium to an optical density of 0.7–0.8 at 600 nm, and expression of recombinant His-tagged C0C2 was induced with 1 mM IPTG (Isopropyl-D-thiogalactopyranoside; Roche Molecular Biochemicals) for 4 h at 37°C or overnight at 30°C and then centrifuged at 4°C for 30 min at 3,000 rpm.

For most preparations a nondenaturing protocol that included no denaturing agents was used. Cells, collected by centrifugation, were lysed in 50 ml of B-PER bacterial protein extraction reagent (Pierce Chemical Co.), with the addition of 0.3 M NaCl, 5 mM 2-mercaptoethanol and "complete, EDTA-free" protease inhibitor cocktail tablets (Roche Molecular Biochemicals). Lysates were sonicated with three 30-s bursts on a Branson Sonifier 450 (Branson Ultrasonics) and clarified by spinning at 20,000 rpm for 15 min.

For the first metal affinity purification, 5 ml Ni-NTA resin (QIAGEN) was equilibrated in "binding buffer," containing 25 mM phosphate, 20 mM Tris-Cl, 10% glycerol, 0.3 M KCl, 5 mM 2-mercaptoethanol, and 0.5% Triton X-100, pH 8.0. Prepared Ni-NTA resin was mixed with clarified cell lysate for 1 h at room temperature, formed into a column, and washed with 500 ml of "washing buffer" consisting of 25 mM phosphate, 20 mM Tris-Cl, 20 mM imidazole, 10% glycerol, 0.3 M KCl, 5 mM 2-mercaptoethanol, and 0.5% Triton X-100, pH 6.0. Purified C0C2 fragment was eluted with the same buffer to which imidazole had been added to a final concentration of 220 mM. Elution fractions were pooled together and dialyzed overnight against 20 mM Tris-Cl, 100 mM KCl, 5 mM 2-mercaptoethanol, and 0.25% Triton X-100, pH 7.0.

For the second metal affinity purification, 5 ml of TALON resin (CLONTECH Laboratories, Inc.) was equilibrated with the same buffer, mixed with dialyzed C0C2 for 15 min at room temperature, formed into a column, washed with 50 ml of the same buffer, and eluted with relaxing solution containing 5 mM 2-mercaptoethanol, no EGTA, and 25 mM imidazole, which is sufficient to elute the fragment.

With a second protocol, employed in a minority of preparations to examine the effect of denaturation and subsequent renaturation, urea was used for elution and then removed. Cells, collected by centrifugation, were lysed under denaturing conditions in Buffer 1: 8 M urea/100 mM sodium phosphate/10 mM Tris Cl, pH 8.0. Further purification on Ni-NTA resin was performed according to QIAGEN protocols. The lysate was clarified by centrifugation at 30,000 *g* for 20 min, and the supernatant was added to a 50% slurry of the Ni-NTA agarose (QIAGEN) in the above buffer at the estimated ratio of 5 mg of protein per 1 ml of resin. It was then mixed for 1 h. The mixture was loaded into a column and washed with the above buffer containing 25 mM imidazole (>10 column volumes). Renaturation of the peptide was accomplished by removing urea with washing of the column with Buffer 2: 50 mM sodium phosphate, 300 mM NaCl, 25 mM imidazole, pH 8.0 (>10 column volumes). Bound recombinant fragment was eluted from the column with the imidazole gradient from 50 to 500 mM in the Buffer 2. Fractions were analyzed by SDS PAGE electrophoresis, and the purest fractions were pooled and run through the D-salt polyacrylamide desalting column (Pierce Chemical Co.) in order to exchange the buffer into the relaxing solution. This step also served as the second purification step. The fractions were analyzed by SDS-PAGE and the purest C0C2 was used in the experiments measuring force.

The purity of each fragment preparation was checked by examining them with SDS PAGE followed by Coomassie blue staining to detect the presence of other peptides and by Western blotting using antibodies specific for C0C1 to confirm the identity of the peptide (Fig. 2). The results show a single band for each fragment on both types of gels. Each fragment migrated with an apparent molecular weight that agreed with the predicted value.

C0C2 on a gel was also identified by a two antibody technique. The primary antibody was His-probe (His-15), rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc.). The secondary antibody was anti-rabbit IgG-peroxidase (Boehringer). The colorimetric enzyme substrate was 1-step TMB-Blotting, (Pierce Chemical Co.).

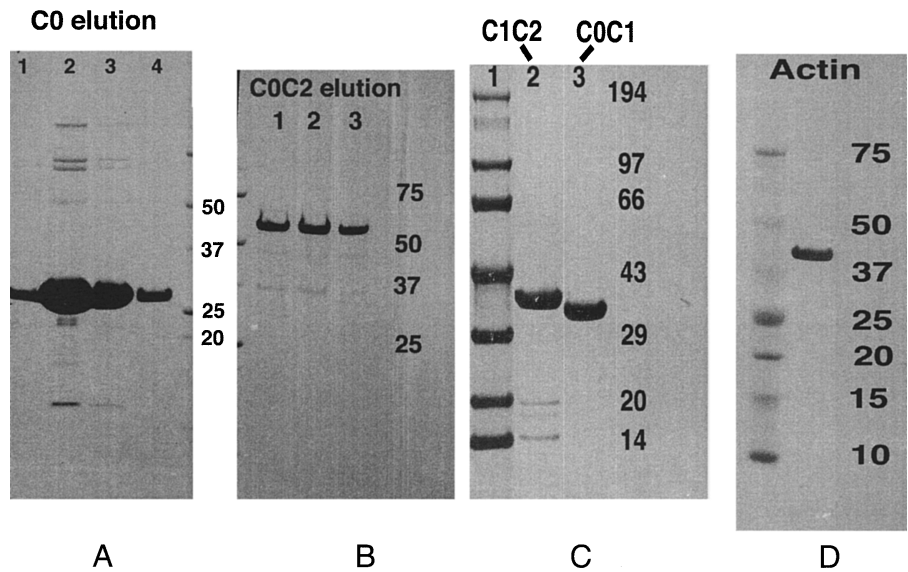


FIGURE 2. Gels of purified fragments and actin stained with Coomassie blue after SDS PAGE. (A) successive fractions of C0 during elution; standards at right. (B) Successive fractions of C0C2 during elution; standards at left. (C) C1C2 and C0C1; lane 1 contains markers and (D) actin.

Expression of Recombinant C0 and C1C2 Fragments

The cDNA sequence of C0 fragment was amplified by PCR, using pMT21-myc-mybpc3 (Flavigny et al., 1999) as a template. Primers were designed on C0 fragment's NH₂ and COOH termini and included BamHI and SalI restriction sites, respectively. The myc sequence was not included in the primer.

Primer 1 was 5'-AAAAGGATCCCCGGAGCCGGGTAAGAAGC-CGGTC-3', and Primer 2 was 5'-AAAAGTCCGACTTAGTCAT-CGGGGGCT-3'. PCR was performed in a 50- μ l mixture using an Expand (HF) PCR System (Roche Molecular Biochemicals) and an Eppendorf Mastercycler. The reaction mixture contained 3 U of Expand High Fidelity thermostable polymerase enzyme mix, 50 ng template plasmid DNA, 50 pmol of each primer, 200 μ l each dNTP in supplied Expand HF buffer. Amplification parameters were an initial 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 1 min annealing at 56°C, and 1 min elongation at 72°C.

