Multiple Structures of Thick Filaments in Resting Cardiac Muscle and Their Influence on Cross-Bridge Interactions

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ABSTRACT Based on two criteria, the tightness of packing of myosin rods within the backbone of the filament and the degree of order of the myosin heads, thick filaments isolated from a control group of rat hearts had three different structures. Two of the structures of thick filaments had ordered myosin heads and were distinguishable from each other by the difference in tightness of packing of the myosin rods. Depending on the packing, their structure has been called loose or tight. The third structure had narrow shafts and disordered myosin heads extending at different angles from the backbone. This structure has been called disordered. After phosphorylation of myosin-binding protein C (MyBP-C) with protein kinase A (PKA), almost all thick filaments exhibited the loose structure. Transitions from one structure to another in quiescent muscles were produced by changing the concentration of extracellular Ca. The probability of interaction between isolated thick and thin filaments in control, PKA-treated preparations, and preparations exposed to different Ca concentrations was estimated by electron microscopy. Interactions were more frequent with phosphorylated thick filaments having the loose structure than with either the tight or disordered structure. In view of the presence of MgATP and the absence of Ca, the interaction between the myosin heads and the thin filaments was most likely the weak attachment that precedes the force-generating steps in the cross-bridge cycle. These results suggest that phosphorylation of MyBP-C in cardiac thick filaments increases the probability of cross-bridges forming weak attachments to thin filaments in the absence of activation. This mechanism may modulate the number of cross-bridges generating force during activation.

INTRODUCTION

The importance of the myofibrillar protein myosin-binding protein C (MyBP-C) in the formation of the thick filament in striated muscle has become more firmly established as the results from several laboratories (Lin et al., 1994; Rhee et al., 1994; Okagaki et al., 1993; Gilbert et al., 1996; Maw and Rowe 1986) have been added to the earlier work of Offer et al. (1973), Craig and Offer (1976), Koretz (1979), and Davis (1988). Normal thick filaments with uniform thickness and length, central bare zones, and periodically arrayed cross-bridges do not form in the absence of MyBP-C. During the development of striated muscle, MyBP-C first appears in the sarcomere and not in the cytoplasm (Lin et al., 1994; Rhee et al., 1994). Thick filaments, sarcomeres, and MyBP-C all appear at the same time.

The cardiac isoform of MyBP-C differs from the two skeletal isoforms in several ways. The most striking difference is the presence of three phosphorylation sites in a region of the molecule located between modules C1 and C2 near the N terminus called the MyBP-C motif, (Kunst et al., 2000). An additional module (C0) is present at the N terminus. The thick filament in cardiac muscle contains a Ca-calmodulin-activated kinase (CAMK) that is specific for phosphorylating MyBP-C (Schlender and Bean, 1991; Hartzell and Glass, 1984). Protein kinase A (PKA) can also phosphorylate cardiac MyBP-C, making the filaments thicker and more loosely packed and increasing the order of the myosin heads (Weisberg and Winegrad, 1998).

Phosphorylation of cardiac MyBP-C appears to play a role in the modulation of contractility (Schlender and Bean, 1991; Hartzell and Glass, 1984; Gautel et al., 1995). Four different transmitters that increase contractility, α- and β-adrenergic agonists, endothelin, and Ca, all increase the degree of phosphorylation of MyBP-C. A fifth type of transmitter, cholinergic agonists, that reduces contractility also decreases the degree of phosphorylation of MyBP-C. When the ATPase activity of cryostat sections of quickly frozen rat ventricle is changed by varying concentrations of α- and β-adrenergic activity, MyBP-C is the only myofibrillar protein whose degree of phosphorylation consistently varies in the same direction (McClellan et al., 1994). All of these changes have been observed in preparations that retain the normal structure of filament lattice. In reconstituted systems of actin and myosin or subfragment 1, which contain the ATPase site, the extent of phosphorylation of MyBP-C has no effect on ATPase activity (Garvey et al., 1988). These apparently inconsistent observations can be reconciled if the effect of phosphorylation of MyBP-C on contractility occurs as a result of a change in the structure of the thick filament and/or the filament lattice.

Functional states of skeletal and cardiac muscles are reflected in the morphology of the elements of the contractile apparatus. The position and attitude of cross-bridges between thick and thin filaments differ during relaxation, rigor, and active contraction (Hirose et al., 1994). The

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organization of myosin molecules within thick filaments from striated muscles is quite specific with respect to their numbers, rotational symmetry, and helical and axial repeats. Nevertheless, both the surface array of myosin heads and the spacing of myosin rods in the backbone of the filaments can undergo considerable shifts with changes in environmental conditions such as alteration in osmotic pressure outside of the thick-thin filament lattice (Irving et al., 1998), nucleotide depletion (Vibert and Craig, 1985), phosphorylation of myosin regulatory light chains (RLC) (Craig et al., 1987; Levine et al., 1996), or MyBP-C (Weisberg and Winegrad, 1996, 1998). Many of these alterations in structure are correlated with changes in functional properties of the muscle.

The contractile properties of cardiac muscle are sensitive to calcium-regulated changes in the degree of phosphorylation of MyBP-C (McClellan et al., 2001). These changes occur in resting cardiac myocytes at intracellular concentrations of calcium below the threshold for activation of contraction. Lowering intracellular calcium concentration in the resting cardiac cells below the normal resting level decreases the value of maximum calcium-activated force (F_{max}), and raising the Ca concentration toward normal induces a recovery in F_{max} measured after the fibers have been chemically skinned. When the Ca concentration is restored to the normal level, phosphorylation of MyBP-C is increased as well.

Using a completely different approach, Kunst and coworkers (2000) have provided important evidence that MyBP-C and its phosphorylation alter the contractile properties of striated muscle. They have shown that fragments of MyBP-C containing the phosphorylation sites can bind to myosin near its hinge when the fragments are unphosphorylated. This interaction decreases F_{max}. Phosphorylation of the three sites on the MyBP-C fragment prevents the binding to myosin and the changes in F_{max}. The binding of the MyBP-C fragment occurs in the region of the myosin molecule where mutations cause hypertrophic cardiomyopathy (Gruen and Gautel, 1999).

In the study reported here we have examined the structure of the thick filaments isolated from cardiac muscle, the effect of phosphorylation of MyBP-C on filament structure, and the interaction of myosin heads with thin filaments. The results indicate that changes in filament structure are associated with different levels of phosphorylation of MyBP-C and that there appears to be a relationship between the structure of the thick filament and the probability of forming weak, non-force-generating bonds between actin and myosin in the resting muscle. The regulation of function of these weak bonds may be a mechanism for modulation of force development.

MATERIALS AND METHODS

Preparation of isolated filaments

Isolated thick filaments were prepared by a modification of the method of Levine et al. (1996; Weisberg and Winegrad, 1996). Hearts were dissected in oxygenated Krebs' solution (McClellan et al., 1994) from euthyroid rats weighing 150–250 g and containing almost entirely the α isoform of myosin heavy chain. The ventricles were opened, pinned out on Sylgard, and allowed to remain in oxygenated incubation solution (50 mM NaCl, 2.5 mM Mg acetate, 1.0 mM EGTA, 5 mM imidazole, 0.5 mM dithiothreitol, 5.0 mM ATP, 0.2 mM sodium azide plus a protease inhibitor cocktail containing 0.001 nM phenyl methyl sulfonyl fluoride, 0.1 mg/L L-*1*-chloro-3-[4-tosylamido]-4-phenyl-2-butanone, 0.1 mg/L *N*α-benzoyl-*b*-arginine methyl ester, 0.05 mg/L benzamidine, 0.01 ml/L aprotinin, 0.02 mg/L leupeptin, and 0.05 ng/500 μl pepstatin) for 1 h at room temperature. The ventricles were then frayed with a sharp scalpel. The tissue with its solution was placed into a refrigerator at 4°C overnight. The following morning the tissue was transferred to 200 μl of fresh incubation solution at room temperature to which 80 μl of 2 mg/ml each of soybean trypsin inhibitor and elastase had been added. After 3 min the tissue was vigorously agitated by shaking and repeated pipetting. The solution with the tissue was centrifuged in a Fisher microcentrifuge (model 59A) for 3 min at a setting of 2.5 (out of 10). The pellet containing the filaments was resuspended in 150 μl of the same solution.

