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Different Head Environments in Tarantula Thick Filaments Support a Cooperative Activation Process

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ABSTRACT Myosin filaments from many muscles are activated by phosphorylation of their regulatory light chains (RLCs). Structural analysis of relaxed tarantula thick filaments shows that the RLCs of the interacting free and blocked myosin heads are in different environments. This and other data suggested a phosphorylation mechanism in which Ser-35 of the free head is exposed and constitutively phosphorylated by protein kinase C, whereas the blocked head is hidden and unphosphorylated; on activation, myosin light chain kinase phosphorylates the monophosphorylated free head followed by the unphosphorylated blocked head, both at Ser-45. Our goal was to test this model of phosphorylation. Mass spectrometry of quickly frozen, intact muscles showed that only Ser-35 was phosphorylated in the relaxed state. The location of this constitutively phosphorylated Ser-35 was analyzed by immunofluorescence, using antibodies specific for unphosphorylated or phosphorylated Ser-35. In the relaxed state, myofibrils were labeled by anti-pSer-35 but not by anti-Ser-35, whereas in rigor, labeling was similar with both. This suggests that only pSer-35 is exposed in the relaxed state, while in rigor, Ser-35 is also exposed. In the interacting-head motif of relaxed filaments, only the free head RLCs are exposed, suggesting that the constitutive pSer-35 is on the free heads, consistent with the proposed mechanism.

INTRODUCTION

Contraction of striated muscle sarcomeres is regulated by Ca^{2+} ions. This control is achieved by molecular switches on the thin actin-containing and thick, myosin-containing filaments. Ca^{2+} activation occurs when Ca^{2+} binds directly either to troponin C on the thin filaments (actin-linked regulation) or to the essential light chain of myosin, or to calmodulin (CaM) (myosin-linked regulation) (1). In the latter case, $Ca^{2+}{}_4$.CaM binds to myosin light chain kinase (MLCK), causing it to be activated (2,3) and in turn to phosphorylate the myosin regulatory light chain (RLC). RLC-phosphorylation is the primary regulatory mechanism in vertebrate smooth muscle, and a secondary (modulatory) mechanism in arthropods (*Limulus*, tarantula, scorpion) and vertebrate striated muscle, acting together with actin-linked regulation. Activation of the thick filaments either by Ca^{2+} binding to the myosin essential light chain (4), or by RLC phosphorylation (3,5,6), causes release of the helically ordered heads from the filament backbone (7), enabling them to cyclically interact with the thin filaments, to produce force and sarcomere shortening.

Determining the molecular structure of the thick filaments is a crucial step for understanding the function of healthy and diseased muscle. Tarantula muscle has emerged as an outstanding model system for understanding the structure and function of the thick filaments. Three-dimensional reconstruction of relaxed, native tarantula filaments showed that the two heads of each myosin molecule interact with each other (8). The interaction is similar to that occurring between the heads in two-dimensional crystals of vertebrate smooth muscle myosin (9–11) and in individual heavy meromyosin and myosin molecules (12), implying that it is an intrinsic property of the two heads and the proximal tail. This motif may have emerged as a small, self-inhibiting unit before the emergence of thick filaments.

The conservation and uniqueness of this two-headed motif between vertebrate smooth and invertebrate striated muscle suggested that it was of fundamental functional importance for preserving the relaxed (off) state in muscle. Analysis of the interaction between the two heads shows that one head (free) physically blocks the actin-binding site of the other head (blocked); this interaction simultaneously blocks the ATPase site of the free head (Fig. 1 *Aa*, *top*). This head-head interaction suggests a simple mechanism to explain relaxation—by switching off the two heads in different ways (8,9). Subsequent studies have demonstrated the presence of this motif in nonmuscle cells (13), and in smooth (14) and striated muscles of other species (15–19), confirming its fundamental importance.

Further analysis suggested an additional intermolecular interaction that would further stabilize the off-state and that crucially involves the long RLC N-terminal extensions

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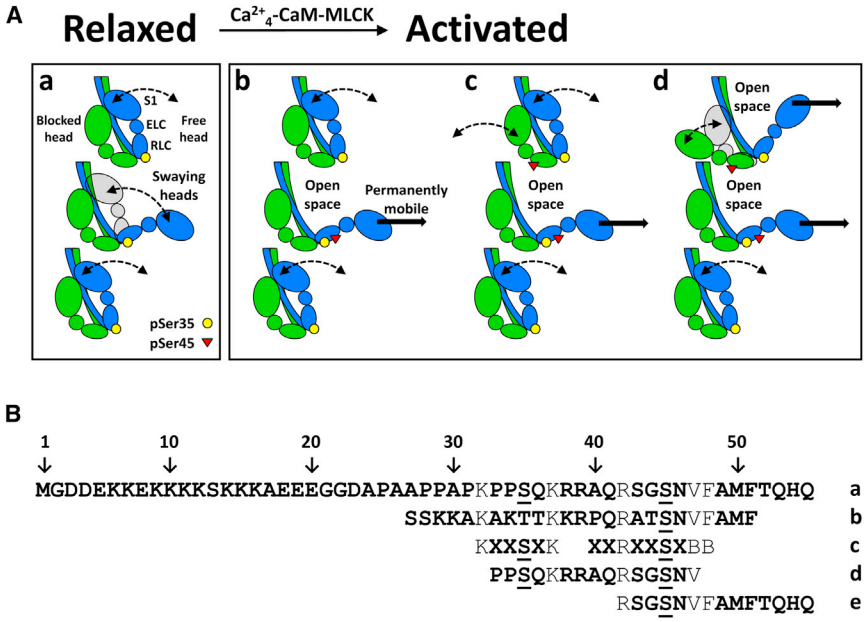