The PCR product was purified with QIAquick PCR Purification Kit (QIAGEN), cut with restriction endonucleases Bam HI and Hind III, electrophoresed on agarose gel and finally purified from the gel using QIAquick Gel Extraction Kit (QIAGEN). For expression of proteins, plasmid pQE-80L containing six histidines on NH₂ terminus (QIAGEN), was cut with restriction endonucleases BamHI and SalI, cleaned with QIAquick Nucleotide Removal kit (QIAGEN), dephosphorylated with calf intestinal alkaline phosphatase (Promega), and finally purified from agarose gel using a QIAquick Gel Extraction Kit (QIAGEN). Ligation of the plasmid and PCR product insert was performed using Rapid DNA Ligation Kit (Roche Molecular Biochemicals) according to instructions.

100 μ l of M-15 (pREP 4) chemically competent cells (QIAGEN) were transformed with the ligation mixture following a standard protocol (20 min on ice, 90 s at 42°C heat shock, 3 min recovery on ice, dilution with 500 μ l of S.O.C. media, 45–60 min incubation in 37°C shaker). The transformed cells were plated onto a LB/ampicillin/kanamycin plate and incubated at 37°C overnight.

Several colonies were picked up and grown in LB/ampicillin/kanamycin media for DNA sequencing and analysis of protein expression. Automated cycle sequencing was performed by the University of Pennsylvania DNA sequencing facility. The sequenc-

ing primer was designed for the promoter area of pQE-80L plasmid. After selecting the appropriate clone, the cells were grown in one liter medium to an optical density of 0.7–0.8 at 600 nm and expression of recombinant His-tagged C0 was induced with 1 mM IPTG (Isopropyl-D-thiogalactopyranoside; Roche Molecular Biochemicals) for 4 h at 37°C or overnight at 30°C and then centrifuged at 4°C for 30 min at 3,000 rpm.

Cells, collected by centrifugation, were lysed in Buffer 1 (lysis buffer): 50 mM NaH₂PO₄, 0.3 M NaCl, 10 mM imidazole, complete protease inhibitors (Roche Molecular Biochemicals) at pH 8.0. The lysate was sonicated three times for 30 s each time and spun at 20,000 for 15 min. The column was then washed with Buffer 2: 50 mM NaH₂PO₄, 0.3 M NaCl, 25 mM imidazole at pH 6.0. Bound and washed recombinant C0 fragment were eluted from the column with an imidazole gradient from 50 to 500 mM in the Buffer 2. Fractions were analyzed by SDS-PAGE electrophoresis with Coomassie blue staining and Western blotting with an antibody against C0C1. The purest fractions were pooled and run through the D-Salt Polyacrylamide Desalting Column (Pierce Chemical Co.). This step also served as the second purification step. Fractions were again analyzed by SDS-PAGE, and the purest C0, at least 98% C0 as determined by SDS-PAGE, was used for further experiments (Fig. 2). The final buffer was relaxing solution with or without 2-mercaptoethanol.

C1C2 was prepared similarly with NH₂ terminus primer: 5'-TATAGGATCCCCGATTGGCCTCTTC-3', containing BamHI restriction site (underlined) and COOH terminus primer: 5'-TATAGTCCGACTTAGGGCTCTTCACAAAC-3', containing SalI restriction site (underlined).

Gifts of C0C1 and C1C2 were given by Dr. Mathias Gautel (King's College, London, UK). The purity of both fragments was checked as described above (Fig. 2).

Preparation of Filamentous Actin

Lyophilized monomeric actin (#A2522; Sigma-Aldrich, or Cytoskeleton, both from rabbit muscle) was polymerized for 1 h at room temperature in 5 mM Tris-Cl, 50 mM KCl, 5 mM 2-mercaptoethanol, 2 mM MgCl₂, and 1 mM ATP. The filamentous actin stock solution (23 μ M) was briefly stored at 4°C. The condition of the actin was evaluated by SDS-PAGE with Coomassie blue staining, Western blotting with antiactin, sedimentation after

high speed centrifugation, binding to α actinin, and failure to bind to bovine serum albumin as measured by cosedimentation.

Immunoprecipitation of Actin–Fragment Complex

5 nM of a given fragment (either C0, C0C1, C0C2, or C1C2) dissolved in 10 μ l of relaxing solution were divided into two aliquots. With fragment C0C2 and C1C2 one aliquot was treated with protein kinase A to phosphorylate the fragment and the other was left completely unphosphorylated. The fragments had a concentration of 5 μ M after this step. Ten micrograms of filamentous actin in 10 μ l of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄) was added to the fragment solution to make an approximately equal concentration of actin and fragment. The mixture was incubated on a shaker for 2 h at room temperature. At the end of this incubation the mixture was divided in two equal volumes for pulldown with anti-actin agarose (see G) or Ni-NTA resin (QIAGEN) that binds to the His-tag (see above). All experiments were performed at room temperature in duplicate.

Ni-NTA Pulldown of the Actin–Fragment Complexes

10 μ l of Ni-NTA resin and 40 μ l PBS at pH 8.0 were added to half of each sample (30 μ l), containing \sim 5 μ g of a fragment and 5 μ g of actin. The mixture was incubated on a shaker at room temperature for 2 h or at 4°C overnight (results were the same for the two protocols). Ni-NTA resin with bound protein and peptide was placed in micro-spin columns (Pierce) and washed extensively with PBS at pH 8. After washing, the protein bound to the resin was eluted with 20 μ l SDS sample buffer, separated on the 4–12% NuPage SDS gel (Invitrogen) and blotted onto PVDF membrane (Millipore). Western Blot was performed using Western Breeze Kit (Invitrogen), according to instructions. The primary antibody was rabbit anti-actin (#A 2066; Sigma-Aldrich).

The presence of actin was measured by Western blots using an antibody against actin. The fragments of MyBP-C were detected with antibodies to C0C1 and C2C5 to confirm the identity of the fragments.

Pulldown with Antiactin Agarose

20 μ l of a 50% slurry of agarose coated with goat antiactin IgG (Santa Cruz Biotechnology, Inc.) were added to the mixture of MyBP-C fragment and actin and also to two control samples that did not contain actin. The mixtures were incubated on a shaker at room temperature for 2 h. Samples were washed in the microspin columns, and the bound protein and peptide eluted with SDS sample buffer. The proteins in the eluate were separated on 4–12% NuPage SDS gel and blotted onto PVDF membrane. A Western Breeze Kit was used according to instructions, and anti-C0C1 custom-made rabbit antibodies were used as the primary.

Cosedimentation of Actin with C0

Cosedimentation of fragments of MyBP-C and actin was performed using the Actin Binding Protein Biochem kit (Cytoskeleton), Beckman TL-100 ultracentrifuge, and TLS-55 swinging bucket rotor with adaptors for Ultraclear 200 μ l tubes. Each fragment was prepared in the actin compatible buffer (5 mM Tris-Cl, 50 mM KCl, 5 mM 2-mercaptoethanol, pH 7.0) at the highest possible concentration and centrifuged at 150,000 *g* for 1.5 h. The supernatant was used in subsequent cosedimentation assays.