Gels showed no loss of actin, tropomyosin, or any of the three troponin subunits during the isolation of the filaments.

In experiments measuring the effect of PKA on filament structure, the filament solution was divided into two parts for control and protein kinase treatment. Calmidazolin, calyculin A, and cAMP were added to each of the two solutions to a final concentration of 10 μM, 100 μM, and 10 μM, respectively. The calmidazolin and calyculin inhibit, respectively, calmodulin and phosphatase. PKA from bovine heart (Sigma, St. Louis, MO) was added to one of the two solutions to a final concentration of 100 μg/ml of solution. Therefore, one solution contained ATP, PKA, and cAMP to phosphorylate MyBP-C, EGTA, and calmidazolin to prevent phosphorylation of the regulatory light chain of myosin (LC2) and calyculin to block phosphatase, whereas the other solution did not contain PKA. The control and phosphorylating solutions were incubated in a shaking water bath at 30°C for 30 min. They were then centrifuged in the microcentrifuge for 3 min, and the pellet was resuspended in solution without PKA or calmidazolin. Then, 20 μl of suspension was placed on carbon-coated grids and washed five times with the same solutions in which they had been suspended. The fluid was removed each time by absorption into a filter paper. The grids were negatively stained in 1% uranyl acetate containing one drop of glycerol per milliliter of solution for 30 s. The uranyl acetate solution was removed by wicking off most of the stain and allowing the remaining liquid to evaporate in air at room temperature.

Thick filaments lying on the carbon over the holes in the grids were viewed with a JEOL 100CX transmission electron microscope operating at 80 kV with an anti-contamination device in use and photographed at ×19,000 and ×36,000 magnification. The electron micrograph negatives were diffracted in a laser optical diffractometer (2 mW helium-neon 632.8 nm) using a diffractometer camera that had been calibrated from diffraction patterns of micrographs of catalase crystals. Measurements of diffraction pattern spacings were made on a large print of the transform. Preparations from 18 different hearts were made, and ~80 micrographs were made from each preparation. Each micrograph contained on average 13 thick filaments. The number of preparations and micrographs were selected to achieve statistical significance for the results.

The relative intensity of specific reflections in an optical diffraction pattern was determined by a direct comparison among optical diffraction patterns from different micrographs (see below). A second method, in which the intensity of the reflection was compared with the average intensity of the reflections along the meridian between 43 and 14 nm to provide an internal standard in each film, gave similar results as long as the exposure and development times were the same.

For measurement of the effect of Ca on filament structure, three sets of cardiac muscles, each consisting of four thin trabeculae and two separated papillary muscles, were isolated from adult rat hearts and allowed to recover for 20–30 min in oxygenated Krebs' solution with a Ca concentration of 2.5 mM (McClellan et al., 2001). One set was maintained in

solution of the identical composition while the remaining two sets were placed in Krebs' solution with a Ca concentration of 1.25 mM. After 120 min, half of the tissues bathed in 1.25 mM Ca were transferred to Krebs' solution with a Ca concentration of 7.5 mM for an additional 10 min. Following these incubations, each of the three sets was chemically skinned with 1% Triton X-100 for 1 h at room temperature in standard relaxing solution (Levine et al., 1996) containing a protease inhibitor cocktail (PIC). The tissues were then rinsed in several changes of relaxing solution with PIC. The thick filaments were separated from the tissues (combined within each set) as described. Droplets of the filament suspensions were adsorbed onto thin-to-medium carbon films on copper electron microscope grids and negatively stained with aqueous uranyl acetate.

Grids were examined and photographed, and their diameter and structure were determined. Images of thick filaments on negatives were subjected to optical diffraction. The numbers of thin-thick filament contacts were counted on prints made from each electron micrograph negative. Measurements were made by two people who did not have knowledge of the experimental protocols for each micrograph.

Measurement of the thick filaments

The structure of thick filaments fell into three groups according to diameter of the filament and degree of order of the myosin heads (see Results). To determine which of three structures each thick filament had, a procedure similar to that already described was used (Weisberg and Winegrad, 1998). Only filaments showing a clear bare zone with a diameter 18–20 nm, a length of ~1.6 μm, and tapered ends were included in the data. Filaments were judged to be ordered if there was a regular periodicity of ~40 nm, myosin heads were not extended from the backbone of the filament at different angles, and the optical diffraction pattern produced by the micrograph of the filament contained clear reflections along the 43-nm layer line (see below). Filaments that did not meet these criteria were considered to be disordered. Ordered filaments were divided into two groups according to their diameters measured along the C zone where the diameter is uniform. Previous work (Weisberg and Winegrad, 1996, 1998) had shown that ordered thick filaments isolated from rat ventricle fall into two distinctly separate populations according to their diameters with average values of ~30 and 34 nm. Because the myosin heads of the ordered filaments lay along the filament, there was no significant difficulty in measuring the diameter. Diameter was not necessary as a criterion in categorizing disordered filaments. The thickness of these filaments was often difficult to establish because of the extension of myosin heads to different degrees at different angles.

The actual measurement of filament diameter was made independently by two different people who were unaware of the experimental protocols of each micrograph and who used different techniques. Electron micrographs were visualized through a microscope with a calibrated scale in one eyepiece at a magnification of up to ×100, or the micrographs were projected onto a calibrated scale. Thickness of the filament at several points was measured, averaged, and allocated to a series of bins from 24–39 nm for determining distribution among structures. There was no difference in the values measured by the two different methods.

The ordered filaments in this study fell into the same two groups as previous studies. In the two independent sets of experiments diameters were 29.8 ± 0.12 nm and 34.2 ± 0.8 nm in one set and 30.6 ± 0.7 nm and 34.4 ± 0.3 in the second set (see Results). Based on this distribution, all ordered filaments with diameters between 32 and 37 nm were considered to be in the thicker group (referred to as loose structure in Results), and all filaments with diameters between 28 and 32 nm were in the thinner group (tight structure). Because the variation of the diameter within each group was only a very small fraction of the average filament diameter, most filaments differed from the two mean values by less than 1 nm.

These same procedures were used for classifying the structure of thick filaments with interactions with thin filaments. In these cases the diameter of the filament was measured outside the zone where interactions between the filaments existed.

Means, standard deviations, and standard errors of the mean were calculated for each parameter measured within each set of cardiac muscle tissues.

Quantitative analysis of optical diffraction patterns

The electron micrographs of over 1000 filaments were subjected to optical diffraction as previously described (Weisberg and Winegrad, 1996, 1998). A mask around the image of the filament in the micrograph was used to eliminate interference from other filaments in the same micrograph and allow only one filament to produce the diffraction pattern. Because the mask itself can produce reflections along the meridian and the equator, and some subjectivity may be involved in separating those from true thick filament reflections, only reflections off the meridian and along well established myosin layer lines were used in judging thick filament structure. The reflections along the 43-nm layer line are produced by the helically arranged myosin heads and are the most useful in evaluating the degree of order of the myosin heads. This layer line normally has stronger reflections than the 14.3-nm layer line also produced by myosin heads (Matsubara 1980). For this reason the degree of order of myosin heads was evaluated by the relative intensity of the reflections along the 43-nm layer line in one quadrant of the optical diffraction pattern.

The intensities of reflections along the 43-nm layer line in the optical diffraction patterns of isolated thick filaments were compared and used as indication of the relative degree of order of myosin heads in the thick filament. Up to three separate reflections along the 43-nm layer line were seen with ordered filaments. To make quantitative comparisons of the intensities of reflections along the 43-nm layer line, each optical diffraction pattern was digitized and analyzed by the NIH Image program. The intensity of each reflection along the 43-nm layer line in one quadrant was measured after the value for the background (where there were no reflections) had been subtracted. There was no significant difference in the background among the diffraction patterns. The intensity of each reflection was multiplied by its area. The sum of the intensity times the area for all of the reflections along the 43-nm layer line in a single quadrant was used as a measure of the degree of order in the filament. As a further check to insure that there was no variability in the method of recording of the diffraction patterns, the intensity of each reflection was normalized to the average intensity of the reflections along the meridian between 43 and 14 nm. This procedure, which provided an internal standard in each film, gave the same results as the direct comparison among diffraction patterns as long as the exposure and development times were the same.

