Myosin Light Chain Phosphorylation Affects the Structure of Rabbit Skeletal Muscle Thick Filaments

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ABSTRACT To identify the structural basis for the observed physiological effects of myosin regulatory light chain phosphorylation in skinned rabbit skeletal muscle fibers (potentiation of force development at low calcium), thick filaments separated from the muscle in the relaxed state, with unphosphorylated light chains, were incubated with specific, intact, myosin light chain kinase at moderate (pCa 5.0) and low (pCa 5.8) calcium and with calcium-independent enzyme in the absence of calcium, then examined as negatively stained preparations, by electron microscopy and optical diffraction. All such experimental filaments became disordered (lost the near-helical array of surface myosin heads typical of the relaxed state). Filaments incubated in control media, including intact enzyme in the absence of calcium, moderate calcium (pCa 5.0) without enzyme, and bovine serum albumin substituting for calcium-independent myosin light chain kinase, all retained their relaxed structure. Finally, filaments disordered by phosphorylation regained their relaxed structure after incubation with a protein phosphatase catalytic subunit. We suggest that the observed disorder is due to phosphorylation-induced increased mobility and/or changed conformation of myosin heads, which places an increased population of them close to thin filaments, thereby potentiating actin-myosin interaction at low calcium levels.

INTRODUCTION

Calcium-induced, calmodulin-dependent activation of tissue-specific endogenous myosin light chain kinase is a sequela of fiber activation common to all types of muscle. The result of kinase activity is the transfer of one or more phosphates from the γ position on ATP to specific residues on the regulatory light chains of myosin molecules (serine 15 in rabbit fast twitch striated muscle; Sweeney et al., 1993). First observed in vertebrate striated muscles (Perrie et al., 1973; Stull and High, 1977; Barany and Barany, 1979), where it is dependent on stimulation frequency (Manning and Stull, 1982; Moore and Stull, 1984), phosphorylation of myosin regulatory light chains has since been demonstrated in many invertebrate striated muscles (Sellers, 1981; Kerrick and Bolles, 1981; Craig et al., 1987; Levine et al., 1991) and in vertebrate smooth muscles (Hartshorne, 1987; Sellers and Adelstein, 1987). The functional effect of this phosphorylation varies among contractile tissues. At one end of the spectrum, the contractile activity of vertebrate smooth muscle is regulated by myosin light chain phosphorylation (Hartshorne, 1987); at the other, vertebrate striated muscle exhibits normal contractility in its absence (Manning and Stull, 1979), but phosphorylation of myosin regulatory light chains modulates contractile function to enhance performance and efficiency (Sweeney et al., 1993). Between these extremes are a variety of invertebrate "dually regulated" muscles (Lehman et al., 1973). In many of these tissues, phosphorylation of myosin regulatory light chains is required, but not sufficient, for contractile activity (Sellers, 1981; Kerrick and Bolles, 1981; Wang et al., 1993).

Several observations have led to the suggestion that phosphorylation of myosin regulatory light chains affects thick filament structure. Both Craig et al. (1987) and we (Levine et al., 1991) reported the disappearance of the helical order of the surface arrangements of myosin heads, characteristic of the relaxed state (Kensler and Levine, 1982; Levine et al., 1983; Crowther et al., 1985; Stewart et al., 1981, 1985) when thick filaments, separated from dually regulated, chelicerate arthropod striated muscles, are exposed to phosphorylating conditions. Sweeney and Stull (1990) demonstrated that phosphorylation of regulatory light chains in skinned fibers of rabbit psoas muscles potentiated the development of tension at low levels of calcium. Most persuasively, Yang et al. (1992) have presented evidence that the magnitude of this effect is inversely proportional to the lattice spacing (lateral distance between thick and thin filaments) in the sarcomere, decreasing at long sarcomere lengths and when the lattice is osmotically compressed. These findings can be explained if phosphorylation of myosin regulatory light chains in mammalian striated muscle affects thick filament structure in a manner similar to that observed in *Limulus* and tarantula thick filaments. We suggest that as a rule, phosphorylation of myosin regulatory light chains perturbs the relaxed orientation of the myosin heads, allowing them to be more mobile than when unphosphorylated (relaxed state). Such increased mobility, seen as disorder, ensures that each head spends more time in the vicinity of the myosin-binding sites on the thin filaments than it does when fully relaxed (dephosphorylated). Proximity to actin pro-

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motes actin-myosin interaction at lower levels of calcium than is seen otherwise. When the myosin molecules are already close to their binding sites on actin, however, as when the thick-thin filament lattice is compressed, the effect of phosphorylation on mobilizing myosin heads is attenuated. In this paper we present evidence for such a structural basis for the observed physiological effects of myosin regulatory light chain phosphorylation in rabbit skeletal muscle.

MATERIALS AND METHODS

Sample preparation

Rabbit psoas major muscle was the source of all of the thick filaments that were used in this study. The filaments were separated from either fresh or glycerinated muscle fiber bundles, and in a parallel set of studies, from halves of transversely bisected glycerinated single fibers, after tension measurements, with and without experimental incubations. Fresh muscle was dissected into small bundles in mincing solution 1 (MS1: 0.1 M NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM NaN_3, 10 mM imidazole buffer, pH 6.8, containing 0.1 mM protease inhibitor cocktail) (Sellers, 1981) or mincing solution 2 (MS2: 70 mM K propionate, 5 mM EGTA, 8 mM $MgCl_2$, 0.5 mM NaN_3, 6 mM 3-(*N*-morpholino)propanesulfonic acid buffer, pH 6.8) and then washed for 4 to 24 h in several changes of relaxing solution 1 (RS1: MS1 plus 2.5 mM ATP) or relaxing solution 2 (RS2: MS2 plus 7 mM ATP). Small bundles of psoas muscle that had been glycerinated in a 1:1 (v:v) mixture of glycerol and double-strength Eastwood relaxing solution (ERS) (Eastwood et al., 1979) for up to 3 months were washed in several changes of ERS, also for 4 to 24 h. Before the last few hours in any of these relaxing solutions, all of the bundles were frayed into brushes (Kensler and Stewart, 1986, 1989, 1993).