To form a tighter pellet during centrifugation, 50 μ l of “Actin Cushion Buffer” (5 mM Tris-Cl, 2 mM MgCl₂ 50 mM KCl 10% glycerol, pH 8.0) was added to the bottom of each tube. For the cosedimentation, 40 μ l of filamentous actin stock solution was mixed with 10 μ l of the prepared fragment solution to a final

concentration of actin of 4.1 μ M. After mixing for 5–30 min at room temperature (the same temperature at which force measurements were made with skinned fibers) the actin plus fragment was centrifuged at 150,000 *g* for 1.5 h. After centrifugation, aliquots of the supernatant and the pellet were separately analyzed by SDS-PAGE with Coomassie blue staining.

The extent of interaction between the fragments and actin was estimated from the absence or the decline in the concentration of the fragment in the supernatant and the concentration in the pellet. Relative densities of the bands were measured by densitometry using the NIH Image program. In conjunction with the measurement of fragment binding to actin, the condition of the actin was checked by its ability to bind α actinin and the absence of binding to bovine serum albumin. Failure to meet these conditions resulted in rejection of the experiment.

Phosphorylation of C1C2 and C0C2 Fragments

Five nanomoles of each fragment were incubated overnight at 30°C in a total volume of 100 μ l, containing: 50 mM Tris-Cl, 2.5 mM MgCl₂, 1 μ M cAMP, 1 mM ATP, 0.1 mM CaCl₂, 1 mM DTT, pH 7.5. Phosphorylated fragments were produced by including 25 units of 3':5'-cyclic AMP-dependent protein kinase, (#P5511; Sigma-Aldrich). A control consisted of kinase without cAMP in the medium. Phosphorylation was measured by IEF gels and Western blotting as previously described in detail (McClellan et al., 2001). Incubation with kinase resulted in a change from total unphosphorylation to >85% phosphorylation.

Sample Preparation for SDS-PAGE of Lysates

For electrophoresis of skinned fibers, the NuPAGE Bis-Tris Gel System (Invitrogen) was used, including precast gradient gels, MES-SDS running buffer, NuPAGE-LDS sample buffer, NuPage reducing agent, and NuPage antioxidant. Prior to electrophoresis, tissue samples were lysed in Tissue-PELB lysis buffer (GenoTechnology) with addition of complete protease inhibitor cocktail (Roche Molecular Biochemicals), okadaic acid (Life Technologies), and kinase inhibitors (Sigma-Aldrich). The total protein concentration in lysates was measured using Coomassie plus protein assay (Pierce Chemical Co.). Approximately 30 μ g total protein was loaded on each lane of the gel. Prior to electrophoresis, washout samples were concentrated generally from 500 μ l to 50 μ l, using ultrafree 0.5 centrifugal filter device, 10 K NMWL (Millipore Corp.). The filter was carefully cleared of all protein after the concentration procedure (McClellan et al., 2001).

Western Blotting

These were performed as already described (McClellan et al., 2001). The relative amounts of MyBP-C and its fragments were estimated from the relative densities of the bands on Western blots after reaction with a polyclonal antibody against C0C1 or C2C5. The concentration of protein applied to each lane and the exposure time of the blots were chosen to produce bands with densities linearly related to the amount of protein. The efficiency of transfer of MyBP-C and of the fragments were compared by measuring several known amounts of each and comparing the densities produced on the gels. The efficiency was the same for MyBP-C and all the fragments. The density and the area of each band were measured with the NIH Image program. Although there are limitations in comparing densities produced by the different peptides or MyBP-C, comparison of density of bands on the same gel produced by the same peptide is valid as long as the densities are kept within the linear range of the relation between amount of peptide and density (McClellan et al., 2001).

Statistics

Populations were compared using ANOVA, chi square, regression analysis, and Student's *t* test. Differences were considered significant when $P < 0.05$. When multiple use was made of the same data, the Bonferroni correction was applied.

RESULTS

Binding of C0-containing Fragments to Actin

Binding of C0-containing fragments of MyBP-C to filamentous actin was examined using three different assays: Ni-NTA resin, which binds His-tagged peptides, antiactin-labeled agarose to pull down actin, and cosedimentation to pull down filamentous actin with fragments that bind to actin. In the first two, the extent of the interaction between filamentous actin and the fragment was determined by analysis of the material eluted from the resin or the agarose. In the cosedimentation assay the extent of binding was determined from the concentration of fragments in the supernatant and in the pellet after centrifugation of a solution with a mixture of filamentous actin and fragment. Binding of fragments containing C0 occurred with each of the three methods. Removal of the His-tag with protease did not alter the binding.

In the immunoprecipitation assays, 0.5–5.0- μM fragments of MyBP-C consisting of C0, C0C1, C0C2, or C1C2 were mixed with 1–5 μM actin. After centrifugation to separate the resin or the agarose, the supernatant was retained for Western blot analysis and the resin or agarose was washed three times with relaxing solution or PBS. SDS was used to elute the protein bound to the resin and the agarose. The presence of actin and fragment in the eluant was confirmed by Western blotting with an antibody against actin or C0C1 (Fig. 3).

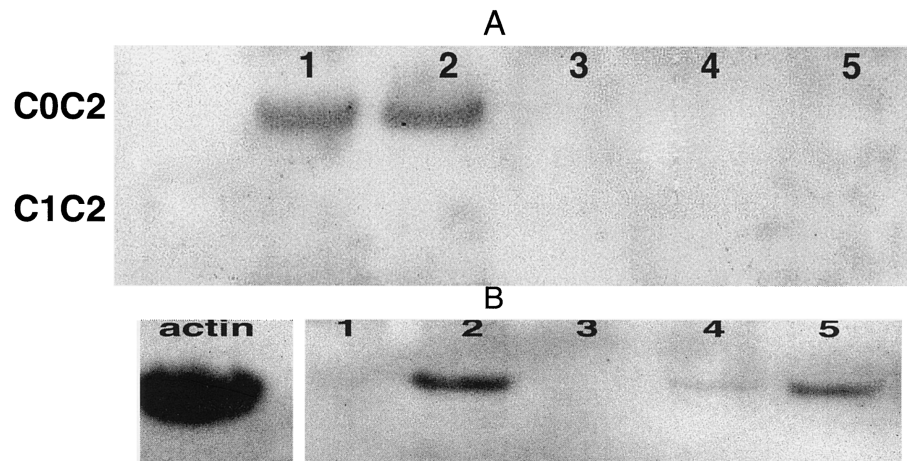
In 16 different experiments both immunoprecipitation methods produced binding of fragments of MyBP-C

to actin when the fragments contained C0. C1C2 did not bind to actin with either of the two protocols. The presence of the C0 module in the fragment was necessary for the fragment to bind to actin. Phosphorylation of the C1-C2 linker (MyBP-C motif) in C1C2 or C0C2 did not alter actin binding (Fig. 3). Denaturation of C0, C0C1, and C0C2 with SDS or urea prevented their binding to actin. Renaturation by removal of urea only partially restored binding (Fig. 3). Actin was bound to C0 less tightly than to C0C1 or C0C2 in as much as washing several times with relaxing solution removed some of the C0 bound to actin, but did not significantly reduce the amount of C0C1 or C0C2 bound. This property appears to be similar to the binding of the COOH terminus of MyBP-C to light meromyosin (LMM), which is stronger when C8 and C9 are included in the fragment even though the binding site is on C10 (Okagaki et al., 1993).