Measurement of phosphorylation of MyBP-C

The extent of phosphorylation of MyBP-C was measured in aliquots of preparations of isolated thick filaments or from preparations treated in parallel according to the same protocols. The several phosphorylated forms of MyBP-C were extracted and identified by Western blotting of isoelectric focusing gels as described elsewhere (McClellan et al., 2001).

Statistics

Populations were compared using ANOVA, χ^2, and regression analysis. Differences were considered significant when $p < 0.05$.

RESULTS

Control preparations of isolated thick filaments

Native thick filaments isolated from rat ventricle exhibit one of three different structures as defined by the thickness of

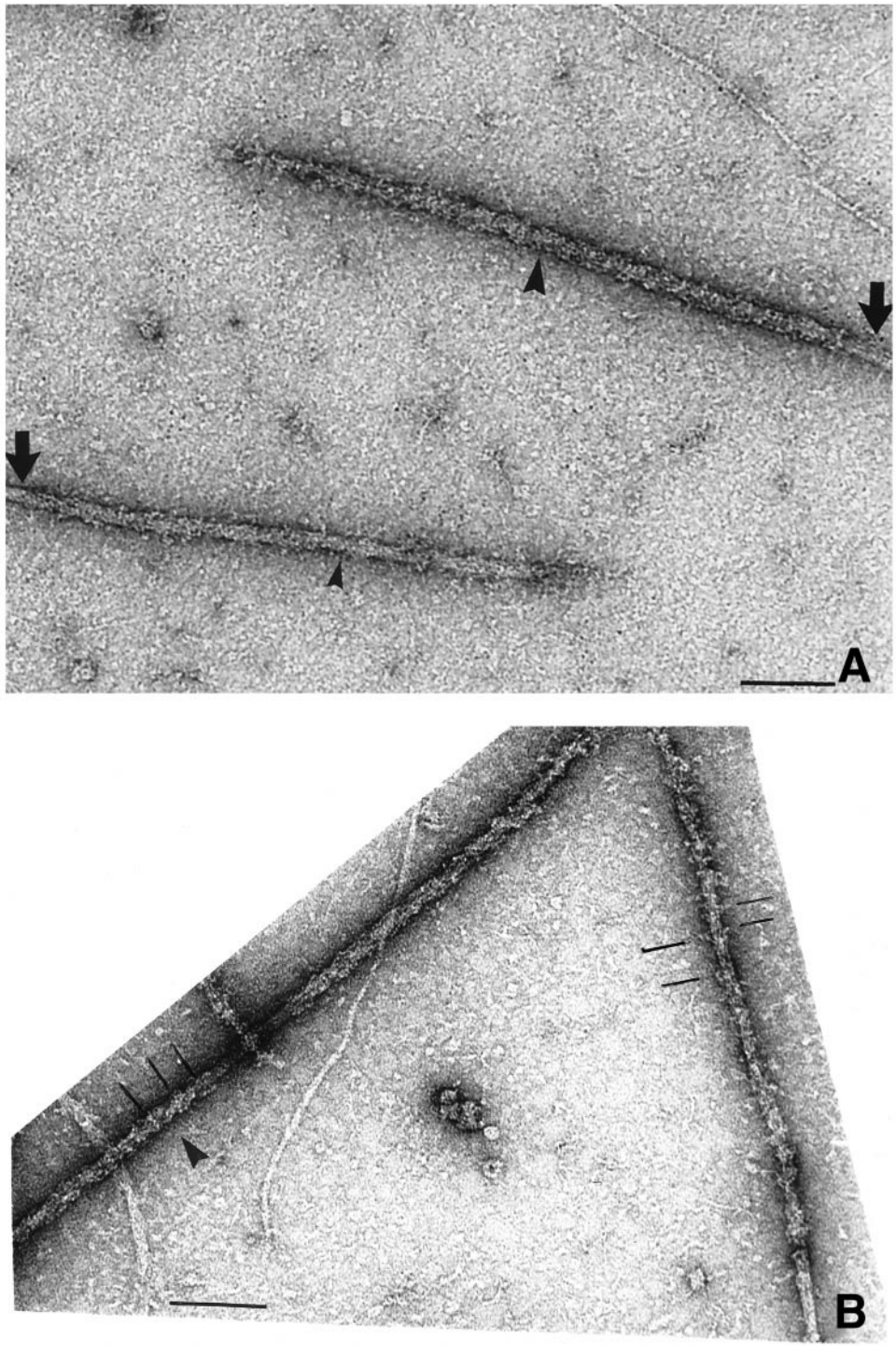
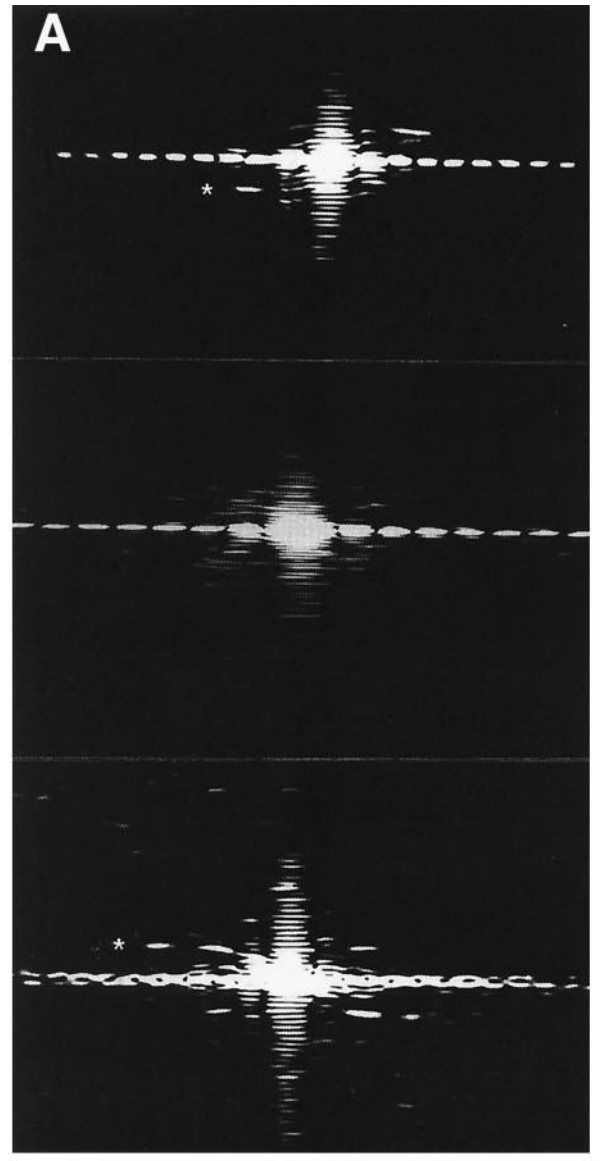
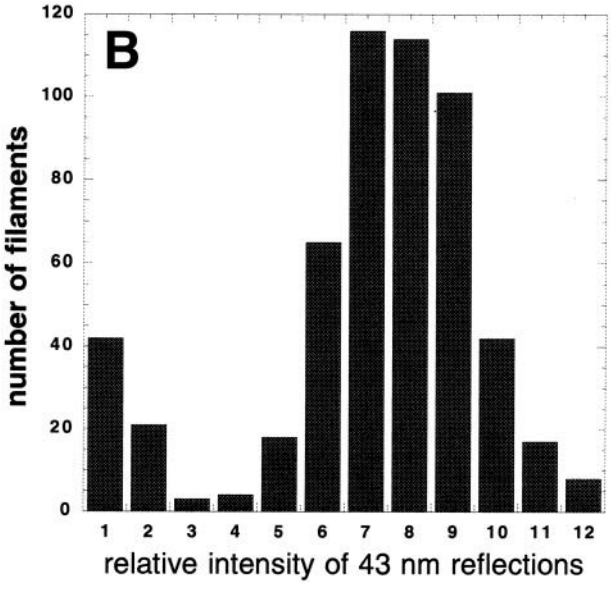


FIGURE 1 Electron micrographs of negatively stained isolated thick filaments showing the three different structures observed. (*A*) Micrograph shows two different thick filaments with tight, thinner (smaller tailless arrow) and loose, thicker (larger tailless arrow) structures from the same preparation. Arrows with tails indicate the bare zone in the filament. (*B*) Two different thick filaments with ordered (loose structure shown with large tailless arrow) and disordered structures from the same preparation. Lines indicate periodicity in the ordered filament and extended myosin heads at different angles to the filament backbone in the disordered filament. Bars, 0.1 μm.

