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A molecular model of phosphorylation-based activation and potentiation of tarantula muscle thick filaments

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Abstract

Myosin filaments from many muscles are activated by phosphorylation of their regulatory light chains (RLCs). To elucidate the structural mechanism of activation, we have studied RLC phosphorylation in tarantula thick filaments, whose high resolution structure is known. In the relaxed state, tarantula RLCs are ~50% non- and 50% mono-phosphorylated, while on activation mono-phosphorylation increases and some RLCs become bi-phosphorylated. Mass spectrometry shows that relaxed-state mono-phosphorylation occurs on Ser35 while Ca²⁺-activated phosphorylation is on Ser45, both located near the RLC N-terminus. The sequences around these serines suggest they are the targets for protein kinase C (PKC) and myosin light chain kinase (MLCK) respectively. The atomic model of the tarantula filament shows that the two myosin heads (“free” and “blocked”) are in different environments, with only the free head serines readily accessible to kinases. Thus PKC Ser35 mono-phosphorylation in relaxed filaments would occur only on the free heads. Structural considerations suggest these heads are less strongly bound to the filament backbone, and may oscillate occasionally between attached and detached states (“swaying” heads). These heads would be available for immediate actin interaction upon Ca²⁺-activation of the thin filaments. Once MLCK becomes activated, it phosphorylates free heads on Ser45. These heads become fully mobile, exposing blocked-head Ser45 to MLCK. This would release the blocked-heads, allowing their interaction with actin. On this model, twitch force would be produced by rapid interaction of swaying free heads with activated thin filaments, while prolonged exposure to Ca²⁺ on tetanus would recruit new, MLCK-activated heads, resulting in force potentiation.

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Keywords

Myosin regulation; myosin regulatory light chain; striated muscle; twitch potentiation; post-tetanic potentiation

Introduction

All muscles are activated by an increase in intracellular Ca^{2+} , leading to actin-myosin interaction, filament sliding, and contraction. In most muscles, Ca^{2+} -regulation is achieved through the Ca^{2+} -sensitive troponin-tropomyosin switch on the thin filaments¹. In many muscles the thick filaments also play a regulatory role, via phosphorylation of the myosin regulatory light chains (RLC) in response to Ca^{2+} -calmodulin (CaM) activation of myosin light chain kinase (MLCK)^{2,3,4,5,6,7}.

Depending on the system, phosphorylation acts either as a switch or a modulator of activity⁴. In vertebrate smooth muscle, some invertebrate striated muscles, and in nonmuscle cells, RLC phosphorylation is essential for activity⁵, while in many vertebrate and invertebrate striated muscles, RLC phosphorylation is not required but functions instead to enhance contractility^{4,7}. For example, in vertebrate fast-twitch skeletal muscles the extent of RLC phosphorylation correlates with the strength of contraction during a low -frequency repetitive stimulus train (staircase potentiation) and with isometric twitch tension following a tetanus (post-tetanic potentiation)⁴. In many muscles more than one site on the RLC is phosphorylated, but the role of these multiple phosphorylations in striated muscle is unresolved^{3,4,7,8,9,10}.

Based on electron microscopic (EM) and other evidence, it has been proposed that the mechanism for these physiological responses involves movement of the phosphorylated myosin heads away from the thick filament backbone towards the thin filaments, enhancing interaction with actin^{3,4,11,9,12,13}. However, a molecular mechanism explaining how RLC phosphorylation produces these movements is lacking⁹. Based on X-ray crystallography, it appears that an initial step in this process may involve changes in interaction between the RLC and essential light chain (ELC) on the heads¹⁴.