An estimate of the half-maximum value for the binding of actin to C0 was derived from the immunoprecipitation results in the following way. The initial concentrations of actin and the fragment were measured spectroscopically, and the threshold amounts of actin and C0-containing fragments for visibility on Western blots were determined. Since neither C0-containing fragments nor actin was detected in the supernatant and the final wash solutions, each was assumed to be present in less than threshold amounts. From these values an estimate of the concentration that gives half-maximum binding was $<10^{-5}$ M.

The binding of C0 and C0C2 to actin was also examined by cosedimentation using four different concentrations of the fragment: 0.1, 0.3, 0.6, and 0.9 μM with 4.1 μM actin (Fig. 4). After centrifugation of solutions containing 0.1, 0.3, or 0.6 μM C0C2 and filamentous actin, no fragment was detected in the supernatant, but a small amount remained in the supernatant with 0.9-

FIGURE 3. (A) Western blot with an antibody to cardiac C0C1 showing binding of actin to C0C2 measured by immunoprecipitation (See MATERIALS AND METHODS). Lanes 1 and 2 show results with a mixture containing C0C2 and actin. Lane 3 contains only actin, and lanes 4 and 5 contain C1C2 and actin. In lanes 2 and 5 the fragments had been phosphorylated before incubation with actin. Labels mark expected positions of C0C2 and C1C2. (B) A Western blot with antiactin showing the binding of actin to natural C0 (lane 2) and natural C0C2 (no exposure to urea, lane 5), but the absence after denaturation with urea (C0, lane 1) and decrease (C0C2, lane 4) after renaturation by removal of the urea. Lane 3 contains only the resin. The lane at left contains only actin.



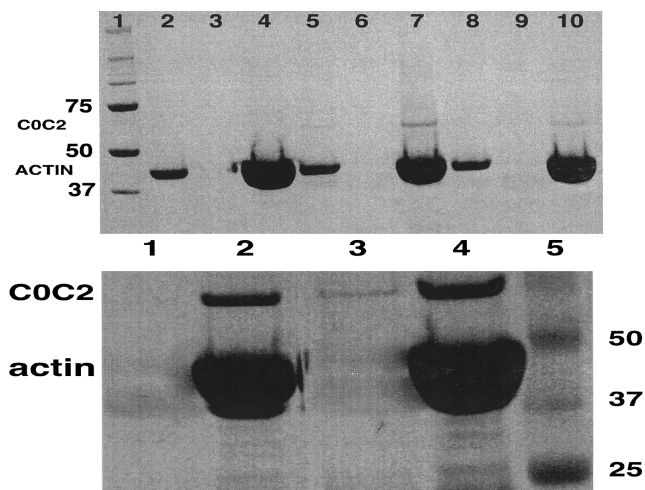


FIGURE 4. (Top) Coomassie blue-stained gels after SDS-PAGE in cosedimentation experiments. Lane 1 contains standards. Lanes 2, 5, and 8 contain the beginning solutions; lanes 3, 6, and 9 the supernatants after centrifugation; and lanes 4, 7, and 10 contain the pellets. In lanes 2–4 the starting solution contained only filamentous actin. In lanes 5–7 and 8–10 the starting solutions contained filamentous actin and C0C2. The actin concentration in all three runs was 4.1 μM . In lanes 5–7 and 8–10 the concentrations of C0C2 were, respectively, 0.3 and 0.1 μM . (Bottom) Cosedimentation showing saturation of actin with C0C2. Lanes 1 and 3 are supernatant and lanes 2 and 4 are pellet. Concentrations of C0C2 in initial solution in lanes 1 and 2 and 3 and 4 are, respectively, 0.6 and 0.9 μM . Lane 5 contains markers with values to the right.

μM fragment. From the concentrations of the fragments and actin in the starting solution, in the supernatant and in the pellet, the relative amounts of the fragment and actin interacting were calculated. The stoichiometry of fragment to actin in the pellet with 0.6 and 0.9 μM C0C2 was not significantly different, indicating that at these concentrations actin had been saturated with the fragment. The stoichiometry of fragment to actin was $\sim 1:7$. C0 was bound less strongly than C0C2 in this assay too.

Reciprocal Binding of C0C1 and C1C2

To evaluate the relationship between binding of the C0-containing fragment of MyBP-C to actin and binding of C1C2 to myosin, two groups of skinned fibers were prepared by soaking cardiac trabeculae in a bathing solution containing 2.5 or 1.25 mM Ca before they were skinned. As measured by IEF gels, trabeculae in the former group contained little completely unphosphorylated MyBP-C (Fig. 5; McClellan et al., 2001). In the latter group most of the MyBP-C was unphosphorylated. Modification of phosphorylation of MyBP-C by variation of the concentration of extracellular Ca before skinning was used because this method affects the phosphorylation of only MyBP-C and does not alter

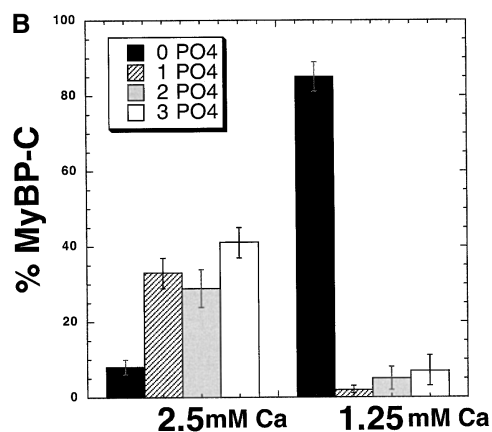
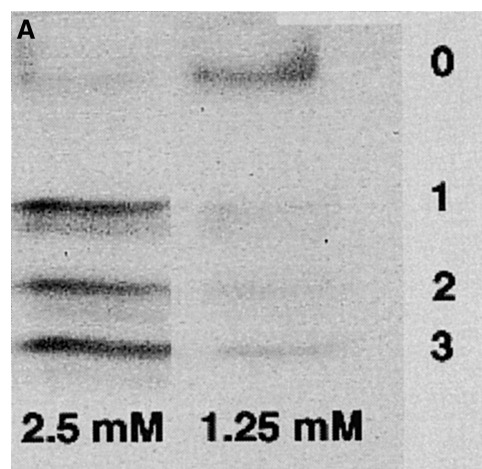
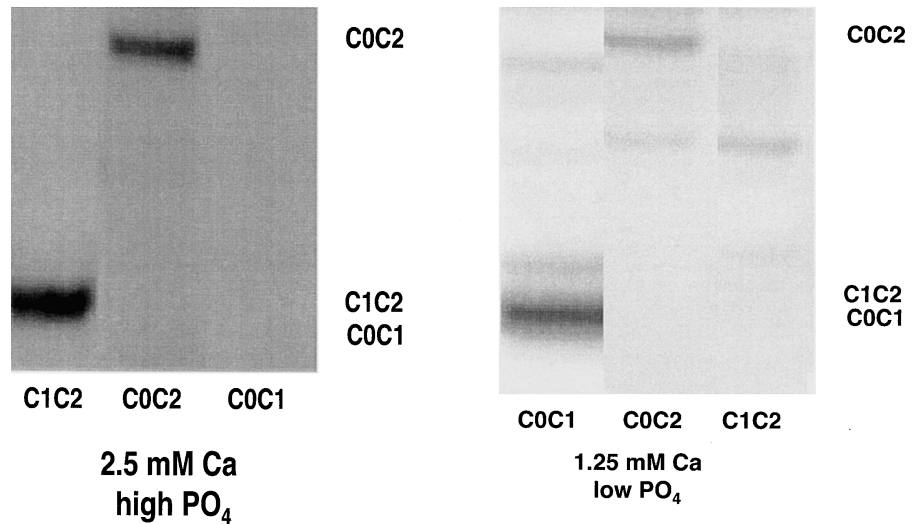