FIGURE 1 (*A*) The cooperative phosphorylation mechanism for activation of tarantula thick filaments (21): in the relaxed state (*a*) free heads have their Ser-35 constitutively monophosphorylated (pSer-35), enabling them to sway away in and out (*swaying heads*, *double arrows*). The blocked heads cannot be released as their Ser-45 are nonphosphorylated. On activation (*b–d*), when Ca^{2+} concentration is high for long enough to activate MLCK, a swaying head is permanently released (*arrow*) by biphosphorylation of its N-terminal extension (*b*). This outward movement of the free head leaves an open space (*b*), allowing access of activated MLCK to phosphorylate the Ser-45 N-terminal extension of the above blocked head (pSer-45), allowing this head to sway (*c*). This phosphorylation hinders the docking back of its partner swaying free head, which thus also becomes mobile even without biphosphorylation (*d*, *top free head*) opening a new space above (*d*). Thus, biphosphorylation of a swaying free head induces the cooperative unzipping of the neighbor blocked and free heads, releasing them from the backbone; and so on along the helix (20,21). The mechanism in Fig. 1 *A* shows the simplest case, in which all Ser-35 of the free heads are monophosphorylated in the relaxed state, i.e., a 1:1:0 un-P/mono-P/bi-P ratio. This ratio changes to 2:3:1 upon activation. According to our statistics (Fig. S1, *A*, *C*, and *D*), the most frequent mechanism, however, corresponds to a 2:1:0 initial ratio (Fig. S1 *D*), in which the free heads of the lower row of myosin interacting heads are un-P, and upon activation could become swaying heads by Ser-45 monophosphorylation (not shown), achieving the same 2:3:1 ratio. As previously proposed (21) this could reflect variations in the constitutive Ser-35 monophosphorylation level of different animals that would allow adjustment of the force potentiation ratio. (*B*) Sequences of the 52 aa long N-terminal extension of the myosin RLC from striated muscle of *Avicularia* tarantula (*a*) with their phosphorylatable serines Ser-35 and Ser-45 (*underlined*) aligned with the 25 aa short N-terminal extension from smooth muscle from chicken with its phosphorylatable Ser-19 (*underlined*) and Thr-18 (*b*); the PKC (*left*) and MLCK (*right*) consensus sequences KXXSXK and XXRXXSXBB (where positively charged K and R amino acids in regular font, X is any amino acid, and B is any hydrophobic amino acid (A,V,F,I,L) also in regular font (*c*) the sequences of the peptides used for producing the anti-pSer-35 (*d*) and anti-pSer-45 (*e*) antibodies.

of the blocked and free heads, where two phosphorylatable serines are present (20) (Fig. 1 *Ba*). Structural studies suggested that both N-terminal extensions are key for the phosphorylation activation of these thick filaments (20) and suggested a molecular model for muscle activation in which the heads are phosphorylated sequentially at the two serines (Ser-35 and Ser-45, Fig. 1 *Ba*) by protein kinase C (PKC, Fig. 1 *Bc*) and MLCK (Fig. 1 *Bc*), respectively, in a way that can explain force development, potentiation, and posttetanic potentiation in striated muscle (Fig. 1, *Aa–d*) (21). According to this mechanism (Fig. 1, *Aa–d*), in the relaxed state most free heads are constitutively monophosphorylated (mono-P) at Ser-35 (pSer-35), which is exposed on the filament surface, whereas Ser-35 of the blocked heads is covered by the free heads and therefore unphosphorylated (un-P); Ser-45 is unphosphorylated on both blocked and free heads (Fig. 1 *Aa*). On Ca^{2+}-activation, MLCK phosphorylates Ser-45 on some heads that were previously constitutively Ser-35 mono-P (and which thus become bi-P), and on some un-P free heads and un-P blocked heads (which become Ser-45 mono-P) (Fig. 1, *Ab–d*).

In this model, only the free head N-terminal extension is exposed in the relaxed state, and thus constitutive phosphorylation at Ser-35 occurs only on this head; similarly only Ser-45 of the free head is accessible to MLCK in the relaxed filament. The model requires two kinases (PKC and MLCK) for the different phosphorylations. Here, we test these predictions as a step toward better understanding the phosphorylation mechanism in the model tarantula system.

MATERIAL AND METHODS

Filament suspensions

Pink-foot (*Avicularia avicularia*) tarantulas were obtained at San Jose de Guaribe (Guárico, Venezuela). Suspensions of thick and thin filaments from freshly dissected relaxed leg muscle were prepared in the presence of Mg.ATP as previously reported (7). Muscles were permeabilized in relaxing solution containing 0.1% saponin, washed twice in relaxing solution (see below), and homogenized. The homogenate was centrifuged for 20 min at 14,000 rpm. The supernatant containing thick and thin filaments was kept on ice and used immediately.

Myofibrils

Grammostola rosea tarantulas were from Ward's Natural Science. Freshly dissected leg muscles were incubated in rigor solution (100 mM NaCl, 1 mM EGTA, 5 mM PIPES, 3 mM NaN_3, 5 mM Na_2HPO_4, 3 mM $MgCl_2$, pH 7.0) containing 0.1% saponin at 4°C for 3 h, and then washed several times for 18 h at 4°C in rigor solution. The high concentration of EGTA (1 mM) was used to avoid Ca^{2+}-dependent phosphorylation by

endogenous MLCK. Myofibrils from these rigor muscles were prepared as previously described (22). These myofibrils, either in rigor solution, or relaxed by incubation for 5 min in relaxing solution (rigor solution plus 5 mM Mg.ATP, pH 7.0), were used for immunofluorescence experiments.

Quick freezing

Avicularia sp. tarantulas were cryoanesthetized for 1 h at 4°C, kept 30 min at −20°C, and then quickly immersed in liquid nitrogen. The legs were cut away in liquid nitrogen and kept in an ice salt mixture. They were then rapidly shaved to remove leg hairs and thus avoid contamination in the mass spectrometry (MS) analysis, and the muscle tissue quickly dissected while on ice. Muscles were then stored in a vial in liquid nitrogen until homogenization in relaxing solution for 6 s using a mechanical homogenizer on ice, followed by immediate precipitation with trichloroacetic acid (see below). All measures were taken to avoid endogenous MLCK activation and therefore Ser-45 phosphorylation.