Most biochemical studies of RLC phosphorylation have been carried out on purified myosin in solution. However, the physiological effects of phosphorylation in intact muscle occur in the context of assembled thick filaments, in which structural factors not present in solution (e.g. intermolecular interactions within a filament) may play a role^{15,9}. The thick filaments of tarantula striated muscle have emerged as a model system that has provided critical insights into thick filament structure at the near-atomic level^{9,15,16}. In the relaxed state, the myosin heads form well-ordered helices that make these filaments suitable for detailed structural analysis^{9,15}. An atomic model of the tarantula filament, based on 3D reconstruction of cryo-EM images, reveals that in the relaxed state the heads of each myosin molecule interact with each other intramolecularly, as well as forming intermolecular contacts along the helices^{9,15}. It is thought that this structural arrangement prevents actin-interaction and myosin ATPase activity, providing a simple structural explanation for the inhibited (relaxed) state^{15,17,18,19}.

In addition to providing structural insights into the relaxed state, tarantula filaments have also proved to be an excellent model for regulation by phosphorylation^{3,11,8,9}. Tarantula RLCs, like those from many species, can be mono- or bi-phosphorylated, and this phosphorylation enhances myosin activity and interaction with actin³. Electron microscopy³ and other techniques¹¹ suggest that phosphorylation causes breaking of the relaxed intra-

and inter-molecular head interactions, allowing release of the myosin heads from each other and from the filament backbone, consistent with the outward movement of myosin heads described above^{3,11 RP,9}.

While these studies have provided important insights into the biochemical and structural basis of phosphorylation-regulation of thick filaments, many questions remain^{4,9}. Here we take advantage of the tarantula system^{15,9} to better understand the structural basis of activation at the level of the intact filament. We use mass spectrometry to identify the two sites on the RLC that are phosphorylated, and sequence analysis to determine the kinases involved. We also use the *in vitro* motility assay and considerations of filament atomic structure to determine the likely time sequence in which the two sites are phosphorylated and how these phosphorylations bring about different levels of filament activity.

Results

To determine which sites on the RLC are phosphorylated, and the effect of this phosphorylation on filament activity, we separated RLCs having different levels of phosphorylation using urea-glycerol gel electrophoresis²⁰; used mass spectrometry to identify the phosphorylated sites present in each band; and used *in vitro* motility assays to determine if the associated thick filaments supported F-actin or thin filament movement.

Identification of RLC phosphorylation sites by Mass Spectrometry

Fig. 1a shows a urea-glycerol gel of RLCs from muscle homogenates in relaxing and activating conditions. In the relaxed state, two equally dense bands are seen (A, B). On activation (indicated by ') a weak additional band appears (C'), band A decreases in intensity (forming A') and B increases (forming B'). These bands have previously been shown to be non-phosphorylated (A, A'), mono-phosphorylated (B, B') and bi-phosphorylated (C')⁸. Thus, upon activation, some non-phosphorylated RLCs become mono-phosphorylated, and some mono-phosphorylated RLCs become bi-phosphorylated. When freshly dissected intact tarantula muscle was quick-frozen in liquid nitrogen, then run on urea-glycerol gels, the non- and mono-phosphorylated bands were again seen, confirming the presence of non- and mono-phosphorylated RLCs in relaxed muscle *in vivo* (Fig. 1b).

In-gel tryptic digestion was performed on the bands cut from these gels (Fig. 1a) and the extracted peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analysis of the data by Mascot (www.matrixscience.com) and also manual inspection identified tryptic peptides of 21 (peptide 1) and 18 (peptide 2) amino acids (aa), each with a serine (Ser35 and Ser45, respectively) that was either phosphorylated or non-phosphorylated (Table 1, Fig. 2). Phosphorylated peptides were initially identified by an increase of 80 Da in the calculated peptide mass, equivalent to the replacement of the hydroxyl group on serine with a phosphate group. Manual inspection of the MS/MS spectrum produced by collision-induced dissociation of the intact peptide shown in Fig. 3 verified each assignment through the neutral loss of the phosphate group as orthophosphoric acid (H₃PO₄; -98 Da) as well as the strong y ion series²¹ which matched the predicted fragmentation pattern of the phosphorylated peptides²².