FIGURE 5. (A) IEF gel of lysate of two muscles stained with anti-C0C1, one soaked at rest in bathing solution containing 2.5 mM Ca, and a second soaked in a bathing solution of the same composition except that Ca was 1.25 mM. The different bands indicate MyBP-C containing 0, 1, 2, and 3 phosphates per molecule of MyBP-C. (B) The average distribution of phosphorylated forms of MyBP-C in eight experiments.

phosphorylation of troponin or the regulatory light chain of myosin (McClellan et al., 2001). Use of protein kinase or phosphatase after skinning would have altered phosphorylation of troponin, which would have complicated the interpretation of the results. All fragments except those containing C1C2 modules and specifically incubated with kinase were completely unphosphorylated.

Since unphosphorylated MyBP-C binds to the S2 region of myosin (S2) but phosphorylated MyBP-C does not (Kunst et al., 2000), distribution of binding of the NH₂ terminus of endogenous MyBP-C between actin and myosin might vary according to the extent of phosphorylation of MyBP-C. Trabeculae from each group ($n = 4$) were incubated in relaxing solution containing a low concentration (0.01 μM) of either C0C1, C1C2, or C0C2. A low concentration, equivalent to <3% of

FIGURE 6. Western blots stained with anti-C0C1 showing reciprocal binding of C0C1 and C1C2 by skinned cardiac muscle occurs depending on the degree of phosphorylation of endogenous MyBP-C. (Right) The intact cardiac muscle was soaked in 1.25 mM Ca at rest for 2 h to produce a low level of phosphorylation after skinning. (Left) The intact cardiac muscle was soaked in 2.5 mM Ca at rest for 2 h to produce a high level of phosphorylation after skinning. Labels at the bottom indicate the fragment added to the skinned fiber preparation before lysing. Labels to the right indicate the position of a particular fragment on the gel. After skinning muscles were then soaked in relaxing solution containing a low concentration of the specified fragment (0.01 μ M) and then briefly washed before lysing and electrophoresis. Note that C0C1 does not bind when phosphorylation is high (2.5 mM Ca). Presumably endogenous C0 is bound to actin under these conditions. C1C2 does not bind when phosphorylation is low (1.25 mM Ca), and endogenous C1C2 is bound to myosin. The faint bands present in gels of trabeculae soaked in 1.25 mM Ca are proteolytic fragments of MyBP-C, which are more common with unphosphorylated MyBP-C.



endogenous protein, was used to avoid significant displacement of endogenous MyBP-C from binding sites on myosin or actin. After 30 min exposure to the fragment, the trabeculae were very briefly washed to remove the excess fragment, exposed to SDS, and electrophoresed on polyacrylamide gels. Western blotting was performed with an antibody against C0C1.

In trabeculae with primarily unphosphorylated MyBP-C and therefore a high degree of binding of endogenous C1C2 to myosin, C1C2 added to the incubation medium did not bind to the trabeculae (Fig. 6). There was, however, a substantial amount of binding of C0C1. The opposite was observed when the trabeculae had a high degree of phosphorylation of endogenous MyBP-C. A substantial amount of C1C2 but no detectable C0C1 was bound. C0C2, which contains the binding sites to both actin and myosin, was bound to trabec-

ulae with little unphosphorylated MyBP-C and to a lesser degree to trabeculae with primarily unphosphorylated MyBP-C. Phosphorylated C1C2 was not studied because Gruen and Gautel (1999) have already shown, and we have confirmed, that phosphorylated C1C2 does not bind to myosin.

These results are consistent with the hypothesis that, in the presence of unphosphorylated MyBP-C, the binding site on myosin at the NH₂ terminus for C1C2 is occupied by endogenous MyBP-C, and in the virtual absence of unphosphorylated MyBP-C, the binding site for C0C1 on actin is occupied.

Effect of C0 and C0C1 Fragments on Contractility

The effect of C0 on contractility was determined by incubating skinned cardiac trabeculae with 0.3–5.0- μ M C0 fragments added to relaxing and then to contrac-

FIGURE 7. Force tracing of a cardiac trabecula that had been skinned after a 2-h exposure to Krebs' solution containing 2.5 mM Ca. Note decrease in F_{max} and increase in force at pCa 5.6 in presence of 3 μ M C0. Changes are reversed within 10–15 min of bathing in a medium without C0. Calibration bars equal 15 min and 0.05 g. Thin arrows pointing down indicate pCa of 6.0, 5.6, and 5.2 in that order. Short thick arrows indicate 5.0 and longer thick arrows indicate 4.5. Short arrows pointing up indicate pCa of 9.0.

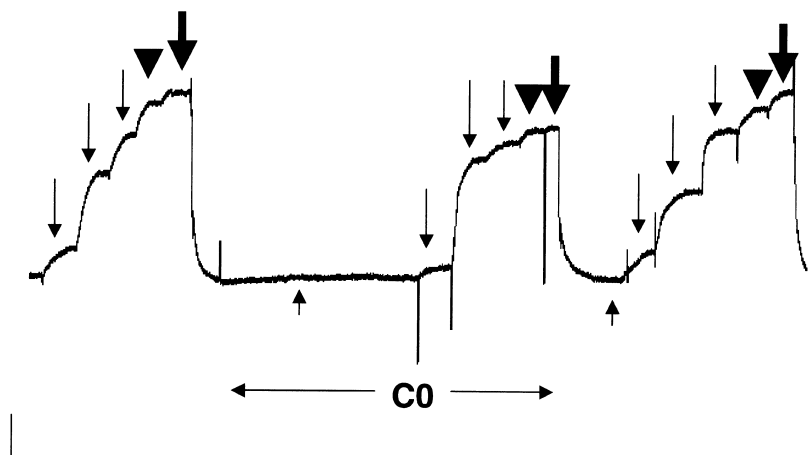


TABLE I
Effect of C0-containing Fragments of MyBP-C on Contractility

	High phosphorylation of MyBP-C	Low phosphorylation of MyBP-C
	<i>mN/mm²</i>	<i>mN/mm²</i>
Before C0 or C0C2	27.1 ± 1.8	16.6 ± 2.0 ^a
During C0C2	19.6 ± 1.3 ^a	15.9 ± 2.3 ^a
During C0	23.1 ± 1.8	16.1 ± 2.2 ^a
After C0C2	18.7 ± 1.7 ^a	15.3 ± 1.7 ^a
After C0	25.3 ± 1.4	15.8 ± 2.0 ^a

^aDifference from high phosphorylation before C0; $P < 0.05$, $n > 12$.

tion solutions (Fig. 7). The trabeculae had been soaked in solution containing either 1.25 or 2.5 mM Ca for 2 h before skinning to vary the degree of phosphorylation of endogenous MyBP-C (Table I). In the first series of contraction solutions containing pCa 9.0 to 4.5, maximum Ca activated force (Fmax) for unphosphorylated skinned fibers was ~30% lower than that for phosphorylated fibers (Table I, $n \geq 16$ for each value; $P < 0.02$; this has already been shown by McClellan et al., 2001). There was, however, no significant difference in the Ca sensitivity of fibers associated with the large difference in phosphorylation of MyBP-C (Fig. 8). (Skinning does not alter the degree of phosphorylation of MyBP-C; McClellan et al., 2001). The solution was then changed to one containing C0 fragments with pCa of 9.0. 30 min later the pCa was decreased in steps from 9.0 to 4.5. After this series of solutions the trabeculae were again exposed to pCa 9.0 to 4.5 without C0 fragments.