Thick filaments were separated from either bundles or fiber segments by a two-step procedure, utilizing elastase, a modification (M. Schrumpf, personal communication) of the method of Magid et al. (1984). Very small bundles of 2 to 10 fibers were incubated for 3 min at room temperature in a 90-μl volume containing 50 μl RS1, 20 μl soybean trypsin inhibitor (Sigma Chemical Corp., St. Louis, MO) (2 mg/ml), and 20 μl elastase (Type III, Sigma Chemical) (2 mg/ml), introduced in the above order and mixed. The muscle was removed from the enzyme-containing medium, placed in a plastic Eppendorf tube containing 60 to 500 μl RS1 (depending on the size of the tissue sample), and shaken vigorously, manually, for 3 min. During this process the tissue fragmented, releasing both thick and thin filaments into the relaxing solution. If the volume of the sample was sufficiently large, a small droplet was taken for observation by phase-contrast microscopy to monitor the degree of fiber disruption. If myofibrils or small chunks of muscle remained, these were dispersed further by moving the contents of the Eppendorf up and down, several times, through a small-bore Pasteur pipette. The latter procedure was performed routinely, immediately before placing droplets of the filament suspension onto carbon films on electron microscope (EM) grids.

Sample incubations

Most incubations and all staining of preparations were performed on EM grids, at room temperature (23–25°C). Several entire experiments were done in a humid atmosphere with the temperature held constant at 24.5°C, without affecting the results in any way. Twice, an aliquot of the filament suspension was placed on ice and rinsed with cold (4–6°C) RS1 before staining, to verify the reported effect of temperature on the ordered array of surface myosin heads on mammalian thick filaments (Wray et al., 1988; Kensler et al., 1994).

Filaments were adsorbed from drops of suspension onto thin to medium (60–120 nm thick) carbon films laid over carbon-stabilized holey formvar supporting films on 300 mesh copper grids (Kensler and Levine, 1982) and rinsed with RS, and a drop of RS was allowed to remain on the grid until just before the incubation step. Several different media were used for incubation. For either the experimental incubation or any of the controls, two drops, each 50 μl in volume, were placed onto parafilm in a humid environment at room temperature (never less than 23°C). In each case, after most of the RS was removed from the grid surface, the grid was inverted on the first dropet and swirled for 10 s, then placed, still inverted, for the remainder of the incubation time on the second drop. After all experimental and control incubations, the grids were rinsed with either full- or one-half-strength RS (Kensler et al., 1994) and then negatively stained with 1% aqueous uranyl acetate containing 0.1% purified glycerol. In several experiments, we included a tannic acid step in the staining procedure (Kensler and Stewart, 1986). Before any experimental or control procedure, native filaments were stained directly from relaxing solution and examined to ensure that they displayed the ordered surface array of relaxed myosin heads.

Experimental incubations on electron microscope grids

The three different experimental media used (E1, E2, and E3) contained, respectively: E1: 96 μl ERS plus $CaCl_2$ to a pCa of 5.0, 2 μl 57 μM calmodulin (CaM), and 2 μl rabbit skeletal muscle myosin light chain kinase (0.13 mg/ml) ($MLCK_1$); E2: this medium differed from E1 only in that it was at a pCa of 5.8, rather than 5.0; E3: ERS lacking both calcium and CaM, and containing MLCK that had been rendered Ca^{2+}-independent by chymotryptic cleavage (Walsh et al., 1982) ($MLCK_2$). The total incubation times for each medium were: E1, 30 s; E2, 5 min; E3, 15 min.

Control incubations on electron microscope grids

Three different control incubations were performed on filaments from the same preparation as the experimentals. In Control 1 (C1), calcium was eliminated from the incubation medium that contained both $MLCK_1$ and CaM. The filaments were incubated in C1 for 15 min. In Control 2 (C2) MLCK was eliminated from the incubation medium that contained CaM and was at a pCa of 5.0. The incubation time for C2 was 5 min. In Control 3 (C3), bovine serum albumin (BSA) was substituted for $MLCK_2$ in the incubation medium, which also lacked both calcium and CaM. The incubation time for C3 was 15 min.

Phosphatase incubation on electron microscope grids

To ensure that any changes in filament structure observed after experimental incubations were due solely to phosphorylation of the myosin regulatory light chains (MRLCs), some grids that had first been incubated on E1 were subsequently rinsed with relaxing solution and then were incubated for 2 min on drops of ERS containing 2 μl of the catalytic subunit of protein phosphatase (2.9 mg/ml)/100 μl RS (Persechini et al., 1985).

Studies on segments of permeabilized single fibers

In one set of studies, permeabilized single fibers were examined for calcium sensitivity at pCa 6.2, before and after phosphorylation and dephosphorylation. Tension levels of untreated fibers were recorded at pCa 6.2. The fibers were then incubated in either MLCK plus CaM at pCa 8.0 for 5 min, and tension was reexamined at pCa 6.2 or (either before or after the incubation in MLCK) in the presence of the catalytic subunit of rabbit skeletal muscle phosphatase for 5 min at pCa 8.0, and tension development reexamined at pCa 6.2. One-half of each fiber was precipitated in 10% trichloroacetic acid and prepared for glycerol-urea gel electrophoresis (Craig et al., 1987), and levels of phosphorylation were estimated on gels.

Gels were stained with either Coomassie blue or silver. In each case, thick filaments were separated from the other half-fiber, by the method described above, and negatively stained for examination by electron microscopy. All procedures were carried out at 25°C.

Electron microscopy and image analysis

Negatively stained grids were examined and photographed in a JEOL 100CX electron microscope operating at 80 kV, with an anticontamination device in use. Micrographs were taken at nominal magnifications of 19,000× and 36,000×. Magnifications were calibrated using a calibration grid (2156 lines/inch). Straight regions of thick filaments on the EM negatives at 19,000× magnification were masked and subjected to optical diffraction, using a 5-mW He-Ne laser emitting at $\lambda = 632.8$ nm. Transforms were recorded on Polaroid type 55 P/N film. The diffraction patterns were enlarged photographically and analyzed for the presence or absence of reflections characteristic of the ordered state of relaxed thick filaments (off-meridional myosin layer lines, particularly the first and the fourth and meridionals with satellite reflections). On transforms from such ordered filaments, the radial positions of the centers of mass of the myosin heads were calculated in one of two ways: either from the distance between the centers of the subsidiaries of the meridional reflection on the third myosin layer line (using $J_0[3.8] = 2\pi r/R$); or, because we know that the filaments are three-stranded, from the radial position of the first maximum on the first myosin layer line (using $J_0[4.2] = 2\pi r/R$). The range of radii to which heads extend from filaments with disordered surfaces was determined by measurements made directly on the EM images, because images of disordered filaments do not produce diffraction patterns with off-meridional reflections derived from the helical arrangement of myosin heads (which is a characteristic of relaxed thick filaments).