the filament and the degree of order of the myosin heads (Figs. 1 and 2). In one structure, thick filaments have an average diameter outside the bare zone of 29.8 $\pm$ 0.12 nm. The myosin heads lie along the backbone of the filament and produce a repeating structure with a periodicity of ~43 nm visible as a mildly convex contour on the surface of the





filament. In the optical diffraction pattern produced by filaments in the micrographs there are reflections on the meridian as well as on layer lines characteristic for helically arranged myosin heads in the filament. This structure has been called the tight structure because it has the narrowest diameter of ordered thick filaments (Fig. 2) (Kensler and Stewart, 1993). In the second structure, myosin heads are extended from the thick filaments at different angles to the backbone of the filament and are highly disordered. The myosin heads arise from the surface of the filament at ~43-nm intervals, but they extend at different angles from the backbone of the filament. Because of this variability in angle, the mass of the myosin heads is not distributed at uniformly repeating intervals. This produces an image with visibly disordered myosin heads and an absence of reflections along the 43-nm layer line (Fig. 2). Therefore, this filament is said to have a disordered structure. The filaments with the third structure are thicker, having an average diameter of 34.2 $\pm$ 0.8 nm. The myosin heads were ordered and not obviously extended. The high degree of regularity in the position of the myosin heads results in strong reflections along the 43-nm layer line in optical diffraction pattern (Fig. 2). Although there is insufficient resolution to determine whether the increase in diameter is solely due to looser packing of the filament backbone or to the position of the myosin heads, the filament appears to be less tightly packed than in either of the other structures. Therefore, these filaments are said to have a loose structure.

It is possible that the increase in diameter of the thick filaments could have been a result of their being flattened on the carbon film by the stain, but this is highly unlikely because both tightly and loosely packed filaments were seen on the same grid square (as shown in Fig. 1).

The relative amounts of each of these structures vary with the speed of isolation of the cardiac tissue and its subsequent incubation. The degree of variation is largely reduced if the time taken during the dissection and removal of the heart is consistent and the isolated tissue is allowed to recover from the dissection in oxygenated, continuously mixed Krebs' solution containing 2.5 mM Ca. Under these conditions the majority of filaments have the tight structure,

FIGURE 2 (*A*) Optical diffraction pattern of filaments with the three different structures. Asterisks indicate the 43-nm layer line. (*Top*) Tight structure showing strong reflections along the 43-nm layer line; (*Middle*) Disordered structure showing weak diffraction pattern; (*Bottom*) Loose structure showing multiple strong reflections along the 43-nm layer line. (*B*) Histogram showing the distribution of filaments separated from control hearts according to the relative density of reflections along the 43-nm layer line in the optical diffraction pattern produced by electron micrographs of individual filaments. Hearts had been soaked in Krebs' solution with 2.5 mM Ca at rest for 2 h. Intensity of the reflections has been arbitrarily divided into 12 bins. Bin 1 contains patterns with no reflections along the 43-nm layer line, and bin 12 contains those with the highest intensity of reflections.

and the least common of the three structures is the disordered structure. The remaining filaments have the loose structure. The total number of thick filaments examined by visualization and optical diffraction of the micrographs to determine the distribution among the three structures exceeded 500. Filaments were classified as loose when their diameters exceeded 32 nm and there were strong reflections along the 43-nm layer line in the optical diffraction pattern. Filaments with diameters less than 32 nm and at least one strong reflection along the 43-nm layer line were considered to be tight. The disordered filaments were easily identified by a combination of myosin heads at different angles to the filament backbone and the absence of any strong reflections along the 43-nm layer line. In the histogram of relative intensity of the reflections (Fig. 2 *B*) there is a clear separation between filaments with disordered myosin heads (bins 1,2) and those with varying degrees of order (bins 5–12). A more rigorous analysis (not shown) indicates that there are probably two separable populations within bins 5–12 with two different levels of order corresponding to tight and loose structures. In the eight preparations used in this study the average distribution of the three structures was 58 ± 5% tight, 12 ± 4% disordered, and 30 ± 4% loose structure (Fig. 2). There was no significant difference in this distribution when only visual inspection was used to assign filaments to one of three structural categories.

PKA-treated filaments

As has already been reported in detail (Weisberg and Winegrad, 1998), the vast majority (92 ± 5%) of thick filaments in which MyBP-C has been specifically phosphorylated with PKA have the loose structure. These studies were repeated (with the same results) to provide a nearly homogeneous population of filaments with the loose structure for study with thin filaments. Treatment with PKA produced 86 ± 4% of maximum phosphorylation of three phosphates per molecule of MyBP-C.

Influence of Ca on thick filament structure

Because a Ca-calmodulin-activated kinase can phosphorylate two thick filament proteins (MyBP-C and RLC), we examined the effect of different extracellular concentrations of Ca on the relative distribution of the three structures of the thick filament. Trabeculae were isolated from rat right ventricle and allowed to recover from the dissection for 20–30 min in normal Krebs' solution without stimulation. The reason for maintaining quiescence was to separate the effects of phosphorylation of MyBP-C from those due to phosphorylation of RLC. In the absence of stimulation and with the maintenance of intracellular Ca below the threshold for activation of contraction, RLC is less than 5% phosphorylated (McClellan et al., 2001). After the period of recovery, quiescent trabeculae were treated in one of three different ways. They were soaked in Krebs' solution containing 2.5 mM or 1.25 mM Ca for 120 min, and then half of the trabeculae soaked in low Ca were bathed in Krebs' solution containing 7.5 mM Ca for an additional 10 min.

The structure of the majority of the isolated thick filaments, judged by the diameter of the filament and its optical diffraction pattern, was different for each of the three protocols (Figs. 3–5). The structure of the vast majority of filaments separated from cardiac muscle that had been exposed to 2.5 mM Ca throughout the experiment was indistinguishable from the tight structure described above. They were generally ordered, with myosin heads arranged along the filament backbone (Kensler and Stewart, 1993). These filaments produced diffraction patterns that displayed myosin layer lines (Fig. 4). A bare zone, free of myosin heads, occupied the central 0.18–0.20 μm, and each intact filament (~1.6 μm long) displayed tapered ends.

In each experiment at least 70% of the thick filaments separated from quiescent cardiac muscle that had been soaked in 1.25 mM Ca for 120 min had a disordered structure (Figs. 3–5). The myosin heads extended from the backbone at various angles and to varying distances, producing disorder of the normally helical arrangement on the surface of relaxed filaments. Myosin layer lines were largely absent from diffraction patterns obtained from images of these filaments and when present were very weak (Fig. 4 *B*). The location, appearance, and extent of the bare zones were the same, regardless of the concentration of Ca in the Krebs' solution. An additional 10-min exposure to 7.5 mM Ca following the 120 min in 1.25 mM calcium had a dramatic effect on the structure of the thick filaments. The myosin heads once more appeared to be well ordered (Fig. 4 *C*), even more so than in filaments soaked for 120 min in solution with 2.5 mM calcium. The periodicity produced by the ordered heads of a helical array was more pronounced than before. Diffraction patterns obtained from images of these filaments had strong reflections along myosin layer lines especially the 43-nm layer line (Fig. 4 *C*). The filaments from muscle bundles soaked in 7.5 mM Ca had significantly greater diameters along their myosin head-bearing limbs than did any of the other filaments (Fig. 5). The diameter, position, and length of the bare zones, however, remained unchanged. These filaments were indistinguishable from the filaments with loose structure described above.