Biochemical procedures

Urea/glycerol PAGE

Avicularia filament suspensions were precipitated using an equal volume of trichloroacetic acid at 6% saturation and centrifuged at 14,000 rpm at 4°C for 5 min. Excess trichloroacetic acid was removed by two washes with cold acetone. Samples were then analyzed with urea/glycerol PAGE. Briefly, RLCs were extracted in urea buffer (8 M urea, 122 mM glycine, 200 mM Tris, and 5 mM DTT), subjected to 200 V electrophoresis according to (23) and stained with 0.25% Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA). Comparative protein standard BSA (bovine serum albumin) was used to quantify protein in samples (24). The gels were scanned with a GE ImageScanner III (GE Healthcare, Life Sciences, Piscataway, NJ) densitometer and processed with a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) to quantify the integrated area under the un-P, mono-P, and bi-P band peaks.

Kinase inhibitors

Kinase activity was inhibited using vertebrate smooth muscle MLCK inhibitors (ML-7, ML-9) or a generic kinase inhibitor (staurosporine). Relaxed or rigor filament suspensions were preincubated at room temperature for 1 h with either 2 μM ML-7 (Sigma-Aldrich, St. Louis, MO, Cat. No. 110448-33-4), 8 μM ML-9 (Sigma-Aldrich Cat. No. 1172), or 0.5–30 μM staurosporine (Sigma-Aldrich Cat. No. S4400). Samples were activated by adjusting the final [Ca^{2+}] to 1 μM (pCa 6.0) in the presence of 5 mM Mg.ATP, and incubating at 37°C for 30 min.

Phosphatases

Smooth muscle phosphatases from turkey and pig aorta (kindly supplied by Dr. Mitsuo Ikebe) were used to dephosphorylate the RLC.

Antibody preparation, Western blots, and immunofluorescence

Antibody preparations

Sequences of peptides containing the phosphorylatable serines (Ser-35, Ser-45) were selected from the tarantula *Avicularia avicularia* RLC sequence (accession no. EU090070, Fig. 1 *Ba*) (20). Rabbit IgGs were produced against the RLC sequences PPpSQKRRAQRSGSNV (Fig. 1 *Bd*) or RSGpSNVFAMFTQHQ (Fig. 1 *Be*) with phosphorylated Ser-35 or Ser-45 respectively for producing anti-pSer-35 or anti-pSer-45. These peptides were synthesized using the F-Moc technique (25), purified by HPLC and their sequences confirmed by tandem MS. Monoepitopic rabbit polyclonal IgG antibodies against tarantula *Avicularia* RLC either un-P or mono-P (pSer-35, pSer-45) were prepared by antigenic subcutaneous immunization of rabbits (see Fig. S2 in the Supporting Material). Both synthetic peptides were strongly antigenic in rabbit, and the IgGs obtained were highly specific against the immunogenic peptide (Fig. S2).

Western Blots

Avicularia RLCs from the urea-glycerol gels of quick-frozen relaxed muscle homogenates were electrophoretically transferred onto nitrocellulose (26) (NC, 0.45 μm; Hybond ECL; Amersham, Amersham Biosciences: GE Healthcare Bio-Sciences, Pittsburgh, PA) at 190 V for 190 min at 4°C in 25 mM TRISMA Base, 192 mM glycine, and 20% methanol. Free-reacting sites on NC were blocked with 5% skimmed milk in 0.01 M phosphate buffered saline pH 8.2 (PBS) containing 1% Tween 20 with constant agitation for 2 h at room temperature. NC strips were cut (2 mm wide), and individual strips were incubated at room temperature with constant agitation for 90 min with the respective serum sample diluted 1:100 in blocking solution. The strips were next washed with PBS containing 0.05% Tween 20 to remove unbound serum components and then incubated for 90 min at room temperature with a secondary antibody (anti-mouse IgG peroxidase conjugate; Sigma A-4937) diluted 1:5000 in blocking solution. Immune reactions were detected by chemiluminescence on Hyperfilm (Amersham Biosciences Hyperfilm ECL, RPN313K) using a peroxidase chemiluminescent substrate (ECL detection system; Amersham).

Immunofluorescence

Myofibrils of *Grammostola rosea* tarantula were prepared in relaxing or rigor solution. For each condition, an aliquot of 50 μl of the myofibril solution was loaded onto a coverglass for 90 s at room temperature. The slide was then washed with rigor or relaxing solution, as appropriate, blocked with 1% BSA (in the corresponding solution) for 30 min at room temperature, and washed with 0.1% BSA. Slides were incubated overnight at 4°C with 1000-fold diluted anti-pSer-35 or anti-Ser-35, and then washed three times with 0.1% BSA. Secondary labeling was done with Alexa Fluor 488 Goat Anti-Rabbit IgG (Invitrogen, Life Technologies Corporation, Grand Island, NY) for 1 h at room temperature. They were then washed three times with 0.1% BSA. To improve the fluorescence signal, one drop of ProLong Gold Antifade Reagent (Life Technologies Corporation, Grand Island, NY) was added on the microscope slide with the myofibril sample and the coverslip was attached. The labeled myofibrils were observed by differential interference contrast and fluorescence microscopy (filters: emission 610 nm, excitation: 560/480 nm) with an Axiovision (Carl Zeiss, Carl Zeiss Inc., Thornwood, NY) fluorescent microscope.