RESULTS

Structure of relaxed, native thick filaments

Thick filaments showing typical relaxed, near-helical order were obtained from both fresh and glycerinated muscle bundles, although the latter required at least 24 h in relaxing solution (changed frequently) to obtain as copious a filament yield as that from fresh tissue after only 4 h in relaxing solution. There was no difference among the different relaxing solutions (RS1, RS2, or ERS) with respect to either filament yield or structure, with either fresh or glycerinated tissue. When exposed to solutions at ≥23°C, the thick filaments appeared ordered in the electron microscope. Native thick filaments that appeared ordered produced transforms with strong myosin layer lines (Fig. 1 *a*). If the sample was kept on ice or exposed to cold relaxing solution just before staining, a marked change occurred in filament structure, seen as a general loss of the relaxed surface pattern and consequent loss of myosin layer lines in the transforms (Fig. 1 *b*). This result is consistent with that reported by Wray et al. (1988) in x-ray diffraction studies of glycerinated rabbit psoas fibers and with the electron microscopic observations of Kensler et al. (1994).

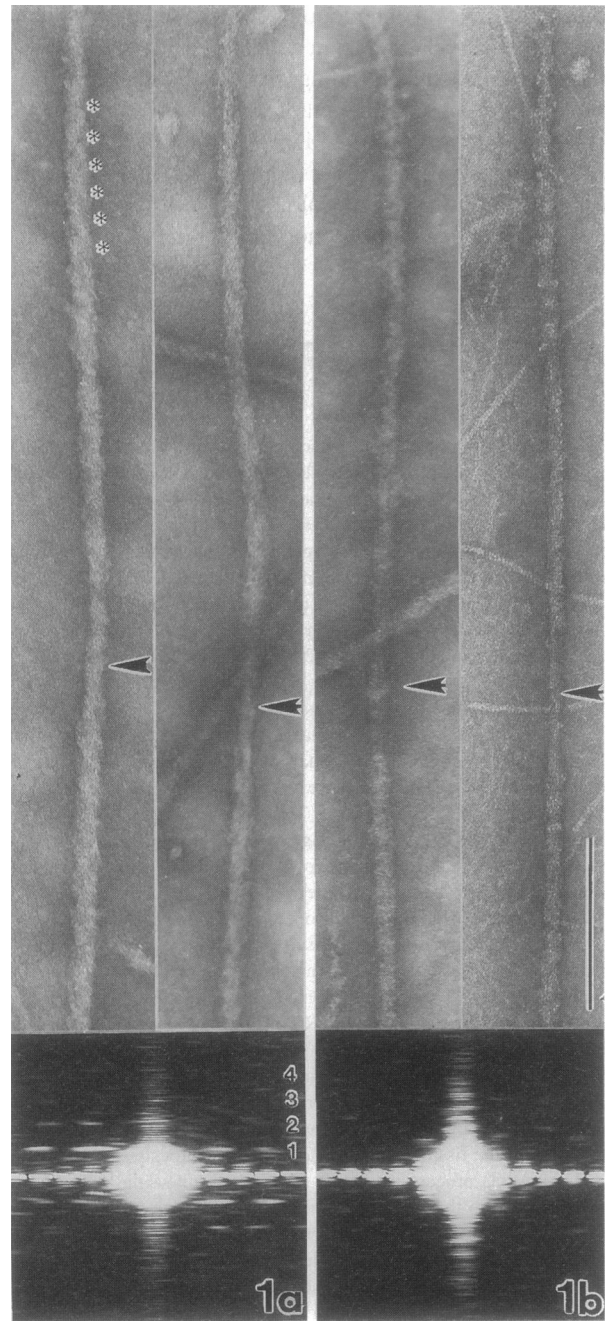

FIGURE 1 Electron micrographs of negatively stained native thick filaments from rabbit psoas muscle and optical diffraction patterns obtained from them. Arrowheads point to bare zones. Bar = 0.2 μm. (*a*) Native thick filaments maintained at a temperature of ≥23°C during the rinsing and staining procedure. (*b*) Native thick filaments were maintained at a temperature of ≤8°C during the rinsing and staining procedure. Note the presence of a repeating, near-helical pattern on the surface of the filaments that were maintained at the higher temperature and the loss of this ordered structure on the surface of the filaments maintained at the lower temperature. The myosin layer lines (numbered) are present on the transform from the former, but absent from that of the latter, filaments.

Structure of thick filaments incubated with myosin light chain kinase on electron microscope grids

Thick filaments in all of the experimental preparations (E1, E2, and E3) showed loss of surface order. In many instances, in all preparations, myosin heads could be seen to extend away from the filament backbones (Fig. 2). The diffraction patterns obtained from such thick filaments