The characteristic y-fragmentation of peptide bonds along the peptide backbone can be correlated to the amino acid sequence (Fig. 3). The peptide sequences (Table 1) deduced from the MS/MS spectra agreed with the RLC sequence previously determined by conventional aa sequencing⁹ (Fig. 2) confirming that these are RLC fragments. No other phosphorylated fragments or phosphorylated serines were observed, and phosphorylated peptides were absent from A and A'. We conclude that the only two phosphorylation sites on the RLC are in the N-terminal fragment (NTF) at Ser35 and Ser45.

Identification of sites phosphorylated in different states

LC/MS scans of peptide 1 (the Ser35-containing peptide) from bands A and B in resting muscle and from bands A', B' and C' from active muscle are shown in Fig. 4, and those from peptide 2 (the Ser45-containing peptide) in Fig. 5. In the relaxed state (Fig. 4a), the 3+ ion peak at m/z 708.7 corresponding to the peptide phosphorylated on Ser35 is prominent in band B but essentially absent from band A; band A shows only the 3+ ion peak at m/z 682, which comes from the non-phosphorylated form of peptide 1. For peptide 2 (Ser45-containing peptide), only the non-phosphorylated form (3+ ion peak at m/z 695.7) is detected in bands A and B (Fig. 5a), showing that Ser45 is non-phosphorylated under relaxing conditions. We conclude that the mono-phosphorylation under relaxing conditions occurs only at Ser35.

Under activating conditions, phosphorylation of peptide 1 at Ser35 is seen in bands B' and C' (Fig. 4b, 3+ ion peak at m/z 708.7), but is absent from band A'. Likewise in peptide 2, phosphorylation is detected at Ser45 (3+ ion peak at m/z 722.4) in bands B' and C' but not in A' (Fig. 5b). Thus, in relaxation a fraction of the RLC (about one half) is Ser35-mono-phosphorylated (Figs. 1, 4a). The rest of the RLC is non-phosphorylated and there is no bi-phosphorylation. Upon activation, additional phosphorylation occurs. This is mainly on Ser45, causing non-phosphorylated RLC to become mono-phosphorylated (Fig. 5bB'), and mono-phosphorylated RLC to become doubly phosphorylated (Fig. 4bC', 5bC').

In vitro motility assay

We used the *in vitro* motility assay to determine the RLC phosphorylation requirements of native thick filaments for supporting motility of F-actin and native thin filaments. To aid in understanding the results we built a setup that allowed visualization of both thick and thin filaments in the same preparation. Differential interference contrast video-enhanced light microscopy of the muscle homogenate revealed thin, ~5 μm -long straight features (Fig. 6a), which are consistent with the presence of tarantula thick filaments (4–5 μm long, 32 nm diameter)²³. Rhodamine/Phalloidin fluorescently labeled rabbit F-actin or tarantula thin filaments were seen as short, straight segments $1.61 \pm 0.49 \mu\text{m}$ (mean $\pm$ standard deviation, $n = 886$; Fig. 6b, Supplementary Movies 1, 3) and $1.94 \pm 0.47 \mu\text{m}$ ($n = 127$) (Fig. 6c, Supplementary Movies 2, 4) in length, respectively.

In the presence of Mg.ATP and EGTA (no Ca^{2+}), native tarantula thin filaments (containing troponin and tropomyosin) showed no movement (Table 2). In the presence of Ca^{2+} , they moved over thick filaments (Fig. 6d,e; Table 2; Supplementary Movie 2 cf. 4) in fast, straight, short patches ($9.78 \pm 1.92 \mu\text{m/s}$, $n = 130$). These straight courses and their length support the conclusion that it is myosin filaments (not molecules) that provided straight tracks for sliding. A sudden stop after these short slides was very common (Supplementary Movies 1, 4). In contrast, F-actin (no troponin/tropomyosin) progressed over thick filaments also in straight, short, fast movements, but independently of the presence ($3.55 \pm 1.29 \mu\text{m/s}$, $n = 321$) or absence ($5.35 \pm 2.05 \mu\text{m/s}$, $n = 574$) of Ca^{2+} (Fig. 6e; Supplementary Movie 1 cf. 3). The observed speeds are comparable to the 4.6 $\mu\text{m/s}$ reported for rabbit fast-twitch back muscle thick filaments²⁴. These results show that Ser35 mono-phosphorylation of the relaxed thick filaments is sufficient for myosin head activity and filament sliding. When tarantula thick filaments are incubated in relaxing medium for extended periods (24 h) following homogenization, their RLCs become partially (~ 10%) bi-phosphorylated. *In vitro* motility assays showed similar results for these filaments as for mono-phosphorylated filaments (Table 2, Supplementary movies 5–8).