MS analysis

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)

Gel bands were excised with a scalpel carefully avoiding keratin contamination. Bands were destained with 250 mM NH_4HCO_3/30% acetonitrile for 10 min, washed with MQ water for 5 min, sliced into 1 mm^3 segments, dehydrated with 90% acetonitrile, and dried for 5 min using a speed vacuum centrifuge, all at room temperature. Gel tryptic digestion (Promega, Sequencing Grade Modified, Promega Corporation, Madison, WI) was carried out for 12 h at 37°C. Peptides were extracted (1% formic acid) and the supernatant loaded through a ZipTip C18 microcolumn (Millipore, EMD Millipore Corporation, Billerica, MA). Samples were eluted with 60% acetonitrile/1% formic acid and mixed with saturated matrix solution (a-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) in 1:1 ratio. MALDI-TOF spectra were collected from spots analyzed on an Autoflex III Smartbeam (Bruker Daltonics, GmbH, Bremen Germany). External calibration was performed with a commercial peptide mixture

(Peptide Calibration Standard, Bruker Daltonics). Data were acquired with the FlexControl software under the following MS conditions: The source was operated in the positive mode with an acceleration voltage of 19 kV, and a delayed extraction time of 60 ns was applied. The reflectron mode was used for the TOF analyzer (voltages of 21.0 and 9.6 kV). Ions were detected over a mass range from m/z 800 to 4000, though according to in silico theoretical trypsin digestion profiles peptides containing either phosphorylation site are clustered between m/z 2000 and 3000. MS and proteomic analysis were performed at the Unidad de Proteómica, Centro de Biología Estructural, IVIC, Caracas, Venezuela.

In vitro motility assay

Avicularia myosin filament activity in relaxing conditions was tested by measuring the sliding of fluorescently labeled filaments, either purified rabbit F-actin filaments (27) or *Avicularia* thin filaments (21). The in vitro motility experiments were done using a flow chamber following (21). The phosphorylation conditions of each filament suspension were assayed by urea-glycerol gels before the motility experiment. 15 μl of the relaxed filament homogenate was introduced into the chamber and flushed with a washing solution (25 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 3 mM NaN_3, 1 mM DTT, 5 mM PIPES, 25 mM Imidazol, 3 mg/ml BSA, 1 mM Mg.ATP; pH 7.5). 500 μl of tarantula thin or rabbit F-actin filaments were fluorescently labeled with 2.5 μl rhodamine/phalloidin (Sigma-Aldrich Cat. No. P-1951) and a 15 μl aliquot was added to the chamber, followed by a 60 μl aliquot of the activating solution (pCa 6.0) for thin filaments. For testing the inhibitors, the thick filament suspension was preincubated with the inhibitor ML-7 or ML-9 for 2 h at 37°C. Movement of the fluorescently labeled filaments was tracked at room temperature (21°–25°C) by video-enhanced microscopy using an epifluorescent/differential interference contrast Nikon (NIkon Instruments Inc., Melville, NY) E-600 light microscope with a digital camera Hamamatsu model ORCA-12ERG (Hamamatsu Photonic Systems, Bridgewater, NJ) connected to a PC running Metamorph software (Universal Imaging, Downingtown, PA). This in vitro motility setup enabled real-time image acquisition and analysis, speed calculations, and statistical analysis of tracked filaments (21).

RESULTS

Our previous studies showed that there is constitutive monophosphorylation of the RLC in quick-frozen relaxed tarantula muscle (21), but did not reveal which serine (Ser-35 or Ser-45) was phosphorylated. We have used MS of quick-frozen live tarantula muscle to answer this question. Our structural model of the tarantula thick filament suggested that in the relaxed state, Ser-35 on the free head is exposed and can therefore be phosphorylated, whereas that on the blocked head is covered by the free head and can therefore not be readily phosphorylated (20,21). We have prepared antibodies that specifically recognize Ser-35 in either the un-P (Ser-35) or mono-P (pSer-35) state, and have used them to assess the exposure of Ser-35 and pSer-35 on the filament. Finally, we have used kinase inhibitors to assess the presence of kinases in the tarantula muscle homogenate that would be necessary for the PKC and MLCK activation mechanism to work.

Western blots identify RLC bands and their phosphorylation state on urea-glycerol gels

As we have shown previously (7,22), the RLCs from filament suspensions in relaxing conditions have different electrical charges (depending on whether or not phosphate is incorporated), and migrate in two separate bands as un-P and mono-P species on urea-glycerol gels (Fig. 2 *A*). After Ca^{+2}-activation a third band appears, corresponding to the presence of bi-P RLCs (Fig. 2 *B*). The average densities of these three bands in the relaxed and activated states are compared in Fig. S1 *A* and show that the ratio of un-P, mono-P, and bi-P species is 74 ± 9 (44): 26 ± 9 (44) in the relaxed state vs. 31 ± 12 (40): 60 ± 11 (40): 10 ± 5 (40) in the activated state. Western blots (Fig. 2 *C*) confirm our previous inferences concerning these bands based on autoradiography and MS (7,20,21): 1), the two bands detected in relaxed muscle represent RLCs, as they were recognized by antibodies to whole RLC (Fig. 2 *C*); 2), Western blots using anti-pSer-35 and anti-pSer-45 show that the mono-P band in relaxed filaments contains pSer-35 but not pSer-45 (Fig. 2 *D*), although in activated filaments it contains pSer-35 or pSer-45 (Fig. 2 *E*). On the other hand, the bi-P band contains both pSer-35 and pSer-45 (Fig. 2 *E*). These results show that only