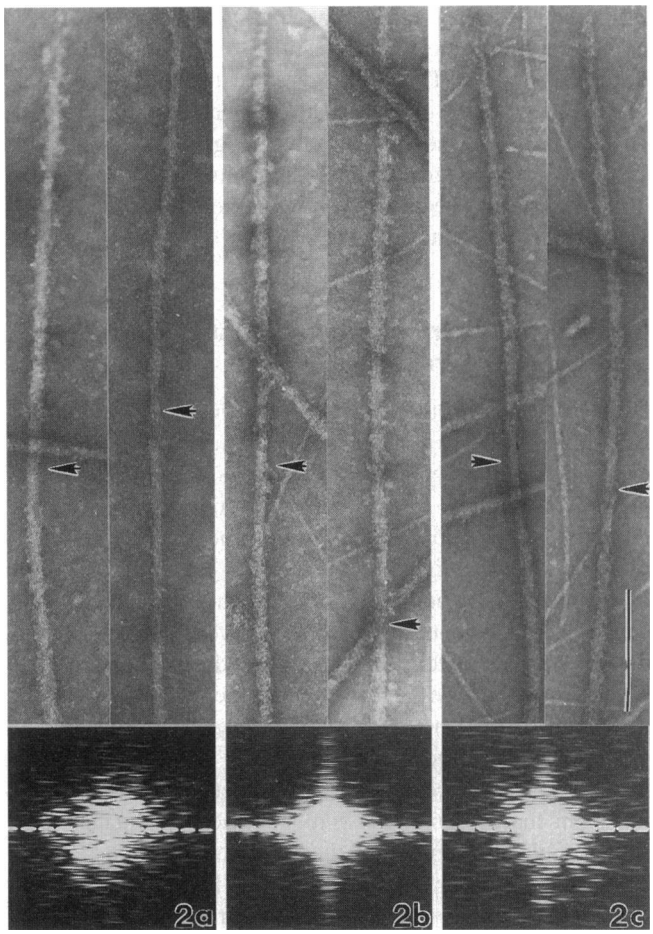

FIGURE 2 Electron micrographs of negatively stained "experimental" rabbit thick filaments that were incubated with MLCK and optical diffraction patterns obtained from these filaments. Arrowheads point to bare zones. Bar = 0.2 μm. (*a*) Thick filaments that were incubated for 30 s with $MLCK_1$ (calcium-dependent kinase) in the presence of calmodulin and at a pCa of 5.0 (E1). (*b*) Thick filaments that were incubated for 5 min, with $MLCK_1$ in the presence of calmodulin, at a pCa of 5.8 (E2). (*c*) Thick filaments that were incubated with $MLCK_2$ (chymotrypsin-cleaved, calcium-independent kinase) in the absence of both calcium and added calmodulin (E3). Note the loss of surface order on all experimental thick filaments, as compared with the native, relaxed structures (Fig. 1 *a*). Tranforms obtained from experimental filaments show loss of off-meridional, myosin-based reflections.

exhibited little or no indication of off-meridional myosin layer lines derived from the helical repeat of myosin heads, characteristic of relaxed thick filaments (Fig. 2). Preliminary experiments indicated that the length of time of exposure to the kinase required to produce the surface disorder differed for each of the experimental solutions, being shortest at a pCa of 5.0 (30 s was more than enough time to produce extensive disorder in E1) and longest when Ca^{2+}-independent kinase was used (considerable disorder was seen after 5 min, but was uniformly the case after 15 min in $MLCK_2$). For the times stated in Materials and Methods, the effect was consistent for each type of experimental procedure.

Structure of thick filaments incubated with control media

The helical surface order associated with the relaxed state was retained by rabbit thick filaments after incubation with all of the different control media (Fig. 3). Optical transforms obtained from these filaments displayed off-meridional myosin-based layer lines similar to those from native, relaxed filaments prepared and stained at ≥23°C (Fig. 3). It is significant that both thick and thin filaments were present on all of the grids. One of the controls, C2, contained solution at a pCa of 5.0, which is sufficient to affect the troponin C on the thin filaments present, derepressing the inhibited state of the latter structures (Sellers and Adelstein, 1987). On grids exposed to C2, we observed that thick filaments lying close to thin filaments were enmeshed in

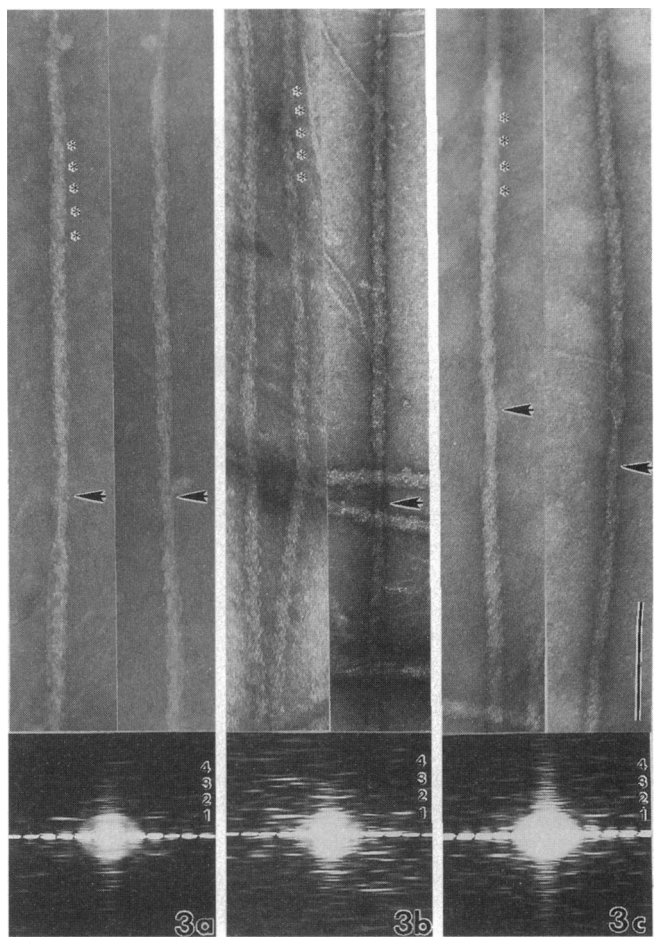

FIGURE 3 Electron micrographs of negatively stained "control" rabbit thick filaments that were incubated with control media. Arrowheads point to bare zones. Bar = 0.2 μm. (*a*) Thick filaments that were incubated with $MLCK_1$ in the presence of calmodulin, in the absence of calcium (C1). (*b*) Thick filaments that were incubated at a pCa of 5.0, in the absence of both MLCK and calmodulin (C2). (*c*) Thick filaments that were incubated in the presence of BSA as a substitute for $MLCK_2$, in the absence of both calcium and added calmodulin (C3). Note the retention of the near-helical ordered array on the surfaces of all of the control thick filaments. The optical transforms likewise show the presence of myosin-based reflections (numbered) typical of the relaxed thick filament structure.

aggregates of thick and thin filaments, whereas those thick filaments lying at some distance from thins retained an ordered appearance (Fig. 4). This behavior is consistent with the thin filament-linked regulation of vertebrate skeletal muscle. On grids incubated with either C1 or C3 media, all of the thick filaments, even those surrounded by thin filaments, retained the surface order typical of the relaxed state.