Discussion

Our goal was to elucidate the molecular mechanism of phosphorylation-based activation in tarantula thick filaments. We found that in relaxed muscle ~50% of the RLCs are mono-phosphorylated at Ser35, and that Ca^{2+} -activation leads to phosphorylation at Ser45 of both non- and mono-phosphorylated RLCs. These findings, together with knowledge of the thick filament atomic structure, lead to a simple molecular model for thick filament activation in which the specific heads that are phosphorylated are determined by their 3D organization on the filament surface. As discussed below, this mechanism may also explain the potentiation of contraction during and following prolonged activation.

The relaxed thick filament

Our results show that relaxed thick filaments (with about 50% non- and 50% Ser35 mono-phosphorylated RLCs; Fig. 1a; 4aA–B), support F-actin and Ca^{2+} -activated thin filament sliding (Fig. 6d,e). This suggests that relaxed tarantula muscle (with 50% phosphorylated heads) would be able to respond rapidly to activation, without additional phosphorylation, simply by the Ca^{2+} -induced switching on of the thin filaments.

Gels from tarantula muscle homogenates incubated for up to 30 min in [^{32}P]- γ -ATP in relaxing conditions³ reveal no γ -phosphate incorporation, implying minimal kinase activity under these conditions and suggesting that the observed Ser35 mono-phosphorylation is present in relaxed muscle *in vivo*. This is supported by our finding of constitutive mono-phosphorylation in quick-frozen live muscles (Fig. 1b). Sequence analysis suggests that this is due to PKC and not MLCK (see below). Similar phosphorylation levels (35–50%³) are present in other relaxed arthropod muscles (*Limulus*²⁵, scorpion²⁶). This constitutive phosphorylation contrasts with “basal” phosphorylation that can occur in rabbit^{27,28} on the MLCK-targeted serine, due to activation of MLCK during tissue handling.

Preliminary experiments show that bi-phosphorylated thick filaments incubated at 37°C for 4 h with potato acid phosphatase lose their bi-phosphorylation but remain mono-phosphorylated and can support F-actin (+/– Ca^{2+}) and tarantula thin filament (+ Ca^{2+}) motility (not shown). In contrast, mono-phosphorylated thick filaments incubated at 37°C for 2 h with alkaline phosphatase from bovine intestinal mucosa lost their mono-phosphorylation and did not support movement of Ca^{2+} -activated thin filaments (not shown). This suggests that constitutive phosphorylation is essential for myosin filament function.

The two phosphorylatable serines of the RLC, Ser35 and Ser45, are found respectively in peptides 1 and 2 of the 52 aa long NTF (Table 1, Fig. 2)⁹, close to the head-tail junction (Fig. 7a). The RLC sequence^{9,29} shows that Ser35 and Ser45 are in sequences that would be targets for PKC and MLCK, respectively (Fig. 2). At least 7 other species apart from tarantula have long NTFs in their striated muscle RLCs (Supplementary Fig. 1, Supplementary Table 1). All of these show two phosphorylatable serines, homologous to the Ser35/Ser45 pair and located in PKC and MLCK target consensus sequences. PKC and MLCK sites are also present in the short RLC NTF of vertebrate smooth muscle myosin³⁰. While the constitutive PKC-dependent Ser35 mono-phosphorylation in relaxed filaments is required for rapid response to Ca^{2+} -activation, our results suggest that Ser45 phosphorylation by MLCK is used to potentiate contraction (see below).