The relative distribution of each of the three structures among filaments from each of the three protocols can be inferred from the diameter of the filaments (Fig. 5). The diameter of filaments from muscles soaked only in 2.5 mM Ca had a Gaussian distribution with a peak at 30 nm, a mean of 30.6 ± 0.7 (n = 17), and a skew toward higher values. The diameter was the same as that of the majority of filaments in the control set described above. The skew toward higher values is due to the presence of a small

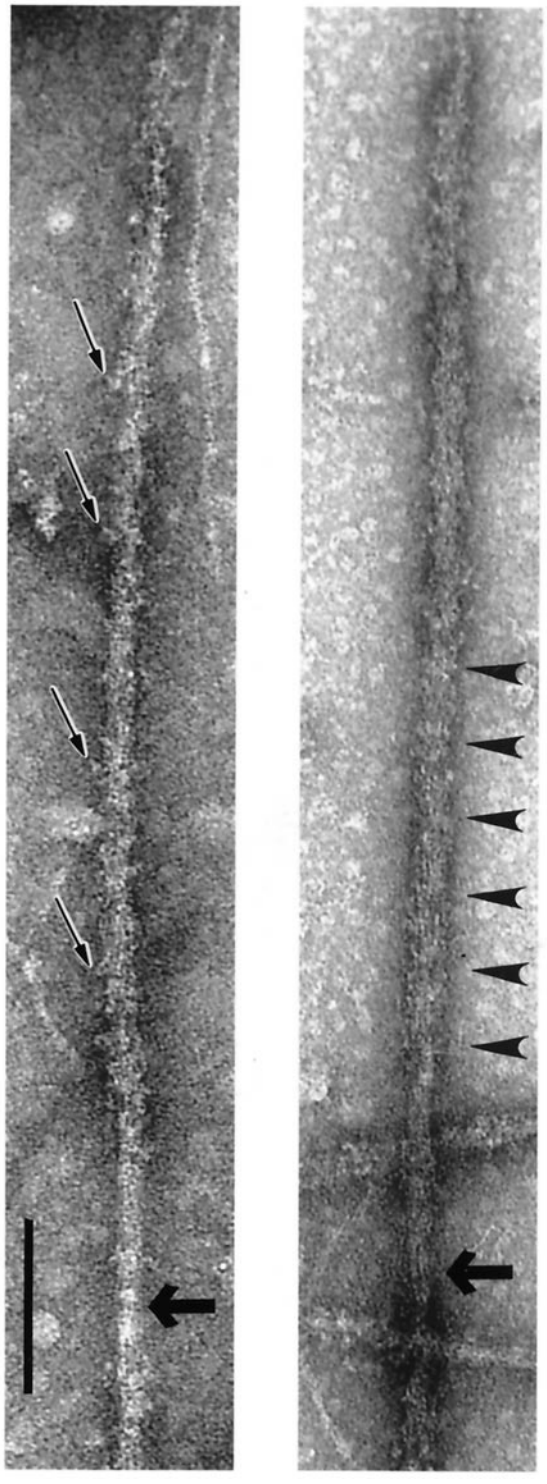


FIGURE 3 Electron micrographs of the predominant structures of negatively stained thick filaments isolated from quiescent rat ventricles that had been soaked for 120 min in Krebs' solution containing 1.25 mM Ca (*left*) and 1.25 mM Ca followed by 10 min with 7.5 mM (*right*). Note the difference in thickness and order between left and right. Thick arrows with tails indicate bare zones. Thin arrows with tails indicate extended myosin heads at different angles to the filament backbone in the disordered filament. Tailless arrows indicate periodicity in ordered filaments on the right. Bar, 0.1 μm

percentage of loose (thicker) filaments. The diameters of thick filaments from tissue that had been soaked for 120 min in 1.25 mM Ca were uniformly distributed between 24 and 39 nm except for a small peak at 30 nm ($n = 31$). Such a broad range of measurements of diameter would be expected with disordered and extended myosin heads forming a variety of angles with the backbone of the filaments. These filaments had either no reflections or weak ones along the 43-nm layer line (Figs. 4 *B* and 5). The peak in diameter at 30 nm is the result of a small fraction of ordered (tight) filaments. The distribution of diameters of filaments from cardiac muscle treated for an additional 10-min in 7.5 mM calcium after an initial 2 h in 1.25 mM calcium differed from both of the other two sets of filaments ($n = 54$). The peak lay between 33 and 36 nm with a mean of 34.4 ± 0.3, and the distribution was normal with a skew toward lower values due to the presence of a small number of filaments with smaller diameter (tight). This is the same average diameter as filaments treated with PKA.

The quantitative measurements of diameter in the micrograph and intensity of 43-nm reflections in the optical diffraction pattern of the three different populations of thick filaments confirm the impression drawn from visual inspection of electron micrograph images of individual filaments. Filaments soaked in 2.5 mM Ca show better surface order than those soaked in 1.25 mM Ca. Ten minutes in 7.5 mM Ca restores helical array to myosin heads and looser packing of myosin in the filaments.

Interactions between thick and thin filaments

Isolated thick and thin filaments were prepared in a solution containing 5 mM Mg ATP without Ca to prevent both formation of rigor links between the two types of filaments and activation of force-generating cycling of myosin heads. There was no significant difference in either number of filaments per square micron or ratio of thin to thick filaments between control and the PKA-treated preparations (Table 1). Thin and thick filaments were counted if at least a half a length (0.5 and 0.8 μm for, respectively, thin and thick filaments) was visible in the micrograph. This result indicated that the type of structure of the thick filament did not influence the concentration of thin filaments in a preparation.

A thin filament was considered to be near a thick filament if it lay within 16 nm for a distance of at least 86 nm, which is six times the periodicity of the myosin heads and twice that of a three-stranded thick filament (the cardiac thick filament is generally agreed to be three-stranded). The value of 16 nm was chosen because it is near the length of an extended myosin head ($\sim$20 nm) and is similar to the separation of thick from thin filaments in the normal filament lattice ($\sim$14 nm). By this criterion, the frequency of thin filaments near thick filaments was significantly greater in preparations treated with PKA ($p = 0.03$; Table 1).