Structure of thick filaments incubated first with MLCK and then with phosphatase

In three separate experiments, after ascertaining (by electron microscopic examination) the disordered appearance of thick filaments that were incubated for 30 s on E1, we first similarly exposed samples from the same preparation (see Fig. 5 a for the relaxed structure) to E1, rinsed them with relaxing solution (see Fig. 5 *b* for the filament appearance after incubation in E1), and then incubated them for 2 min in the presence of protein phosphatase catalytic subunit, to dephosphorylate the myosin regulatory light chains before negative staining. Thick filaments treated in this way displayed the ordered arrangement of surface myosin heads characteristic of the relaxed state (Fig. 5 *c*). Transforms obtained from images of these filaments also displayed the off-meridional reflections typical of those from native, relaxed thick filaments (Fig. 5 *c*).

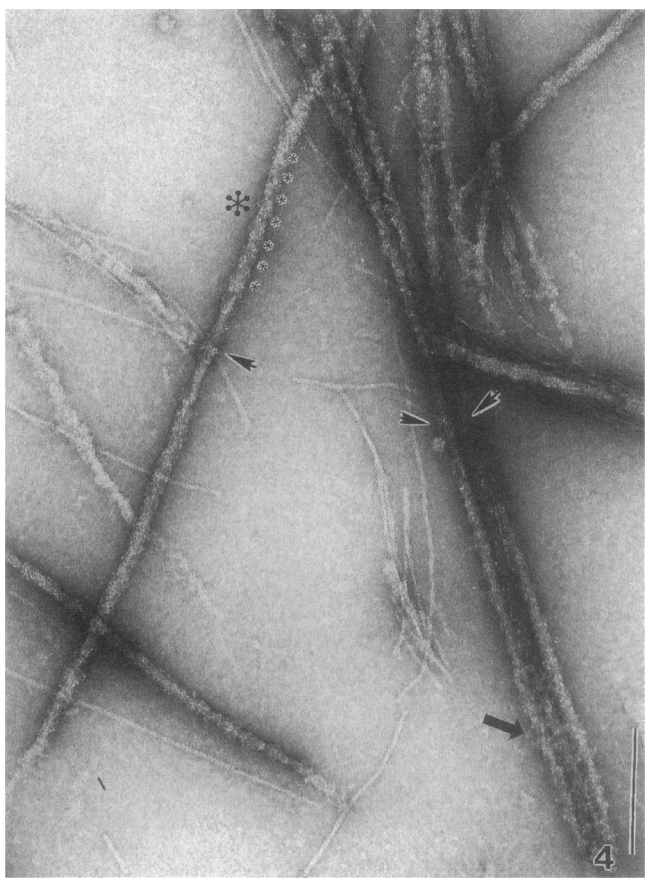

FIGURE 4 Electron micrograph of a field of negatively stained thick and thin filaments that were incubated in the presence of calcium (pCa = 5.0) as in Fig. 3 *b* (C2). Arrowheads point to bare zones. Bar = 0.2 μm. Note the ordered surface structure on the filaments on the left side of the image (indicated by asterisks), which are well separated from any thin filaments. The thick filaments on the right side of the image are clumped and appear disordered. Thin filaments are found in close proximity to (even between) them (black arrow) and appear to be connected to these thick filaments by projections (myosin heads) from the latter.

Measurements from optical transforms and electron micrographs

Measurements of meridional, layer-line, and other off-meridional reflections can only be made on transforms obtained from ordered thick filaments. Measurements of diffraction patterns from images of native thick filaments, those from each control incubation and those exposed to phosphatase after incubation in E1, showed little or no difference among them (Table 1). The radial positions of the centers of mass of the myosin heads were calculated from the diffraction patterns or measured directly on the EM images, in the case of disordered thick filaments. In all measurements calculated from transforms of filaments with ordered surface structure, the centers of mass of the myosin heads were estimated as lying at radii between 13.4 and 13.5 nm from the center of the filament backbone. This is not significantly different from the figure of 13.3 nm reported by Kensler (Kensler and Stewart, 1993; Kensler et al., 1994) from direct measurements from electron micrographs of negatively stained native rabbit skeletal muscle thick filaments. The weak to absent myosin-based layer lines in transforms obtained from images of filaments that had been incubated in MLCK precluded the collection of similar data from filaments with phosphorylated myosin regulatory light chains. However, when myosin heads could be seen clearly in electron micrographs of disordered filaments, the radial distance between them and the centers of the thick filament backbones was measured directly. This distance varied, ranging between 16.1 to 19 nm, even on the same filament (Table 1).

Results of studies on single, permeabilized fibers

Permeabilized fibers that were taken from bundles glycerinated using any one of the relaxing solutions showed variable levels of phosphorylation of MRLCs with no further treatment. Most fibers, however, had levels of phosphorylation of no more than 10%, as estimated from gels. These fibers produced low tension at pCa 6.2, which was potentiated up to 100% after 5 min of incubation in MLCK and CaM. Filaments separated from such fibers appeared well ordered in this "unphosphorylated" state. After phosphorylation in the fiber, the separated thick filaments appeared disordered (Fig. 6). One glycerinated preparation had higher levels of phosphorylation (~40%) before incubation with MLCK, as seen on glycerol-urea gels (Fig. 7).