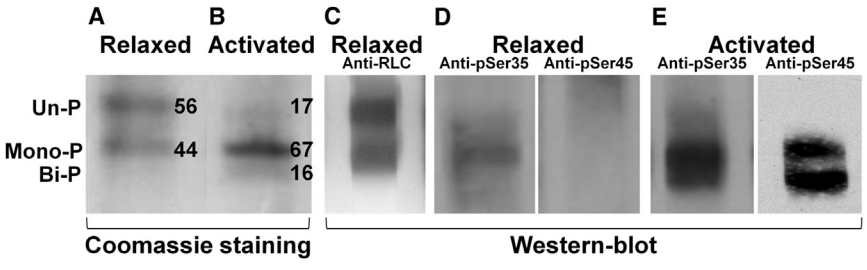

FIGURE 2 Urea-glycerol gels of filament suspensions from quick-frozen relaxed *Avicularia* muscle stained with Coomassie blue showing the RLC migration pattern: (*A*) In relaxing conditions the RLCs have two different electrical charges due to phosphate incorporation, migrating in two separate bands as unphosphorylated (un-P) or monophosphorylated (mono-P) RLCs. This confirms that mono-P is constitutive in live muscle (21). (*B*) After Ca^{+2}-activation a third band corresponding to the migration of biphosphorylated RLCs (bi-P) appears. (Numbers in *A* and *B* show their relative densities.) (*C*) Western blots using an anti-RLC antibody, which labels the two bands (un-P and mono-P) of the relaxed state (*A*). This shows that the two bands seen in live muscle are *Avicularia* myosin RLCs. (*D* and *E*) Western blots of relaxed (*D*) or activated (*E*) filaments (cf. (*A*) and (*B*), respectively), using antibodies specific for pSer-35 or pSer-45. In relaxing conditions (*D*) the pSer-35 antibody only labels the mono-P band, whereas the pSer-45 antibody does not label any band. This shows that the constitutive mono-P is due to pSer-35 only. In contrast, on activation (*E*); both antibodies label both the mono-P and bi-P bands. On the right of each urea-glycerol gel band (*A* and *B*) is shown the relative density (see Material and Methods) reflecting the relative number of RLC molecules present in the band.

pSer-35 is present in relaxed muscle, whereas on activation pSer-45 phosphorylation appears, either as mono-P from previously un-P RLC, or as bi-P, from previously mono-pSer-35 RLC.

pSer-35 is constitutively present in live relaxed muscle

Urea-glycerol gels of muscle homogenates from quickly frozen intact relaxed muscle show the presence of the un-P and mono-P bands (Fig. 2 *A* and Fig. 3 *A*) indicating that mono-P is constitutively present in this condition, the closest (in our hands) to the relaxed state in vivo. Using MS MALDI-TOF we identified which serines were phosphorylated. Tryptic digestion of the un-P band shows that it does not contain any phosphorylated peptides, whereas the mono-P band contains pSer-35 but not pSer-45 peptides (Fig. 3 *A*), consistent with the Western blot finding that pSer-35, but not pSer-45 is present in relaxed muscle homogenates (Fig. 2 *D*). The presence of pSer-35 in relaxed myofibrils was additionally detected by immunolabeling with anti-pSer-35, and visualizing by immunofluorescence. The relaxed myofibrils showed immunolabeling only with anti-pSer-35 and not with anti-Ser-35 (Fig. 4 *A*), supporting the presence of constitutive Ser-35 phosphorylation in intact relaxed myofibrils, and implying that pSer-35 is exposed.

Ser-45 becomes monophosphorylated only on activation

As shown previously, Western blots of urea-glycerol gels show that on activation only Ser-45 phosphorylation occurs, appearing in both the mono-P and bi-P bands (Fig. 2, *B* and *E*). To verify these results and the ability to detect pSer-45, MALDI-TOF analysis of these bands was carried out, showing that after activation the mono-P band is composed of a mix of peptides with either pSer-35 or pSer-45, and the bi-P band is composed of peptides with both pSer-35 and pSer-45 (Fig. 3 *B*) (20). This confirms our previous results using the LC/MS/MS Q-TOF technique (21).

Ser-35 is hidden and pSer-35 is exposed in the relaxed state

We carried out immunofluorescence observations to analyze whether Ser-35 and/or pSer-35 were exposed on the filament surface and thus accessible to antibody. Only pSer-35 was labeled under relaxing conditions, suggesting that only pSer-35 (and not un-P Ser-35) is exposed in relaxed muscle (Fig. 4 *A*). In contrast, under rigor conditions, similar labeling was found for both un-P Ser-35 and pSer-35 (Fig. 4 *B*). The rigor experiment shows that anti-Ser-35 can indeed label myofibrils, but that labeling depends on the state of the muscle: thus, lack of labeling in relaxed myofibrils