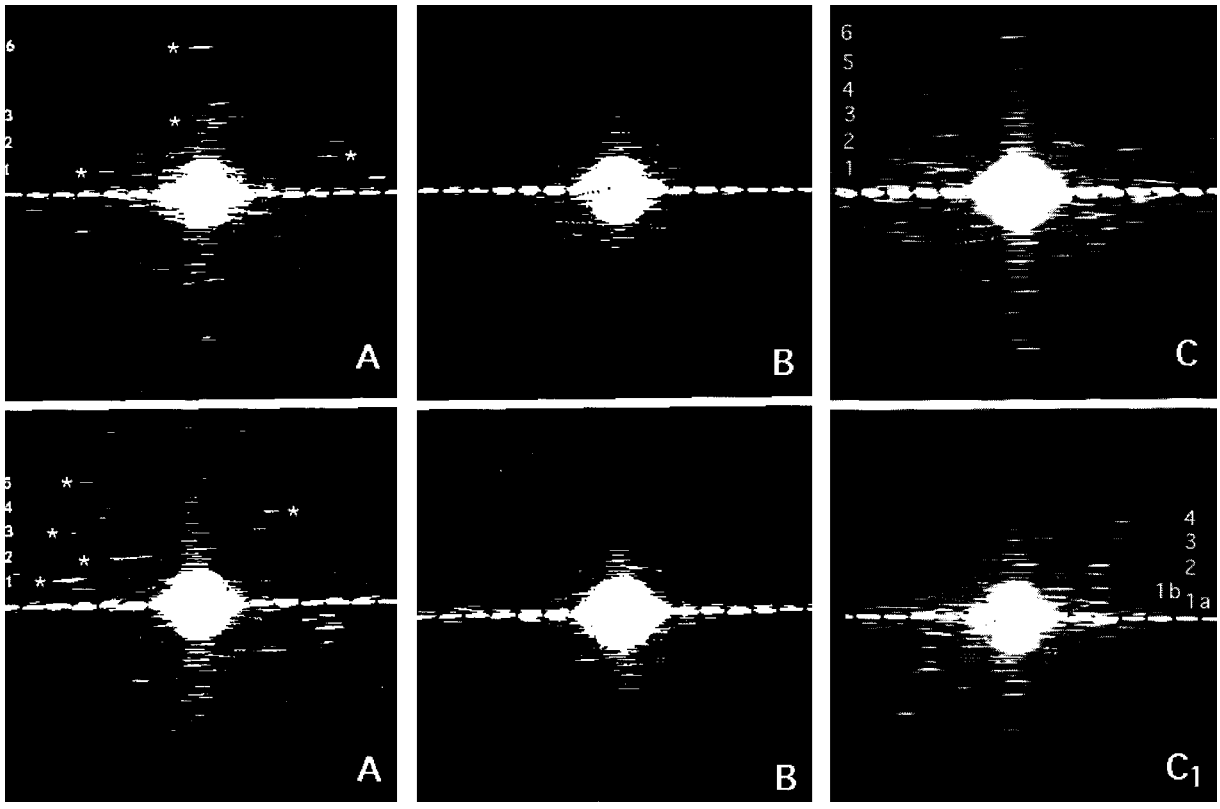


FIGURE 4 Optical diffraction patterns of negatively stained thick filaments isolated from quiescent rat ventricle that had been soaked for 120 min in Krebs' solution containing 2.5 mM Ca (*A*), 1.25 mM Ca (*B*), and 1.25 mM Ca followed by 10 min with 7.5 mM (*C*). Two examples from the most common structures in each protocol are shown. Myosin layer lines in the diffraction pattern are indicated by numbers in *A* and *C* and asterisks in *A*. The myosin layer lines are absent in *B*. The presence of reflections along myosin layer lines in *A* and *C* but not *B* indicates orderly arrangement of myosin heads in *A* and *C* but not in *B*. In *C*, two different examples of optical diffraction patterns are shown. One is from an ordered thick filament (*C*), and the second is from an ordered thick filament with cross-bridges extended to the thin filament (C_1). Myosin layer lines are present in the diffraction pattern from both thick filaments. In the diffraction pattern from the thick filament with cross-bridge connections to a thin filament (C_1) there is an additional layer line (*1b*) consistent with a repeat periodicity along the thin filament (~36–39 nm).

An interaction between thick and thin filaments was considered to exist if a thin filament was parallel to a thick filament with no more than 16-nm separation between the two filaments for a distance of at least 86 nm. Structures with approximate periodicities of 14 or 43 nm had to run between thick and thin filaments (Fig. 6).

Among the PKA-treated preparations, 644 thick filaments were examined, of which 58 had parallel thin filaments nearby (Tables 1 and 2). Ninety-two percent of thick filaments parallel to nearby thin filaments had cross-bridges extending to the surface of the thin filaments with 43-nm periodicity (Figs. 6 and 7). Of the 3017 thick filaments from untreated preparations examined, 147 had parallel thin filaments nearby. Only 47% of the thick filaments parallel to and near thin filaments in the untreated preparations had cross-bridges extending to the thin filaments with a 43-nm periodicity (Fig. 7). This difference is highly significant ($p < 0.01$).

The untreated preparations contained a mixture of all three types of structures, including some thick filaments with the loose structure. The relative amount of each type of structure was estimated by measurement of the thickness, visual estimate of the degree of order, and the intensity of the reflections along the 43-nm layer line in the optical diffraction pattern (Table 2). Twenty-nine percent of the parallel thick and thin filaments with tight structure had myosin heads periodically extended to the thin filaments, and eighty-nine percent of the loose structures had similar interactions. Because of the disorder of myosin heads in the disordered thick filament, it is more difficult to judge whether there was a similar interaction. Sixteen percent were considered to have interactions, but this could be an overestimate.

In 83% of the thick filaments in the PKA-treated preparations with myosin heads extended to the thin filament, the myosin heads on the opposite side were well ordered but not extended (Fig. 6). They lay as convex densities along the backbone of the filament. This observation strengthens the interpretation that the myosin heads were not merely extended, but were interacting with the thin filaments.

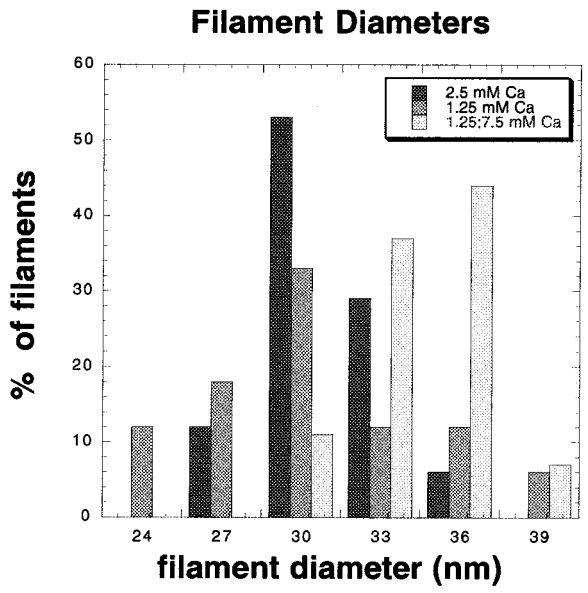


FIGURE 5 Bar graph showing the distribution of thickness of thick filaments among thick filaments isolated from hearts soaked for 120 min in Krebs' solution containing 2.5 mM Ca, 1.25 mM Ca, or 1.25 mM Ca followed by 10 min with 7.5 mM. Each thick filament was placed in the bin with the closest value from a series of bins of different thickness. Note the difference in the thickness of filaments with different structures and the primarily random distribution of the thickness of filaments soaked in 1.25 mM Ca, although there is a peak at 30 nm. Diameters of filaments soaked in 2.5 mM Ca have a Gaussian distribution, peaking at 30 nm and skewed toward the higher values. Diameters of filaments soaked in 1.25 mM Ca also have a peak value at 30 nm but are widely distributed. Diameters of filaments soaked first in 1.25 mM and then 7.5 mM Ca are significantly different from the others ($p < 0.001$) with a mean of 34 nm and a skew toward lower values.

Among the nearby thick-thin filament pairs, the frequency of structures connecting the two filaments also differed with the concentration of Ca during the incubations before separation of the filaments (Fig. 7). Particularly striking was the difference produced by 10 min in 7.5 mM Ca after 120 min in 1.25 mM Ca. The occurrence of cross-bridges linking thick and thin filaments separated from trabeculae exposed to different concentrations of Ca was quantified using the same criteria as described above. The effect of the Ca concentration on both the proximity of thick and thin filaments and interactions between nearby thick-thin filament pairs was pronounced. Of 11 micrographs of filaments from muscle soaked in 2.5 mM Ca, 10 showed neighboring thick and thin filaments. In 6 of these there were periodic links connecting the thick and thin filaments. Of 29 micrographs of filaments from muscles that had been soaked in 1.25 mM Ca, only 8 had nearby thick and thin filaments with periodic links running between them. When the soak in 1.25 mM Ca was followed by 10 min in 7.5 mM Ca, the results were quite different. Of 24 micrographs, 22 had neighboring thick and thin filaments with periodic structures connecting the two. The number of periodic repeats along the filaments varied from 4 to 12, with a majority having the higher values. The percent of interacting filament pairs was increased from 36% to 91% by the 10 min in 7.5 mM Ca.

TABLE 1 Frequency of parallel thick and thin filaments

	Total number of thick filaments	Ratio thin/thick	Number of parallel pairs	% parallel
Untreated	3071	1.59	147	4.8
PKA treated	644	1.28	58	9.0*

*$p < 0.05$

Thus, the frequency of interactions between myosin heads on thick filaments with thin filaments was significantly greater when the thick filaments showed greater helical order regardless of whether the degree of order was induced by Ca or PKA ($p = 0.0034$; Fig. 7). This was the case after the muscle had been soaked in 2.5 mM Ca or in 7.5 mM Ca after a period in 1.25 mM Ca. Because ordered myosin heads in the tightly structured filaments were more likely to have interactions with thin filaments than extended disordered myosin heads, the distance of the myosin heads from the axis of the thick filaments appears to be less important than the degree of helical order of the heads.

Optical diffraction patterns of paired thick and thin filaments with periodic connecting structures contained reflections along an ~36-nm layer line (Fig. 4 C_1), which is a characteristic period for thin filaments. Optical diffraction of separate thick and thin filaments did not contain this reflection. The reflection, present only when there were periodic structures that appeared to be cross-bridges, indicates that cross-bridges are probably attached to actin in the thin filament in a site-specific manner.