C0 fragments increased Ca sensitivity in both groups of skinned fibers. With phosphorylated trabeculae, C0 fragments caused an increase in Ca sensitivity at submaximal force that almost completely reversed within 20 min after the fragments had been removed (Figs. 7 and 9). The threshold was 0.8–1.0 μM , and the maximum effect occurred at 3–4 μM . The extent of change in Ca sensitivity was substantially less but equally reversible when the trabeculae had only a low degree of phosphorylation of MyBP-C (Fig. 9). The largest increase in Ca sensitivity occurred with Ca concentrations that normally produce low levels of force. The result was a dramatic increase in force over a small range of Ca concentration producing a large value for the Hill coefficient.

During exposure to 3- μM C0 fragments, Fmax of phosphorylated skinned fibers decreased by $15 \pm 7\%$ ($n = 18$) and recovered when the fragments were removed (Fig. 7). The significance was borderline with $P < 0.06$ but >0.05 (Table I). The effect of the more strongly binding C0C1 on Fmax was unequivocal. With 2 μM C0C1, Fmax of phosphorylated skinned fibers decreased by $31 \pm 8\%$ ($n = 6$, $P < 0.05$). The greater effect with C0C1 than C0 is consistent with the stronger

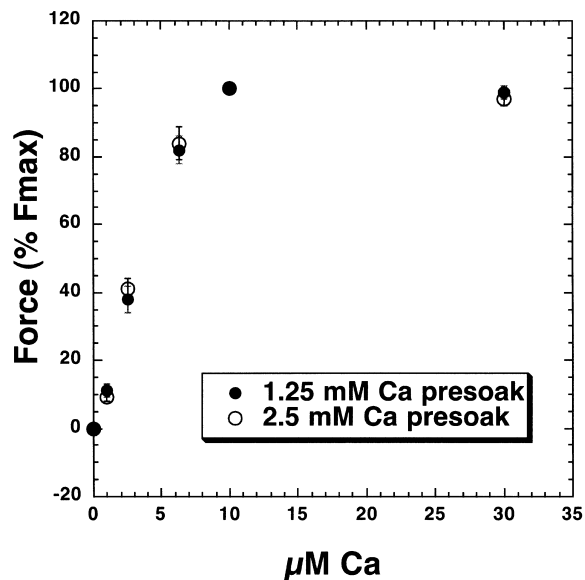


FIGURE 8. Force/[Ca] relation of trabeculae skinned after 2 h soak in Krebs' solution containing either 1.25 mM (filled circles) or 2.5 mM (open circles) Ca. There is no significant difference.

binding of C0 when it is present in a fragment with additional modules. In trabeculae with primarily unphosphorylated MyBP-C there was no significant change in Fmax with the same concentrations of C0C1 that were effective with phosphorylated MyBP-C.

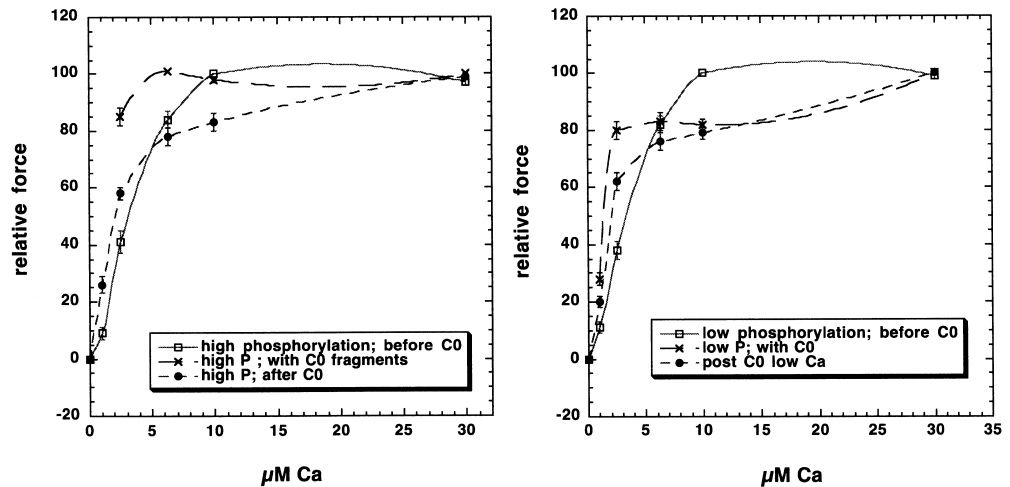
Dephosphorylation of MyBP-C decreases Fmax in skinned cardiac fibers by increasing C1C2 binding to the S2 region of myosin (Kunst et al., 2000). If endogenous C0 binding to actin enhances contractility and binding of endogenous C1C2 to myosin depresses contractility, then addition of a high enough concentration of C0-containing fragment to disrupt the interaction between endogenous C0 with actin should have a substantially larger effect on the contractility of skinned fibers with the greater phosphorylation of MyBP-C. This difference was observed (Table I).

Effect of C0C2 on Contractility

The interaction of C0 and C1C2 binding when the two peptides were present in one protein, as they normally are, was examined by exposing skinned trabeculae to C0C2. Fibers with an approximately equal amount of totally phosphorylated and unphosphorylated MyBP-C were studied, using a protocol similar to that used for C0. Contractions with pCa from 9.0 to 4.5 were recorded before, during, and after exposure to 2 μM C0C2.

The contractile response was complex (Fig. 10). A pCa of 6.0 normally produces a small amount of tension, but in the presence of 2 μM C0C2 tension developed very rapidly to a value close to Fmax. The response of the Ca sensitivity resembled that produced by

FIGURE 9. Addition of 3- μ M C0 fragments to solution bathing skinned rat cardiac trabecula increases Ca sensitivity. The effect of C0 was compared on two different types of preparations: trabeculae with a high (left) and trabeculae with a low (right) degree of phosphorylation of endogenous MyBP-C. In the former, C0 of a majority of MyBP-C are bound to actin, and in the latter a majority of C1C2 are bound to myosin. Force is expressed as percent of maximum. Open squares indicate before addition of C0; x indicates with C0; and filled circles indicate after C0 removal.