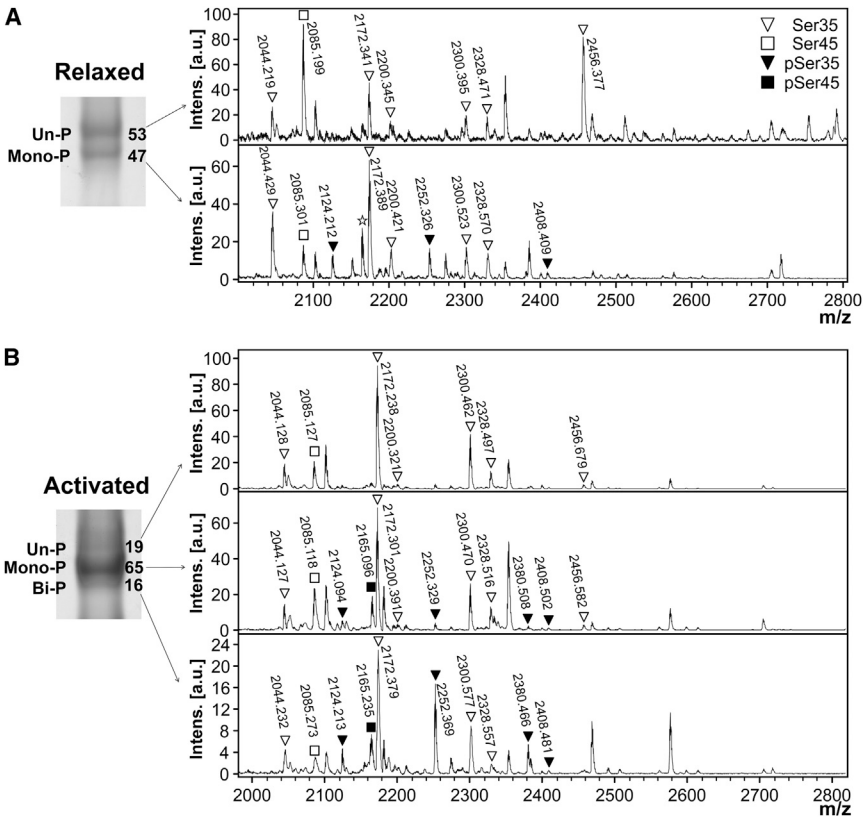

FIGURE 3 MS spectra of the bands of the urea-glycerol gels of a filament homogenate from quick-frozen relaxed *Avicularia* muscle (*A*) and after activating it (*B*). The bands (*left*) were excised and analyzed using MALDI-TOF (*right*). With the un-P bands only un-P Ser-35 or Ser-45 are observed. With the mono-P band of relaxed muscle only pSer-35 (but not pSer-45) is observed. These results show that pSer-35 is present in the quickly frozen relaxed intact muscle. On activation, besides pSer-35 the pSer-45 appears in the mono-P band, whereas in the bi-P band both pSer-35 and pSer-45 are observed. The individual peaks, resolved to a minimum of 1 Da and corresponding to all RLC peptides derived by trypsin digestion containing either phosphorylation site, are annotated as follows: Ser-35: open triangles, pSer-35 solid triangles, Ser-45: open squares, pSer-45 solid squares. The predicted sequence for each detected RLC peptide m/z signal is shown in Table S1. The peak denoted with a star corresponds to a trypsin autolysis fragment (m/z 2163.056). On the right of each urea-glycerol gel band (*A* and *B*) is shown the relative density (see Material and Methods) reflecting the relative number of RLC molecules present in the band.

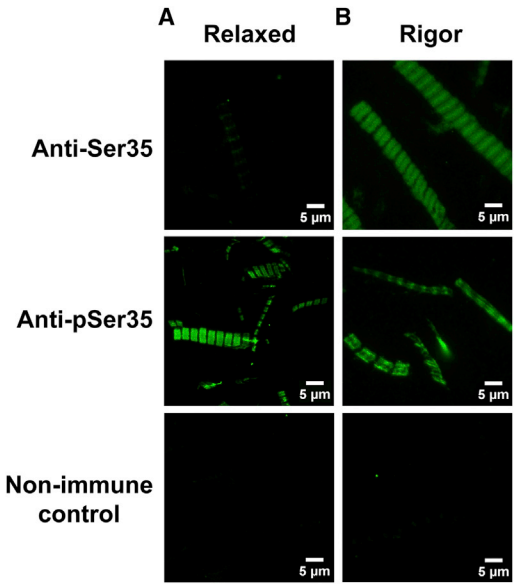

FIGURE 4 Immunofluorescence micrographs of tarantula *Grammostola rosea* striated muscle myofibrils prepared in relaxing (*A*) or rigor (*B*) conditions, immunolabeled with antibodies (anti-Ser-35, anti-pSer-35 or nonimmune control). The staining patterns of the relaxed myofibrils (*A*) show good labeling against pSer-35 but little labeling against un-P Ser-35. This suggests that pSer-35 is constitutively present in relaxed myofibrils and that only pSer-35 (not un-P Ser-35) is exposed for labeling in relaxed muscle. In contrast, in rigor (*B*) similar labeling is found both for un-P Ser-35 and pSer-35, suggesting that both Ser-35 and pSer-35 are now equally exposed. Nonimmune control: nonspecific IgGs were used as a specificity control using nonimmunized rabbit serum.

was not due to a poor antibody but more likely to lack of accessibility of Ser-35. The results therefore suggest that previously hidden Ser-35 becomes exposed by the change in head configuration going from relaxed to rigor, and supports the conclusion that in the relaxed state most exposed Ser-35 are phosphorylated.

MLCK-like kinase activity is present in the tarantula muscle homogenate

We investigated the presence of endogenous kinases in the filament homogenate by assaying the effect of two MLCK inhibitors; ML-7 (28) and ML-9 (29) and a generic kinase inhibitor (staurosporine) (30). Relaxed preparations were incubated first with each inhibitor, and then the preparation activated with Ca^{2+} to assess their inhibitory effect. Staurosporine, ML-7, and ML-9 all inhibited phosphorylation (Fig. 5) suggesting the presence of a MLCK-like activity. Their inhibitory effects are compared in Fig. S1 *B*.

Only the swaying heads are required for F-actin or thin filament sliding

It has been proposed that only the Ser-35-phosphorylated swaying heads (Fig. 1 *Aa*) are required to support quick force production in single twitches or twitch summation, and that Ser-45 mono-P potentiates force by recruiting additional Ser-45 mono-P heads (Fig. 1, *Ac* and *d*) (21). Here, we investigated the effect of Ser-45 phosphorylation on motility by testing the effect of ML-7 and ML-9 on filament sliding. Before each motility assay, samples were analyzed by urea-glycerol gels to assess their phosphorylation state. As a control, we found that F-actin filaments can slide on intact relaxed tarantula thick filaments irrespective of the presence of Ca^{2+} (Movies S1 and S2), at similar speeds to those reported before (without Ca^{2+}: 5.35 ± 2.05 μm/s, $n = 574$; with Ca^{2+}: 3.55 ± 1.29 μm/s, $n = 321$) (21). This is because