EM has shown that in the helically ordered relaxed state, the two heads of each myosin molecule in tarantula^{9,15} and other thick filaments^{31,32} interact asymmetrically with each other (“interacting-heads motif”, Fig. 7a). In this structure, one head (“blocked”) is unable to bind to actin, while the other head (“free”) cannot carry out ATP turnover^{15,17,18,19}. These

interactions place the two heads in different structural environments, raising the possibility that one head may be sterically targeted for the constitutive Ser35 mono-phosphorylation of relaxed filaments. Fig. 7a–c shows that the phosphorylatable serines of the free and blocked heads are not equally accessible to kinase: both free head serines are exposed on the surface (red spheres, Fig. 7a–c), while those of the blocked head are hidden below (magenta spheres, Fig. 7b). Therefore the ~50% constitutive Ser35 mono-phosphorylation detected by gels and mass spectrometry appears most likely to occur only in the free heads; if so, then the majority of the free heads would be mono-phosphorylated on Ser35, while the less accessible blocked heads would be non-phosphorylated. It is these constitutively Ser35-phosphorylated free heads that would presumably support F-actin sliding in relaxing conditions and that in muscle would respond immediately to Ca^{2+} -activation.

Analysis of the thick filament 3D structure suggests that blocked and free heads both have multiple interactions with other sites on the filament (the partner head in the same molecule, the heads of neighboring molecules, and the myosin tails)^{9,15}. The number of interactions of the blocked head (7) is greater than that of the free head (5)⁹, suggesting that the free head may be more weakly tacked down on the filament than the blocked head. This is supported by EM studies of isolated myosin molecules showing that the free head is less strongly attached to the S2 tail of myosin than is the blocked head³³. Double electron resonance EPR methods reveal heterogeneity in the orientation of the two myosin RLCs in vertebrate smooth muscle in the relaxed state³⁴, consistent with the mobility of the free head that we are proposing. We conclude that the free heads spend their time in an equilibrium, swaying in and out (by Brownian motion) from their positions on the filament surface (“swaying” heads, Fig. 8a). As the free heads have similar density to the blocked heads in the 3D reconstruction, we conclude that the equilibrium is biased toward the docked state, and that the free heads spend most of their time in the ordered, backbone-attached configuration. On the occasions when they detach, they would be available for immediate actin interaction upon thin filament activation. Structural evidence for a population of disordered heads in relaxed muscles also comes from X-ray diffraction³⁵, EM^{31,36}, X-ray diffuse scattering³⁷, EPR/STEPR³⁸ and polarized fluorescence³⁹. Changes in the “11” equatorial X-ray diffraction reflection of frog (fast-twitch)⁴⁰ and fish (slow-twitch)⁴¹ striated muscles show that, upon activation, heads reach actin filaments in ~30 ms, consistent with the presence of loosely attached heads in these muscles.

The activated thick filament

On Ca^{2+} -activation of tarantula filaments (with endogenous MLCK and CaM), we find that 50–90% of non-phosphorylated RLC becomes mono-phosphorylated, while <50% of mono-phosphorylated RLC becomes bi-phosphorylated (Fig. 1a)⁸. In band B' (Fig. 1), mass spectrometry detects phosphorylation on Ser35 (Fig. 4bB') and Ser45 (Fig. 5bB'). As band B' is mono-phosphorylated, these represent *alternative* phosphorylation sites on different RLCs. The same sites are phosphorylated in band C' (Fig. 4bC', 5bC'), but in this bi-phosphorylated case, *both* sites must be phosphorylated on the same RLC. These results (Fig. 4b, 5b) agree with autoradiography³ showing rapid *de novo* [³²P]- γ -phosphate incorporation into bands B' and C' after 2 min activation. These data suggest that Ca^{2+} -CaM-MLCK-activated phosphorylation occurs only on Ser45 (of previously non- and mono-phosphorylated RLCs), consistent with Ser45 being in a consensus sequence that would be targeted by MLCK (Fig. 2). New Ser35 mono-phosphorylation by MLCK is unlikely because Ser35 is in a target consensus sequence for PKC (not MLCK), which is not Ca^{2+} activated⁴². Any *de novo* Ser35 phosphorylation could be detected by isobaric mass spectrometry experiments (in progress) using ¹⁸O-ATP.