C0. With C0C2, tension quickly began to decay and dropped by $26 \pm 9\%$ ($n = 8$ trabeculae; $P < 0.01$). This lower value remained after C0C2 had been removed from the bathing medium in spite of several changes of solution. At this point the residual content of C0C2 in the muscle was measured by Western blotting (Fig. 10 B) and compared with the density of a series of calibration values to provide absolute amounts. 10 nmoles of C0C2 per g of tissue were present 60–70 min after the removal of C0C2 from the solution ($n = 4$; $P < 0.01$). This corresponds to a stoichiometry of $\sim 1:1.5$. Such an effect on contractility from this low level of binding is not unreasonable in view of the finding of Moos et al. (1978) that even one molecule of MyBP-C per 20 actins can have a major effect on the physical properties of the filaments. In contrast, C0 was washed from the muscle with a half-time of ~ 10 min (unpublished data). As measured by Western blotting no myosin or MyBP-C was lost from the muscle during this period (unpublished data).

The results can be summarized as an initial rapid “C0-like” effect on Ca sensitivity followed by a persistent decrease in F_{max} associated with a persistent binding of C0C2 by the muscle. The decrease in F_{max} was probably due to the combined effects of C0C2 binding to myosin and actin. The slower washout and longer effect on contractility of C0C2 are consistent with the stronger binding of C0C2 than C0 found above.

In skinned fibers with a high degree of phosphorylation of MyBP-C, incubation in a solution containing C0C2 produced a significant decrease in F_{max} that did not reverse during 60 min after the removal of C0C2 (Table I). The threshold concentration for the changes in contractility was 0.5μ M, and maximum effect was achieved with 3–5 μ M. C0C2 had no significant effect

on F_{max} of skinned fibers with a low degree of phosphorylation.

DISCUSSION

By decreasing the affinity of C1C2 for myosin S2, phosphorylation of MyBP-C in cardiac muscle can modify the structure of the thick filament and modulate contractility (Gautel et al., 1995; Weisberg and Winegrad, 1996, 1998; Kunst et al., 2000). The mechanism of the change appears to involve an alteration in the number of myosin heads bound to actin in the nonforce generating, loosely bound state that precedes the entry of the cross-bridge into the force generating cycle (Levine et al., 2001; McClellan et al., 2001).

Moos et al. (1978), Hartzell (1985), and Yamamoto (1986) have shown that skeletal MyBP-C can bind to actin in the micromolar range. By forming stable actin-myosin aggregates in solution, MyBP-C binding to actin stimulates actin activated but not Ca-, Mg-, or EDTA-activated myosin ATPase activity. In spite of these observations of skeletal MyBP-C, a role for the MyBP-C interaction with actin in the normal function of heart muscle has not been elucidated. MyBP-C binds strongly to I bands (Moos, 1981) and, in transfected cardiac myocytes, to a region within the A bands, but C0 does not bind to myosin in solution (Kunst et al., 2000).

Here we have demonstrated that there is a site on the C0 module of MyBP-C that binds actin with an affinity similar to that found for skeletal MyBP-C in myofibrils. Isolated C0 modules bind to filamentous actin, but the affinity of C0 for filamentous actin and for skinned fibers is greater when it is present in a larger fragment such as C0C2. This is apparent in immunoprecipitation and cosedimentation assays as well as in the magnitude and the duration of the effect of C0-containing fragments on

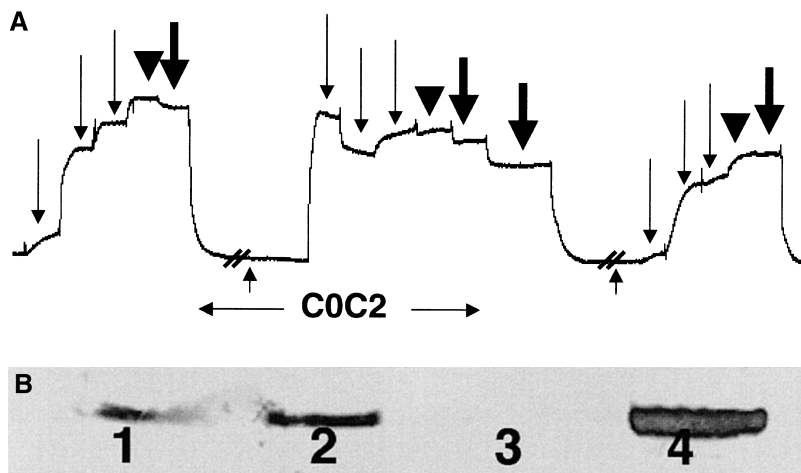


FIGURE 10. (A) Force tracing of a skinned cardiac trabecula before, during, and after soaking in 2 μ M C0C2. Calibration lines equal 0.05 g and 20 min. Thin arrows pointing down indicate pCa of 6.0, 5.6, and 5.2 in that order. Short thick arrows indicate 5.0 and longer thick arrows indicate 4.5. Short arrows pointing up indicate pCa of 9.0. (B) Western blot of lysate of trabeculae stained with anti-His tag to demonstrate retention of C0C2 after 60 min in solutions without C0C2. In lanes 1 and 2 the trabeculae had been exposed to C0C2 for 75 min before the 60 min without C0C2. Lane 3 is the control, in which the 75 min soak solution did not contain C0C2. Lane 4 is C0C2.

contractility. The binding of C0C2 is still due to the presence of C0 because in these studies C1C2 did not bind to actin. The enhancement of binding of the C0 module by the presence of adjacent modules in the fragment is similar to what has been observed with the C terminus binding to LMM (Okagaki et al., 1993). The binding site is in C10, but the presence of C8 and C9 increases the affinity. For this reason the quantitative studies of C0 binding to actin have been performed with C0C2.

In cardiac muscle, exogenous C0 at a low concentration that does not alter contractility does not bind to trabeculae when the MyBP-C is phosphorylated and endogenous C0 is presumably bound to actin. C0 does bind to actin when MyBP-C is not phosphorylated and is bound to S2 through its NH₂ terminus. This suggests that C0 added at low concentration to skinned cardiac trabeculae binds to the same actin as endogenous C0 in phosphorylated MyBP-C even though these actins are only a small fraction of the total population in the thin filaments. Under these conditions other actin molecules appear to have a lower affinity for C0 and do not bind C0. The presence of regulatory proteins in thin filaments in skinned fibers could produce heterogeneity of actin monomers that results in different affinities for MyBP-C, particularly since one of the subunits of troponin binds to actin, and tropomyosin can block sites on actin.

C0 binding to filamentous actin, in which there are no regulatory proteins, appears to saturate with a stoichiometry of \sim 1:7. Moos et al. (1978) found a similar stoichiometry of 1:5 using skeletal MyBP-C. This ratio is quite similar to the periodicity of the regulatory proteins in native thin filaments and is suggestive that either all actin molecules in a thin filament are not equivalent or the binding of C0 or MyBP-C to an actin monomer decreases the affinity of other monomers in the same filament for C0. Moos et al. (1978) came to a similar conclusion that the binding of one skeletal MyBP-C molecule to filamentous actin blocked the interactions of up to five, presumably adjacent, molecules of actin

with MyBP-C. We have also found a similar limit in the amount of C1C2 bound to skinned fibers. The stoichiometry at saturation is \sim 1:4 (unpublished data).

In higher concentrations, exogenous C0C2 decreases F_{max} but only when MyBP-C is phosphorylated and bound presumably to actin and not to myosin. At these concentrations C0 appears to displace endogenous MyBP-C and disrupt the interaction between endogenous C0 and actin, decreasing development of force. C0 binding to actin enhances contractility only when it is part of the intact MyBP-C with its COOH terminus bound to LMM.