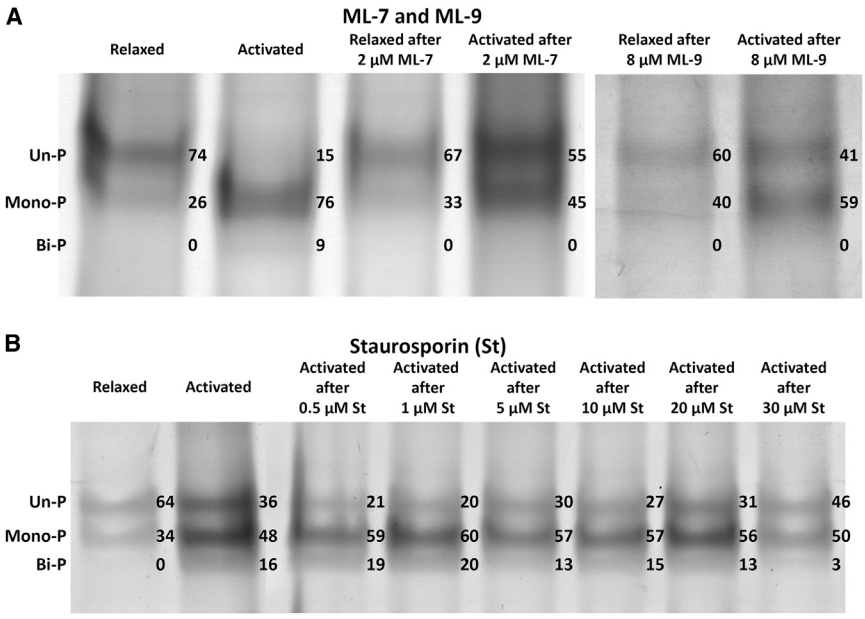

FIGURE 5 Urea-glycerol gels of the filament homogenate prepared in relaxing conditions, assaying the effect on RLC phosphorylation of smooth muscle MLCK inhibitors ML-7 and ML-9 (*A*) and the generic kinase inhibitor staurosporine (*B*). On activation 2 μM ML-7 or 8 μM ML-9 inhibit phosphorylation (*A*), whereas staurosporine (St) shows a dose-dependent action and at 30 μM inhibits phosphorylation (*B*). On the right of each gel band (*A* and *B*) is shown the relative density (see Material and Methods) reflecting the relative number of RLC molecules present in the band.

F-actin filaments lack the troponin-tropomyosin Ca^{2+}-switch. In contrast, tarantula thin filaments, which do have this switch, only slide in the presence of Ca^{2+} (Movies S3 and S4) at similar speed to those reported before (no movement without Ca^{2+}; with Ca^{2+}: 9.78 ± 1.92 μm/s, $n = 130$) (21). We found no significant effect of ML-7 on thin filament sliding in the presence of Ca^{2+} (10.24 ± 2.77 μm/s, $n = 28$, Movie S5). Its effect on F-actin sliding was difficult to test because ML-7 specifically affects F-actin (not shown) but not thin filaments (31); however, this disturbance by ML-7 could be avoided if the incubation time with ML-7 is below 15 min, which allowed measuring the sliding speed (without Ca^{2+}: 4.06 ± 1.44 μm/s, $n = 54$, Movie S6, with Ca^{2+}: 4.83 ± 1.19 μm/s, $n = 38$, Movie S7). We also found no effect of ML-9 on the sliding of tarantula thin filaments in the presence of Ca^{2+} (8.01 ± 2.23 μm/s, $n = 157$, Movie S8), nor on the sliding of F-actin in the absence of Ca^{2+} (4.15 ± 1.35 μm/s, $n = 50$, Movie S9). We were not able to test the effect of staurosporine due to the large quantities necessary to perform the motility assays. These in vitro motility results show that motility is supported mainly by the swaying heads; the extra heads recruited by Ser-45 mono-P are not essential for motility, and may only potentiate force, which is not measured in these experiments.

DISCUSSION

pSer-35 is constitutively present in intact relaxed muscle

Urea-glycerol gel autoradiography revealed the lack of [^{32}P]γ-phosphate incorporation into tarantula thick filaments on incubation with [^{32}P]γ-ATP in relaxing conditions (7), implying minimal kinase activity and suggesting that the observed mono-P band in the urea-glycerol gels is already present in relaxed muscle in vivo. Sequence analysis suggested that there are two phosphorylatable serines (Ser-35 and Ser-45), targeted by either PKC or MLCK (Fig. 1 *Bc*), and that the mono-P on Ser-35 was due to PKC and not MLCK (21). As MLCK is not activated in relaxing conditions (low Ca^{2+}), we conclude that—because there is no new [^{32}P]γ-phosphate incorporation—the PKC is also not activated in these conditions. We previously showed that the mono-P band was also present in quick-frozen relaxed intact muscle suggesting that mono-P was constitutive (21), however we did not identify which serine was phosphorylated in this case. Here, we demonstrated by MS of quickly frozen relaxed muscle (Fig. 3 *A*) that it is only pSer-35—and not pSer-45—that is already present in the intact relaxed muscle. Thus, pSer-35 mono-P is constitutive and there is no constitutive pSer-45. This is supported by Western blots, which showed that the mono-P band of quick-frozen muscle has only pSer-35 (Fig. 2 *D*), and by immunolabeling of relaxed myofibrils showing the presence of pSer-35 (Fig. 4 *A*).

On activation only Ser-45 is phosphorylated

On Ca^{2+}-activation, autoradiography reveals a rapid (<2 min.) de novo [^{32}P]γ-phosphate incorporation into the second and third bands (7). Urea-glycerol gels and MS provided evidence that this resulted from MLCK phosphorylation of Ser-45, either as mono-P or bi-P, of previously un-P and mono-P RLCs (21). The urea-glycerol gels presented here (Fig. 2, *B* and *E*), and MS (Fig. 3 *B*) confirm these results. Therefore, on activation only new pSer-45 phosphorylation appears, either as mono-P or bi-P. New Ser-35 mono-P by MLCK is unlikely because Ser-35 is in a target consensus sequence for PKC (not MLCK, Fig. 1 *Bc*), which is not Ca^{2+}-activated.

Blocked head Ser-35 is hidden and free head Ser-35 is exposed and constitutively phosphorylated in relaxed filaments

The immunofluorescence results (Fig. 4) show that exposure of Ser-35 and pSer-35 in the thick filament is related to the state of the muscle. Mainly pSer-35 RLCs (and not un-P Ser-35 RLCs) are available for antibody binding in relaxed muscle (Fig. 4 *A*). In contrast, both are available in rigor (Fig. 4 *B*), where the heads are away from the filament backbone. The specific head-head interactions that characterize relaxed filaments are broken in rigor as the heads attach to the thin filaments. The thick filament atomic model suggests that in the relaxed state these interactions cause the RLC N-terminus of the blocked heads to be hidden under the free head, whereas the free head N-terminus is exposed (20). The immunolabeling supports the concept that the free heads in the relaxed state are phosphorylated on Ser-35, and that few free heads are not phosphorylated. Exposure of blocked head RLC in rigor would explain the labeling of un-P Ser-35 RLC in this state. The multiple antibody binding assay shown in Fig. S2 supports our conclusion that differences in labeling in different states are not due to different affinities of the antibodies, but to availability of epitopes. The results overall support a model for the relaxed filament in which the free head Ser-35 is exposed and constitutively phosphorylated (by PKC), whereas the blocked head RLC is hidden, and therefore not available to kinases and not phosphorylated. This is consistent with the proposal that the Ser-35 mono-P free heads (swaying heads) are less strongly attached to the filament and available for immediate interaction with actin when muscle is activated (Fig. 1 *Aa*) (21). Recent results on the orientation of myosin heads and their rotational motion are in agreement with this interpretation (32).