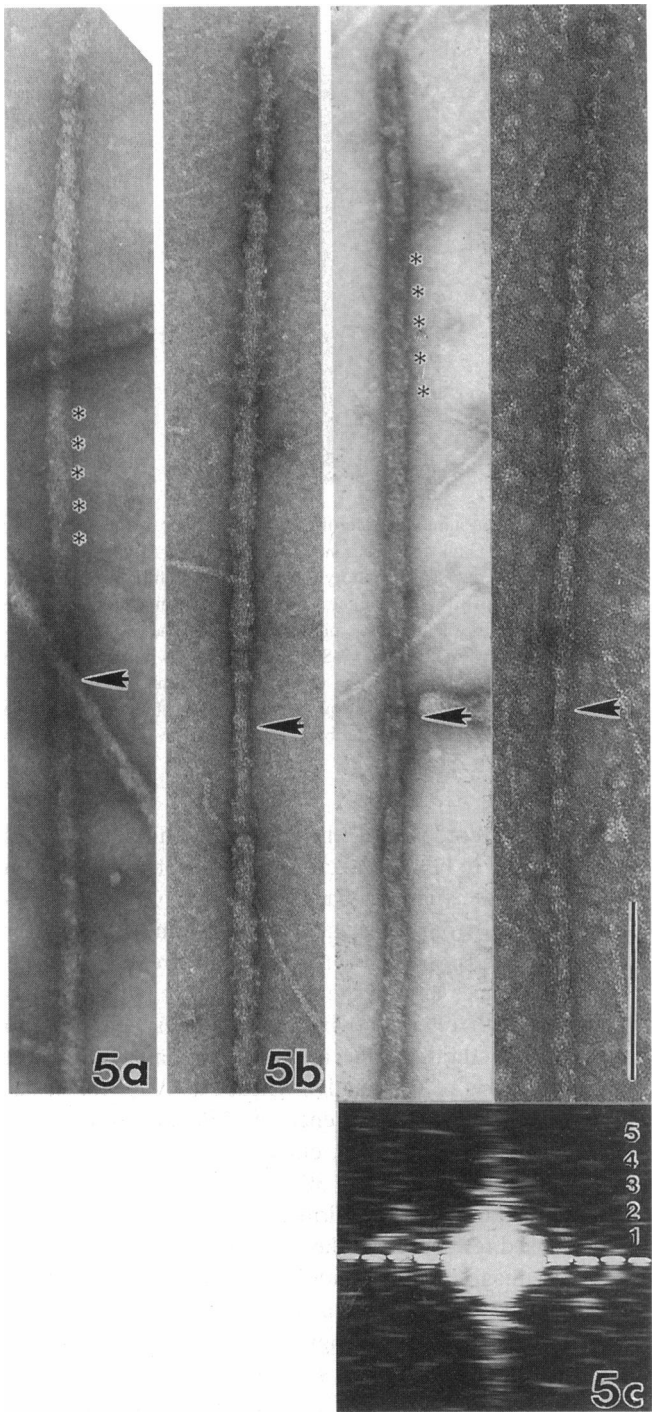

FIGURE 5 Electron micrographs of negatively stained rabbit thick filaments, during the course of a phosphorylation/dephosphorylation experiment. Arrowheads point to bare zones. Bar = 0.2 μm. (*a*) Native thick filament before incubation with MLCK. Note the presence of relaxed surface order (*asterisks*). (*b*) Thick filament after 30-s incubation with $MLCK_1$ in the presence of calmodulin, at a pCa of 5.0. Note the complete loss of surface order due to phosphorylation of MRLCs. (*c*) Thick filament that had been incubated first as in *b*, then incubated for 2 min in rabbit skeletal muscle phosphatase, before negative staining. Note the reappearance of the ordered surface array (*asterisks*) after dephosphorylation of the myosin regulatory light chains. The optical transforms obtained from such filaments also display myosin-based, off-meridional reflections (numbered) associated with the relaxed thick filament structure.

TABLE 1 Disposition of myosin heads on rabbit skeletal muscle thick filaments

Treatment of filaments	Radii of centers of mass (mean [nm] ± SD [no.])	Filament appearance
Native, relaxed	13.46 ± 0.43* [17]	Ordered
$MLCK_1$, CaM, pCa 5.0	16.2 to 19#	Disordered
$MLCK_1$, CaM, pCa 5.8	16.1 to 18.7#	Disordered
$MLCK_2$ alone	16.1 to 18.4#	Disordered
$MLCK_1$, CaM, no Ca^{2+}	13.45 ±0.26* [16]	Ordered
pCa 5.0 alone	13.54 ± 0.24* [11]	Ordered
BSA alone	13.37 ± 0.14* [14]	Ordered
Phosphatase, after $MLCK_1$, CaM, pCa 5.0	13.54 ± 0.35* [11]	Ordered

*Calculated from optical transforms.
#Measured directly on electron micrographs.

This increased level of MRLC phosphorylation was evident on the tension record: higher tensions were produced at pCa 6.2, and little to no further potentiation was seen after incubation with MLCK and CaM. Furthermore, filaments separated from fibers with this intrinsically high level of phosphorylation appeared disordered by electron microscopy. Incubation of fibers from this preparation with the catalytic subunit of skeletal muscle light chain phosphatase, for 5 min, lowered the degree of tension developed at pCa 6.2, and restored the ordered, helical array of surface structure to the filaments. Rephosphorylation by incubation with MLCK and CaM (at pCa 8.0) again doubled the calcium sensitivity of the fiber at pCa 6.2 and disordered the thick filament surface structure.

DISCUSSION

The results presented here indicate that the disordered surface array of myosin heads on thick filaments separated from rabbit psoas muscle observed after incubation with either intact MLCK in the presence of calcium and CaM, or chymotrypsin-cleaved, Ca^{2+}-independent MLCK, was due solely to the phosphorylation of the regulatory light chains of the component myosin molecules.

Retention of the relaxed filament structure after incubation with intact enzyme in the absence of calcium (C1) demonstrates a requirement for the activated CaM-MLCK complex for light chain phosphorylation. The inability of calcium (at a pCa of 5.0), in the absence of added MLCK (C2), to affect the relaxed organization of myosin heads of those thick filaments that were not surrounded by, or in close proximity to, thin filaments, while promoting extensive interaction between myosin heads on thick filaments and adjacent thin filaments, demonstrates that our thick filament preparations were free of endogenous MLCK and CaM, and furthermore, supports the premise that the direct effect of calcium on contractile activity in rabbit (and all vertebrate skeletal muscle) is restricted to thin filaments (by binding to troponin C of the regulatory complex). Additionally, skeletal muscle MLCK is soluble and easily removed from skinned fibers and myofibrillar preparations (Per-