Relation between filament structure and phosphorylation of MyBP-C

In view of the apparent importance of MyBP-C in the formation of thick filaments and the effect of PKA-induced phosphorylation of MyBP-C on the structure of the thick filament, the frequencies of given thick filament structures at different degrees of phosphorylation of MyBP-C were compared with electron microscopy and optical diffraction. Two types of experiments provided the data: measurements of phosphorylation and determination of structure carried out on the same set of trabeculae and measurements on trabeculae from different sets that had been treated in the same fashion. Exposure to PKA or to different concentrations of extracellular Ca and to 0.1 μM isoproterenol was used to produce the different structures and levels of phosphorylation. The results with the two types of data were not significantly different and therefore have been lumped together (Table 3; Fig. 8). There is an excellent direct correlation between the relative amount of unphosphorylated,

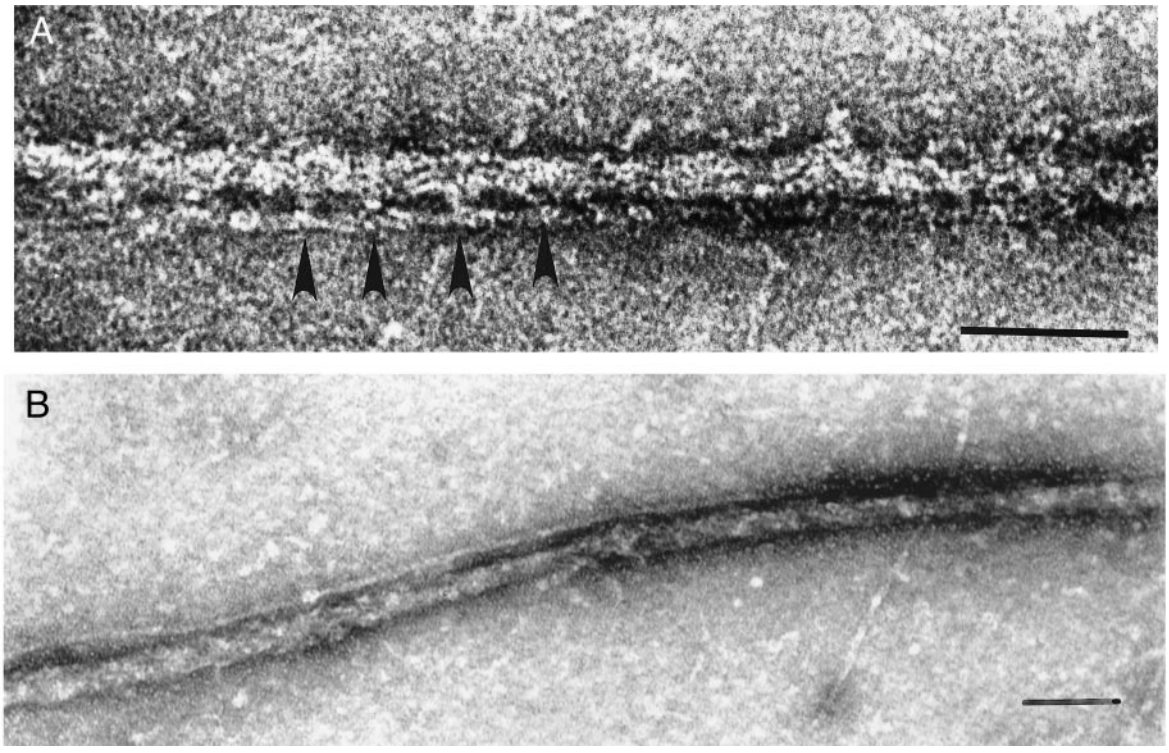


FIGURE 6 Electron micrographs of thick and thin filaments lying parallel and close to each other. (*A*) Thick filament with loose structure showing cross-bridges extended to the thin filament with 43-nm periodicity (*arrows*). Cross-bridges on the side of the thick filament away from the thin filament are not extended. (*B*) Thick filament with tight structure showing parallel thick and thin filaments close together but with no periodic cross-bridges extending from the thick to the thin filament. Bar, 0.1 μm; ionic strength = 0.05 mM.

monophosphorylated, and di- plus tri-phosphorylated MyBP-C and the relative amount of, respectively, disordered, tight, and loose structures. There is also an excellent inverse correlation between the relative amount of disordered and loose structures and the relative amount of, respectively, di plus tri- phosphorylated and unphosphorylated MyBP-C.

Fig. 8 shows the shape of the relation between the relative amount of each of the structures and the relative amount of phosphorylated MyBP-C. As the phosphorylation increases, the fraction of thick filaments with disordered structure falls exponentially and the amount of loose structure rises exponentially. The relative amount of the tight structure rises, reaches a maximum, and then falls.

TABLE 2 Frequency of interactions between thick and thin filaments

	% of the total thick filaments	Number of parallel pairs	% of parallel pairs interacting
Untreated tight	58	69	29
Untreated disordered	12	6	16
Untreated loose	30	72	89*
Average of total untreated			47†
PKA-treated loose	84	58	92*

*Significant difference ($p < 0.05$) from tight or disordered filaments.
†Significant difference ($p < 0.05$) between averages of untreated and PKA-treated filaments.

DISCUSSION

Thick filaments in resting mammalian cardiac muscle can assume three different structures, all of which can be present at the same time in the tissue. Because filaments are isolated from a population of cardiac myocytes, it is not possible to determine whether all three structures exist in a single cell at the same time. It is, however, likely that the distribution among nearby cells is very similar because actomyosin ATPase activity and its response to stimulation with β-adrenergic agonists or PKA are normally uniform among the myocytes (Winegrad et al., 1986). The relative proportion of the three structural forms can be influenced by the events immediately before and during the removal of the heart from the animal and by the conditions of incubation of the isolated tissue. The existence of three different structures was suggested in a previous study (Weisberg and Winegrad, 1998) by the distribution of filament thickness. The current work provides a more complete description of these different structures and the functional implications of the different structures.

There is an excellent correlation between the degree of phosphorylation of MyBP-C and the structure of the thick

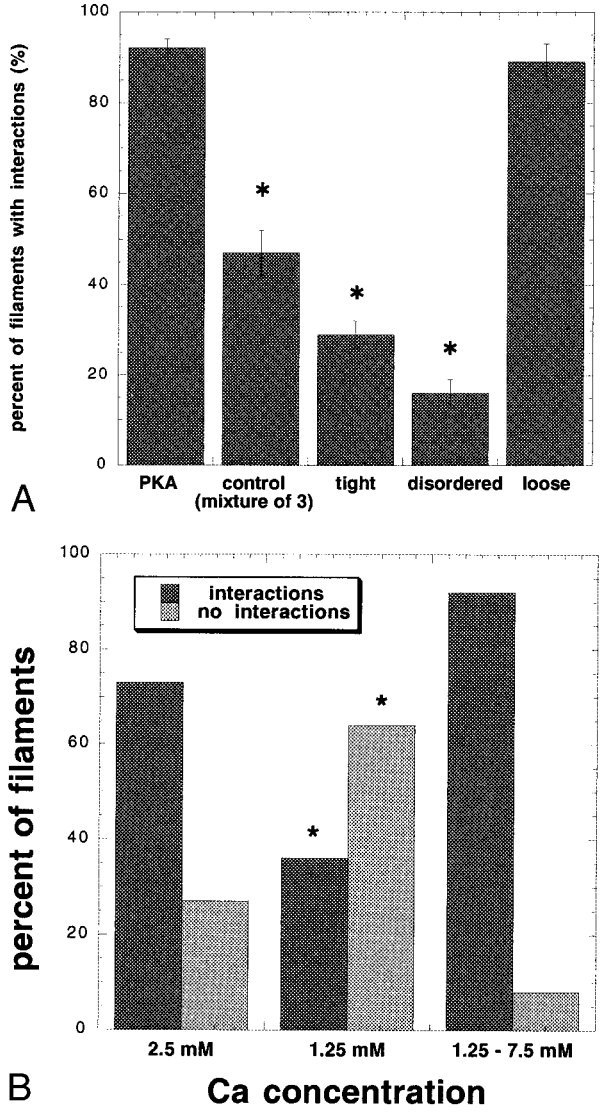


FIGURE 7 Bar graphs showing the percentage of thick filaments with cross-bridges extended to parallel nearby thin filaments in preparations. (*A*) Treated with PKA, mixture of three structures in the control, and individual structures ($n > 200$); (*B*) Exposed to 2.5, 1.25, and 1.25 followed by 7.5 mM Ca ($n > 60$). *Significant difference from the PKA treated.