Molecular dynamics simulations of chicken smooth muscle RLC NTF⁴³ predict that it is disordered when non-phosphorylated, but becomes better ordered (more α -helical) and

elongated on phosphorylation; our simulations of the longer tarantula NTF (in preparation) also suggest similar changes going from the non-phosphorylated to the mono-phosphorylated state and then to the bi-phosphorylated state. The difference in the shapes of the NTFs of the blocked (non-phosphorylated) and free (mono-phosphorylated) head RLCs observed in the 3D-map of the relaxed tarantula thick filament⁹ are consistent with these predictions.

Following the switching mechanism proposed by Himmel *et al.*¹⁴ for molluscan myosin regulated by Ca^{2+} , we suggest that these changes in NTF structure when the free or blocked head RLCs become bi- or mono-phosphorylated on Ser45 could modulate the intrinsic flexibility of the regulatory domain heavy chain. Formation of critical links between the RLC C-lobe and the ELC N-lobe by phosphorylation could make the regulatory domain heavy chain intrinsically more rigid in the activated state¹⁴ (this contrasts with the suggestion that in single heads –not HMM- phosphorylation may cause the regulatory domain heavy chain to become more flexible⁴⁴). Stiffening of the lever arm could contribute towards breakage of the off-state head-head interaction¹⁴, as well as ensuring an effective crossbridge power-stroke. In addition to this intra-molecular effect of NTF phosphorylation, the NTF disorder-to-order transition may weaken some of the inter-molecular interactions between myosin heads⁹, allowing them to move away from the filament backbone.

A structural mechanism for phosphorylation-activation in tarantula striated muscle

The preceding observations together with the tarantula thick filament atomic model^{15,9} suggest a simple structural mechanism for phosphorylation activation in tarantula (Fig. 8), which depends on simple steric/accessibility factors to control which heads can become phosphorylated and thus able to interact with actin to generate filament sliding.

In the relaxed state, the free heads have their Ser35 constitutively mono-phosphorylated, allowing them to sway in and out (Fig. 8a, dotted white arrows), supporting thin filament movement (see Supplementary Movie 10). The free head RLC NTF (yellow) is thought to cover domain 1 of the lower (N-) lobe of its partner blocked head RLC (Fig. 7a, c, 8a), which may hinder the release of this head⁹, contributing to its immobility. Fig. 7d shows that electrical charges of the opposed surfaces of domain 1 and the free head RLC NTF are complementary⁹ over a large central surface (Supplementary Movie 9), stabilizing this interaction. The electrostatic interaction between the free head RLC NTF and the blocked head RLC domain 1 (Fig. 7d) would facilitate docking back of the free head after its occasional movements away from the filament backbone.

On activation, when Ca^{2+} concentration is high for long enough to activate MLCK, phosphorylation of Ser45 can occur. Steric constraints suggest that the free (mono-phosphorylated) heads will be phosphorylated first (thus bi-phosphorylated, Fig. 8b, yellow), as free head Ser45 is exposed on the surface of the filament (Fig. 7a–c, 8a). In contrast, access of MLCK to Ser45 (magenta) on the blocked head NTF (brown) is hindered in two ways: this serine is hidden below the blocked head RLC and free head NTF (Fig. 8a, yellow), and the positioning of the free head motor domain of the next head below (further from the bare zone) would block the approach of MLCK (Fig. 8a, Supplementary Fig. 2a). Phosphorylation of free head Ser45 causes elongation and stiffening of its NTF, which would hinder the docking back of this head after swaying, making it permanently mobile (Fig. 8b, white arrow; Supplementary Fig. 2b) until its NTF is Ser45 de-phosphorylated on relaxation. Release of the free head creates a space sufficient to allow access of MLCK-CaM to the RLC NTF of the blocked head above (closer to the bare zone; Fig. 8b, white dotted circle; Supplementary Fig. 2c). This allows Ser45 mono-phosphorylation of blocked heads, which become swaying heads (Fig. 8c), able to interact with actin. Removal of the free head NTF upon bi-phosphorylation may also electrically destabilize the blocked head RLC and its

NTF (Fig. 7d), making it less constrained, and adding to the ability of the blocked heads to sway.