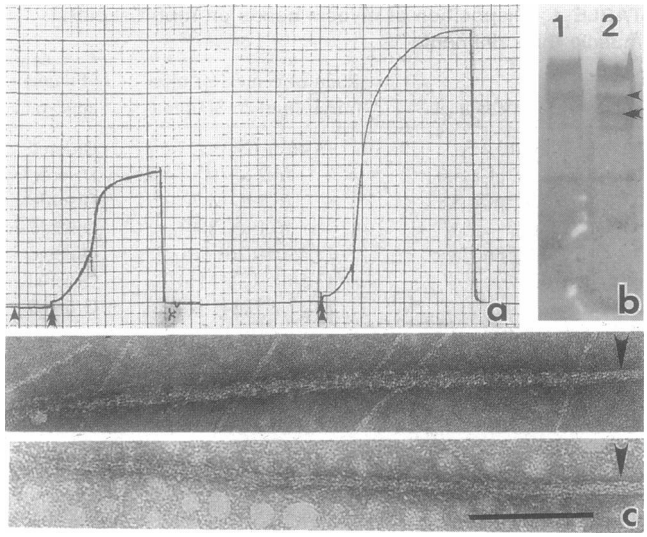

FIGURE 6 Phosphorylation in a single permeabilized fiber. (*a*) Tension record from a single fiber. Single arrowhead: untreated fiber in relaxing solution (pCa 8.0). Double arrowhead: fiber in activating solution (pCa 6.2). Asterisk: incubation in MLCK and CaM at pCa 8.0. Vertical = tension; horizontal = time. Note 100% increase in tension developed at pCa 6.2 after incubation in MLCK and CaM. (*b*) Silver-stained, glycerol-urea gel of fiber segments as in *a*. *Lane 1:* Segment of untreated fiber. Note the presence of only unphosphorylated MRLCs. *Lane 2:* Segment of fiber, as in Lane 1, after incubation in MLCK and CaM. Note the presence of phosphorylated MRLCs. Single arrowhead: Unphosphorylated MRLC bands. Double arrowhead: Phosphorylated MRLC bands. (*c*) Thick filaments separated from fiber segments as in *a* and *b*. Top filament: Separated from untreated fiber segment. Note the ordered surface array of myosin heads. Bottom filament: Separated from fiber segment after incubation in MLCK and CaM (*a* and *b*). Note the disordered appearance of the thick filament surface. Arrowheads: Bare zones. Bar = 0.25 μm.

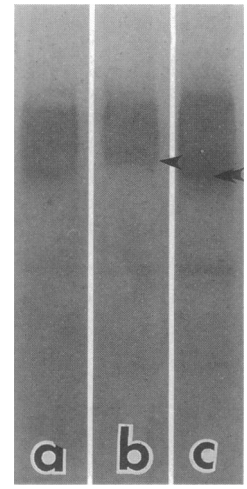

FIGURE 7 Silver-stained, glycerol-urea gel of fiber with intrinsically high level of MRLC phosphorylation. *Lane a:* Segment of untreated fiber. Note the presence of both phosphorylated and unphosphorylated MRLCs. *Lane b:* Segment of fiber as in Lane a, after 5 min of incubation with the catalytic subunit of skeletal muscle phosphatase. Note the presence of only unphosphorylated MRLCs. *Lane c:* Segment in fiber as in Lane b, after 5 min of incubation with MLCK and CaM. Note the rephosphorylation of MRLCs. Single arrowhead: Unphosphosphorylated MRLC bands. Double arrowhead: Phosphorylated MRLC bands.

sechini et al., 1985), and so could not have been activated to affect the separated filaments. Our data show that, even if myosin light chains bind calcium, as suggested by Metzger and Moss (1992), such binding does not produce the structural changes of thick filaments that we describe as a result of light chain phosphorylation. The latter effect is virtually identical to the structural change elicited by calcium binding to myosin on thick filaments of scallop striated adductors (Vibert and Craig, 1985), the sole mode of regulation in these muscles (Kendrick-Jones et al., 1970; Szent-Gyorgyi et al., 1973). Finally, lack of any structural change after incubation with the nonrelated protein, BSA, as a substitute for $MLCK_2$ (C3), demonstrates that under the conditions used in this study, the response of myosin heads on thick filaments was specific to phosphorylation of the regulatory light chains.

Our contention that the disordered state of myosin heads on rabbit thick filaments, seen after exposure to experimental media (E1, E2, and E3), at $\geq$23°C, is a direct result of light chain phosphorylation, is also strongly supported by the reordering of the filaments' surface array observed after removal of the bound phosphate by incubation of disordered, (presumably) phosphorylated, thick filaments with protein phosphatase. The centers of mass of the myosin heads of such reordered, dephosphorylated, thick filaments were estimated to lie at an average distance of 13.5 nm from the center of the filament backbone, which is not significantly different from the distance of 13.4 nm estimated for the myosin heads on native, relaxed structures.

The differences among the three different phosphorylating media, with respect to incubation times necessary to produce a structural change in thick filaments, were most likely reflections of the relative MLCK activity. Intact enzyme ($MLCK_1$), in the presence of CaM and moderately high calcium (pCa 5.0) (E1), elicited a response most rapidly. After exposure to E1 for 30 s, nearly all thick filaments appeared disordered, as confirmed by the disappearance of myosin-based reflections in the Fourier transforms obtained from such images. These conditions are the closest to those that are likely to occur in vivo, as a result of fiber activation. An increase of an order of magnitude (to 5 min) in exposure time was needed to achieve a similar response with $MLCK_1$ and CaM, at lower levels of calcium (pCa 5.8) (E2). This result is probably due to a decrease in calcium concentration and hence decreased Ca^{2+}/CaM for enzyme activation. A threefold further increase in incubation time (to 15 min) was required for Ca^{2+}-independent $MLCK_2$ (E3) to produce complete disorder of thick filament structure, which was probably related to a decrease in MLCK activity upon partial proteolysis.