The mechanism of action of MyBP-C and its phosphorylation on contractility remains obscure because MyBP-C is concentrated in only one part of the sarcomere and has a low stoichiometry in its interactions with actin and myosin. Yet fragments of MyBP-C can modify force by $>$ 50% (Kunst et al., 2000). The facts that phosphorylation of MyBP-C only alters myosin-actin interaction (ATPase and force) in organized myofibrils and phosphorylation of MyBP-C changes the structure of the whole filament argue in favor of a mechanism in which the overall structure of thick filaments can be modulated by interactions in a limited region of the filament (Weisberg and Winegrad, 1996; Winegrad, 1999; Levine et al., 2001).

When the binding sites on C0 and C1C2 are both present on the same peptide, the change in contractility resembles the combination of the effects of the two separate peptides except that unphosphorylated C0C2 binds with a greater affinity than C1C2 or C0. The amount of C0C2 bound to skinned fibers was more when endogenous MyBP-C is phosphorylated and the C1C2 site was open than when it was dephosphorylated and the actin site was open. This suggests that the affinity of the C1C2 site for myosin is greater than the affinity of C0 for actin. This interpretation is consistent with the persistence of the effect on contractility from C0C2 compared with C0.

Proline-rich C0C1 vs. PEVK of Titin

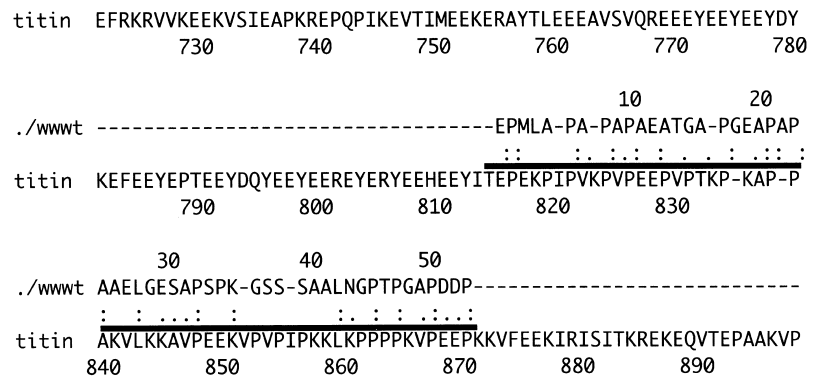


FIGURE 11. Comparison of the amino acid sequence of the proline-rich region of C0 with the sequence of the PEVK region of titin indicates a high degree of homology (58%).

These results suggest that phosphorylation determines where the NH₂ terminus of MyBP-C binds and the location of the NH₂ terminus, in turn, modulates contractility through an influence on the formation of weak bonds between actin and myosin (Levine et al., 2001). If this is the case, phosphorylated MyBP-C could act as a load on the contraction. At first thought this should have an undesirable effect on the development of force. However, with cardiac muscle (skeletal isoforms of MyBP-C do not contain C0 or phosphorylation sites) such a load could serve a useful function if cardiac MyBP-C is compliant. Some of the energy generated during systole would be stored in the compliance to assist in the filling of the heart during diastole. In the absence of phosphorylation of the motif between C1 and C2, C1C2 would bind to myosin, and the compliance of MyBP-C would not be in parallel with the force generators and not store energy for release during diastole.

There are four independent observations in the literature that support such a mechanism. Hofmann et al. (1991b) observed that partial removal of MyBP-C from isolated cardiac cells increased maximum velocity of unloaded shortening (V_{max}) at submaximal activation, an effect they attributed to removal of an internal load. Harris et al. (2002) observed impairment of diastole in their MyBP-C knockout preparations of heart. The rate of relaxation of an isolated heart is also enhanced by phosphorylation of cardiac MyBP-C (Hartzell, 1984). Calaghan et al. (2000) have found that excess S2, which would be expected to bind strongly to endogenous MyBP-C, slows relaxation. In addition, the rate of diastolic filling of the ventricles becomes progressively more important as the heart rate increases, and β adrenergic stimulation, which is one of the major mechanisms for increasing heart rate, also increases phosphorylation of MyBP-C.

For cardiac MyBP-C to store energy effectively during systole, it must not detach and reattach to actin during the contraction because detachment and reattachment would dissipate as heat the energy stored in the compliance. By analogy with the compliant molecule titin,

cardiac MyBP-C may have sufficient compliance and length for cardiac MyBP-C to remain attached to the same actin throughout a contraction. The amino acid sequence of the proline-rich region of C0 and C1 has a high level of homology with the compliant PEVK region of titin (58%, Fig. 11), and cardiac MyBP-C contains tandem Ig domains, the other major source of compliance in titin (Kellermayer et al., 1998; Trombitas et al., 1998; Granzier and Labeit, 2002). Under normal conditions, myocardial cells shorten 150–200 nm per half-sarcomere during a contraction that ejects ~50% of the ventricular contents. Since cardiac MyBP-C contains ~1,300 amino acid residues, its maximum length should be ~1,300 × 0.37 or 481 nm, but about half that length (C5–C10) probably forms a “collar” around the thick filament (Moolman-Smook et al., 2002), leaving a maximum of ~240 nm to connect actin to LMM during the contraction. This value is almost twice the required length to maintain attachment to the same actin, but the force required to extend MyBP-C the necessary 150 nm is not known. The distance between the surfaces of the thick and thin filaments is ~12–13 nm (the length of a skeletal MyBP-C is ~35 nm; Offer et al., 1973). Therefore, it is likely that cardiac MyBP-C is heavily folded in the resting muscle. Although these calculations demonstrate feasibility, in the absence of detailed information about the physical properties of cardiac MyBP-C, the existence of a compliance that aids diastolic filling can only be speculative.

Three different interventions should modify endogenous C0 interaction with actin and alter contractility: (a) disruption of endogenous C0 interaction with actin by exogenous C0 at a sufficient concentration; (b) extraction of MyBP-C; and (c) incubation with exogenous C1C2. All three result in similar changes in F_{max} and Ca sensitivity that are dependent on the state of phosphorylation of endogenous MyBP-C. All three probably have the same final pathway.

Disruption of C0 interaction with actin in *in vitro* preparations modifies Ca sensitivity, but an increase or a decrease in phosphorylation of MyBP-C in intact cells

does not change Ca sensitivity or the Hill coefficient. There is an important difference between decreasing endogenous C0-actin interactions with added C0 fragments in skinned fibers and decreasing phosphorylation of MyBP-C in intact cells. In the first case, the phosphorylated NH₂ terminus of endogenous MyBP-C will not bind to myosin when the endogenous C0 interaction with actin is disrupted by added C0 fragments. In the second case disruption of the C0-actin interaction is produced by the dephosphorylation of the motif, which will allow binding of the NH₂ terminus to myosin.

The results of this study indicate that phosphorylation of the NH₂ terminus of MyBP-C determines the distribution of binding between actin and myosin, which in turn regulates two properties of the force generators, the magnitude of a parallel compliance and the probability of myosin heads entering the force generating cycle. Through this mechanism, the strength of developed force, the power of the contraction of the heart and possibly the rate of diastolic filling can be varied. Myofibrillar proteins not directly involved in generating force can regulate cardiac contractility.

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