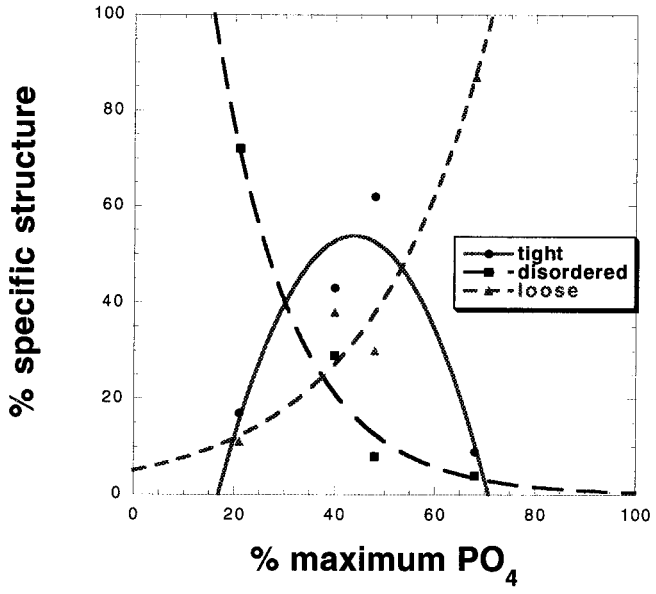


FIGURE 8 Relation between the percent of maximum phosphorylation ($3PO_4$/MyBP-C) and the relative percentage of each of the three structures of the thick filament.

filament. Phosphorylation of MyBP-C has been varied by exposure of isolated thick filaments to PKA plus cAMP and by exposure of intact cardiac muscle to isoproterenol or to different concentrations of extracellular Ca before isolation of the thick filaments. Both PKA and a CAMK that is present in the thick filament can phosphorylate MyBP-C (Hartzell and Glass, 1984; Schlender and Bean, 1991), although in the intact cell the pattern of phosphorylation produced by each of the two kinases is not necessarily the same. The CAMK can add the first phosphate, but PKA apparently cannot (McClellan et al., 2001). The presence of a specific site that must be phosphorylated before the other two can be phosphorylated has already been shown (Gautel et al., 1995).

TABLE 3 Values for the correlation coefficient for the relation between thick filament structure and phosphorylation of MyBP-C

	0 PO_4	1 PO_4	2 or 3 PO_4
Disordered	**0.98**	0.67*	**0.96***
Tight	0.22	**0.91**	0.04
Loose	**0.85***	0.01	**0.83**

$n = \geq 30$ filaments for each point in the correlation analysis. Bold numbers indicate high correlation.
*Degree of inverse correlation.

In the absence of phosphorylation of MyBP-C, the thick filament has a disordered structure. Addition of the first phosphate produces order and the tight structure. A further increase in order of myosin heads and a looser packing of myosin follows the addition of a second and third phosphate to each MyBP-C. At the level of our resolution we are not able to detect any significant difference in the structure of the thick filament produced by the increase in phosphorylation from two to three. Consequently, they have been lumped together. These changes in structure with phosphorylation are not due to changes in the phosphorylation of RLC because there is less than a 5% change in its phosphorylation associated with these changes in structure (McClellan et al., 2001).

When isolated thick and thin filaments were parallel and lay near each other, extension of a portion of the thick filament to the thin filament was sometimes seen. In most cases a distinct periodicity of ~40 nm existed between the connecting structures near the surface of the thick filament,

strongly suggesting that the structures were myosin heads extended from the thick to the thin filaments. Where these connecting structures were present a reflection in the optical diffraction pattern on the 36–38-nm layer line was often seen in addition to the 43-nm layer line. Thick-thin filament pairs never produced this reflection in the absence of the periodic structures connecting thick to thin filaments. The presence of this reflection with an actin periodicity indicates that the extended myosin heads have a site-specific interaction with actin in the thin filament. The lack of extension of the myosin heads on the side of the thick filament away from the thin filament is additional support for this interpretation. The probability of multiple interactions between myosin heads and thin filaments is significantly enhanced by phosphorylation of MyBP-C and the formation of the loose, more ordered structure. The presence of parallel nearby thick and thin filaments without the repeated periodic structures, seen most commonly with the tightly structured thick filaments, is probably due to an occasional myosin head attaching to actin as might occur with lower probability of interaction between filaments.

At the time of their staining, thick and thin filaments were incubated in a solution containing 2.5 mM MgATP, 0 Ca, and 1.0 mM EGTA to prevent the formation of rigor links or actively cycling cross-bridges. The ionic strength was kept near physiological to permit the formation of weak attachments of cross-bridges to thin filaments. This weakly bound state is non-force-producing and exists before cross-bridges with bound ADP and Pi release Pi to form a strongly bound state that leads to generation of force and movement. In view of the conditions under which the interactions between cross-bridges and thin filaments were observed, it is likely that the interactions consisted of weak, non-force-generating bonds, similar to what Matsubara et al. (1979) observed during diastole in rhythmically contracting mammalian heart.

Formation of the weakly bound state may be a rate-limiting step in the cycling of cross-bridges in cardiac muscle or simply determine which myosin heads will enter the force generating cycle. An increase in the concentration of weakly bound cross-bridges would increase the number of force-generating cross-bridges when the muscle is activated by Ca. Such a mechanism would explain the relationship between phosphorylation of MyBP-C and contractility when contractility is changed by stimulation with α- or β-adrenergic agonists, endothelin, cholinergic stimulation, or Ca. The prevailing opinion is that ADP release is rate limiting in the force-generating cycle of cross-bridges (Goldman 1987; Siemankowski et al., 1985). The studies that have shown ADP release to be the rate-limiting step have been carried out under conditions where change in thick filament structure of the type described above would not have been expected to occur.

Lin et al. (1991), in their first demonstration that quiescence of heart muscle changes its response to activator Ca, noted that there is a parallel effect on actomyosin ATPase activity but no change in the maximum velocity of unloaded shortening. This combination of effects suggests that the rate of attachment of the myosin heads to the thin filament is being regulated without change in the rate of detachment. Such a mechanism would explain why maximum force and rate of ATP hydrolysis change in the same direction together without any change in velocity.

In reconstituted systems of actin and myosin lacking the steric constraints of an intact filament lattice, the weakly bound state should form more easily and be less dependent on phosphorylation of MyBP-C. In intact cardiac muscle, on the other hand, enhancement of maximum Ca-activated force from increased formation of weak bonds between actin and myosin would be expected with phosphorylation of MyBP-C.

In the preparations of thick and thin filaments treated with activated PKA, the inhibitory subunit of troponin (TNI) should also be phosphorylated. This will not affect the structure of the thick filaments, but it could modify the interaction of myosin heads with thin filaments if phosphorylation of TNI has other effects besides its decrease in the affinity of troponin C for Ca. Decreased affinity normally diminishes the likelihood of myosin head binding to actin. In this study, EGTA was present in the solutions, and therefore Ca binding should not have been a factor. When thick filament structure was changed by varying extracellular Ca concentration, TNI phosphorylation should not have changed.

Disordering of the arrangement of surface myosin heads and declining contractility are, in these experiments, coincident with dephosphorylation of MyBP-C. Rephosphorylation leads to recovery of both contractility and order. These new data allow one to make an important distinction that had not been possible. On the basis of our previous work (Weisberg and Winegrad, 1998) and that of others, it was not possible to determine the relationship between contractility and the specific structures of the thick filament. We had suggested that the extended myosin heads in the disordered filament structure might have had a higher rate of attachment and therefore produce a larger force because of the smaller separation of myosin heads from actin. From the new data, which describe transitions between different structural forms and the different values for F_{max} (McClellan et al., 2001), it is clear that thick filaments in the disordered state have the lowest level of force generation of the three structures. The presence of the helically ordered array of surface myosin heads on the thick filament appears to be more important than the distance between the thick and thin filaments in determining the number of cross-bridges generating force simultaneously, although distance may be an important parameter as well (McDonald and Moss, 1995).

Ca-regulated changes in thick filament structure that modulate the maximum level of force production of con-

traction fit well with changes in contractile activity that occur with Ca-induced alterations in excitation-contraction coupling. Increase in cytoplasmic Ca concentration does not only lead to greater Ca binding of the ion by troponin C and greater thin filament activation. The increase in cytoplasmic Ca also alters thick filament structure to produce a more rapid rate of cross-bridge attachment and greater force production as a result of an enhanced duty cycle. Lowering cytoplasmic Ca moves cross-bridges away from the thin filament, decreasing the probability of formation of the weakly binding state.

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