The results of our studies on single, permeabilized fibers confirm that the degree of calcium sensitivity exhibited at pCa 6.2 and the disordered structure of the thick filaments are both reflections of the amount of MRLC phosphorylation: fibers with $\leq$10% phosphorylation of MRLCs develop

little tension at low calcium, and thick filaments from these fibers have ordered surface arrays of myosin heads. After phosphorylation of the MRLCs in the fibers, increased calcium sensitivity is seen at pCa 6.2, and the filament structure is disordered. The former situation is restored by incubation in phosphatase. Intrinsic MRLC phosphorylation of about 40%, however, seen in one permeabilized fiber preparation, is sufficient to permit high tension production at low calcium and disorder of thick filament structure, without additional incubation in MLCK and CaM. Because 100% phosphorylation is not required for this effect, some degree of cooperativity probably operates to ensure an adequate response of the system to a triggering level of MRLC phosphorylation. Again, both high levels of tension development at pCa 6.2 and filament disorder were reversed after incubation with phosphatase, further supporting our hypothesis. Finally, because only dephosphorylated (or up to 10% phosphorylated) thick filaments display the ordered, helical array of surface myosin heads, it is most likely that the dephosphorylated state was present on all of the filaments that had ordered structures and on which we performed our phosphorylation/dephosphorylation studies on electron microscope grids.

One possible mechanism for the structural and physiological effects of phosphorylation of myosin regulatory light chains is that such phosphorylation puts negative charge on the phosphorylatable serine (serine 15) (Sweeney et al., 1993), which is situated adjacent to positively charged residues in the N-terminal region of the peptide (Saraswat and Lowey, 1991; Yang et al., 1992). In the unphosphorylated state, this positively charged region may form ionic interactions with negatively charged regions of either light or heavy chains in neighboring myosin heads or underlying myosin rods in the filament backbone. Such interactions may stabilize the relaxed (ordered) organization of the myosin heads on the filament surface. By neutralizing the overall positive charge of the regulatory light chain N-terminus, phosphorylation of serine 15 may effectively free the myosin heads from their association with other myosin molecules. "Unbound" heads would exhibit increased mobility, which would produce the disordered appearance of the thick filaments. More mobile, phosphorylated heads are likely to spend proportionally more time in proximity to myosin-binding sites on the actins of thin filaments and are thus more likely to be involved in actin-myosin interaction, than the stable, unphosphorylated species. Physiologically, the increased population of heads near thin filaments is expressed as potentiation of tension development at low levels of calcium (pCa 5.8, 5.9; Sweeney et al., 1993).

Even though the increased mobility of the myosin heads may require neutralization of charged residues in the amino-terminal region of the myosin regulatory light chain, this in itself may not be the only mechanism that operates to produce either the structural or physiological effects observed. The state of hydrolysis of the nucleotide in the active site of myosin affects the disposition of myosin heads on thick filaments (Wray et al., 1988; Schrumpf and Wray, 1992). Head orientation, in turn, may reflect the degree of flexure or "twist" of domains within the subfragment 1 (S1) about an "internal hinge," as modeled by Rayment et al. (1993a), based on their crystallographic solution of S1 structure (Rayment et al., 1993b). Wray and his colleagues (Schrumpf and Wray, 1992, 1995; Wray, 1995) examined the effect of a variety of myosin ATPase inhibitors, including phosphate analogs such as vanadate (Goodno, 1979) and metallofluorides, on the myosin layer lines in x-ray diagrams of skinned fibers. They found that the ordered pattern of reflections, characteristic of relaxed thick filament structure, is associated with the presence of ADP·P_i or an intermediate form of this complex, bound to myosin in the active site (M·ADP·P_i). On the other hand, ATP or ADP complexed with myosin in the active site (M·ATP; M·ADP) both disorder the array of myosin heads on thick filaments. Interestingly, fluoride ion inhibits an endogenous phosphatase and maintains at least partial phosphorylation of the regulatory light chains (Schrumpf and Wray, 1995), thus stabilizing a disordered (phosphorylated) state. Recent explorations of the effect of fluoride on tarantula thick filaments at different temperatures suggest that myosin light chain phosphorylation may primarily effect the kinetics of ATP hydrolysis in the active site of myosin, causing the predominant nucleotide state to be one that produces a myosin head conformation associated with a disordered appearance of the filament surface array (Schrumpf and Wray, 1992, 1995). If light chain phosphorylation pushes the reaction so that the active sites are largely populated by ADP, this would, according to the original model of Rayment et al. (1993a), result in cleft closure and straightening of the flexure of domains about the "internal hinge." In the newer model, based on x-ray diffraction of *Dictyostelium* S1 complexed with MgADP·BeFx and MgADP·AlF_4 (Fisher et al., 1995), a 1.0-nm rotation between the upper and lower domains of the myosin head coupled with a 1.0-nm "straightening" of the lower domain is postulated. Both models predict movement of myosin heads from their relaxed (bent) orientation with respect to the filament backbone. Heads containing ADP would also be highly likely to associate with myosin-binding sites on thin filaments, because M·ADP is a species associated with the "strongly" binding state between myosin and actin (Schoenberg, 1988; Brenner, 1990). Unpublished, structural investigations at a resolution of ~35 Å by cryoelectron microscopy and image analysis suggest that there are no discernible differences between phosphorylated and dephosphorylated skeletal muscle S1s in rigor (M. Whittaker et al., 1995). This is not surprising, however, because rigor heads are already in the most "strongly" binding state. If operative, such a mechanism, involving nucleotide-dependent changes in myosin head conformation, would also explain the potentiation of tension development at low calcium. Thus, the results described here provide a major impetus for detailed analysis of the effect of lattice spacing within the overlap region of the

A-band on the potentiating effects of myosin light chain phosphorylation. This study is currently under way.

Whichever mechanism is responsible for the effects of myosin regulatory light chain phosphorylation in rabbit skeletal muscle, the overall result is that myosin heads become more accessible to actin than in the relaxed state. Their increased accessibility (via either increased mobility or by straightening of an internal hinge) is maintained until they are dephosphorylated. MRLC phosphorylation occurs in a few seconds with repetitive stimulation of muscle contractions at physiological frequencies (Sweeney et al., 1993), whereas minutes are required for dephosphorylation, even in resting muscle with inactive kinase. Such dynamics may allow myosin regulatory light chain phosphorylation to function as a form of "muscle memory," because the degree of activation at a specific calcium concentration would depend on the recent contractile history of the tissue. Therefore, a recently active muscle, with myosin heads still in the phosphorylated (disordered) state, would respond more rapidly to submaximum sarcoplasmic calcium levels than would a long-term relaxed muscle with helically ordered myosin heads.

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