Is there a preset number of constitutive pSer-35 heads?

We attempted, unsuccessfully, to dephosphorylate the constitutively phosphorylated Ser-35 using smooth muscle

phosphatases from turkey and pig aorta (not shown). The smooth muscle phosphatase—SMP IV—also did not remove the mono-P band completely (22). The failure to find a phosphatase that would dephosphorylate pSer-35 suggests that the number of swaying heads in a muscle could remain constant, possibly functioning as a mechanism to set the required number of heads for supporting quick activation, whereas phosphorylation of Ser-45 by MLCK could be used to recruit additional heads for force potentiation (21). Similar force enhancement by MLCK phosphorylation of the RLC occurs in mammalian muscle (3,5,6).

There is endogenous MLCK in the muscle homogenate

The inhibition of Ser-45 phosphorylation by the general kinase inhibitor staurosporine (Fig. 5 *B*) suggests the presence of a kinase involved in the Ser-45 phosphorylation. Inhibition by ML-7 and ML-9, which are specific smooth muscle MLCK inhibitors (Fig. 5 *B*), suggests that this kinase is MLCK-like, i.e., that it is specific to the myosin RLC. The smaller effect of ML-9 is in agreement with reports that ML-7 is more selective for smooth muscle MLCK (29).

Only the swaying heads are required for the sliding of F-actin or thin filaments

It has been shown (21) that relaxed thick filaments support F-actin filament sliding in the absence of Ca^{2+}. This suggested the presence of heads that are not intrinsically switched off at low Ca^{2+}, i.e., heads that are not locked down on the thick filament and are available to interact with F-actin even in the absence of Ca^{2+} (so-called swaying heads) (21). This is similar to the situation in vertebrate striated muscle. Electron micrographs of negatively stained smooth muscle myosin molecules suggest that the free heads are more mobile than the blocked heads (12), supporting the proposal that it is the swaying free heads (Fig. 1 *Aa*) that support initial filament sliding when muscle is activated. Our results show that neither ML-7 nor ML-9 affect the sliding of tarantula thin filaments in the presence of Ca^{2+} (Movies S5 and S8). Thus, phosphorylation of Ser-45 is not required for sliding of tarantula thin filaments on thick filaments, supporting the results obtained with F-actin. Preliminary experiments on bundles of myofibrils of relaxed, demembranated tarantula muscle, which are Ser-35 monophosphorylated, demonstrate rapid, reversible contraction and shortening upon Ca^{2+} addition, giving functional support to the conclusion that the swaying heads can reach out and generate force in the presence of Ca^{2+} before any MLCK phosphorylation occurs. We conclude that constitutive pSer-35 phosphorylation alone is sufficient for movement of F-actin or tarantula thin filaments by the swaying heads. We suggest that the extra heads recruited by Ser-45 mono-P are not essential for motility, but may potentiate force, which is not measured in our motility experiments.

The activation mechanism

The mechanism in Fig. 1 *A* is a plausible explanation for all our current evidence on phosphorylation activation of tarantula thick filaments. It requires 1), Ser-35 mono-P swaying heads for quick force production in single twitches (Fig. 1 *Aa*), and 2), a molecular switch activated by Ser-45 phosphorylation that recruits additional heads for force potentiation (Fig. 1, *Ab–d*). Our immunofluorescence and other results show that in the relaxed muscle there is constitutive Ser-35 mono-P on the free heads (Fig. 1 *Aa*) and that the un-P Ser-35 are hidden. This is consistent with the thick filament model (20) in which the free head N-terminal extension is exposed on the filament surface, whereas the blocked head N-terminal extension is hidden (by the free head). Previously, we also showed that in activated muscle only new pSer-45 appears; either as bi-P (consistent with the bi-P of the Ser-35 mono-P swaying heads in Fig. 1 *Ab*) or mono-P (consistent with the mono-P on Ser-45 of the blocked heads in Fig. 1 *Ac*) (21). The mechanism sequentially releases first the free and then the blocked heads in response to Ser-45 phosphorylation (Fig. 1, *Ab–d*), similar to a watch escapement mechanism. Our results are consistent with the requirement of an endogenous Ser-45 MLCK and complementary phosphatase (21). Thus, our results support the basic aspects of the cooperative phosphorylation mechanism for the activation of the tarantula thick filament (21).

Why does the tarantula RLC have two phosphorylatable sites?

The endogenous MLCK/MLCP pair reversibly controls the Ser-45 phosphorylation level. According to the cooperative phosphorylation mechanism (21) the pSer-35 heads should behave just like unphosphorylated heads in vertebrates (i.e., these swaying heads can interact with F-actin in a similar way to unphosphorylated vertebrate heads). Therefore, an unsolved puzzle is why tarantula has two phosphorylation sites when one appears to be constitutively switched on by a specific kinase and ATP, instead of, for example, by substitution with a negatively charged residue such as aspartate or glutamate (33). We have proposed that although the overall function of phosphorylation in arthropod and vertebrate skeletal muscle appears to be similar, an important difference may be that tarantula muscle can be better switched off, by inactivating its blocked head and possibly by regulating the number of monophosphorylated free heads (via an activated PKC and complementary phosphatase). If PKC and its complementary phosphatase are active only at specific times, this would make it possible to set specific basal numbers of pSer-35 swaying heads. This mechanism could be a way to increase force when needed, or save

ATP when not needed, which would be energetically advantageous in an animal that spends large amounts of time immobile, like when moulting, with only occasional bursts of energy required for capturing food or avoiding predators.

SUPPORTING MATERIAL

One table, two figures, nine movies, and reference (34) are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)01016-3.

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