Ser45 phosphorylation of the blocked head NTF makes it less compact, hindering the docking back of its partner swaying free head, which could make this head mobile too, even if only Ser35 mono-phosphorylated (Fig. 8d). This could produce – along the filament and towards the bare zone – a cooperative unzipping of adjacent myosin interacting-heads, which starts with bi-phosphorylation of a free head followed by mono-phosphorylation of the blocked head above, and continues without the requirement that bi-phosphorylation of each free head must occur for neighboring blocked head phosphorylation to be possible. Possible cooperativity is suggested by the gels, which show a greater level of new mono-phosphorylation (band A going to B') than of bi-phosphorylation (band B' going to C') upon activation (Fig. 1a).

Finally, this structural model would suggest that upon relaxation, when both Ser45s become dephosphorylated, the blocked head would dock back *first* on to the backbone (to avoid steric clashes; Fig. 8d), and its partner free head would then dock back on top of it, locking the blocked head with its NTF (Fig. 8a).

Relationship of phosphorylation to activation, potentiation and post-tetanic potentiation of force in tarantula striated muscle

RLC phosphorylation and dephosphorylation are too slow to control rapid twitch contraction and relaxation. This rapid mechanism is provided by the combination of the thin filament Ca^{2+} -troponin/tropomyosin switch and the thick filament swaying heads (see Supplementary Movie 10). In contrast, RLC Ser45 phosphorylation by MLCK appears to provide a modulatory – but slow – mechanism to increase the number of heads available for interaction with actin (beyond the constitutive Ser35 mono-phosphorylated heads), leading to force potentiation in prolonged contractions. This is suggested by ATPase³ and force⁴⁵ measurements in chelicerates. The model in Fig. 9 summarizes how phosphorylation may control different levels of tarantula muscle activity.

In relaxed muscle (Fig. 9a), Ser35 of most of free head RLCs is constitutively phosphorylated (the number could be adjusted by PKC and its phosphatase partner). These heads sway in and out from the filament surface (Fig. 9a, double arrows), the equilibrium being towards the “in” position. This mixture of blocked and free heads agrees with ATP turnover measurements in tarantula relaxed myofibrils which suggest that blocked heads exhibit a “super-relaxed” (very slow) turnover rate, while free (swaying) heads have a normal relaxed rate^{46,47}. Upon release of Ca^{2+} into the cytosol (Fig. 9b), free heads that are “out” interact with activated thin filaments, producing a single twitch (Fig. 9b; Supplementary Movie 10). A second Ca^{2+} pulse would recruit additional swaying heads that were initially in, producing a second, stronger twitch without extra phosphorylation (Fig. 9b, twitch summation). With prolonged activation (Fig. 9c), some Ser35-phosphorylated free heads become Ser45 phosphorylated by MLCK (thus bi-phosphorylated and mobile). Neighboring blocked heads along the helix can then become Ser45 mono-phosphorylated, allowing them to interact with thin filaments as well. These additional heads would lead to force potentiation in prolonged contractions (e.g. tetani, Fig. 9c). Assuming that dephosphorylation by MLCP is relatively slow, the newly phosphorylated heads will remain ready for subsequent contraction after Ca^{2+} decreases (Fig. 9d). A new twitch induced just after a tetanus will therefore be bigger than a twitch elicited before (post-tetanic potentiation; Fig. 9e). The Ser45 phosphorylation “labeling” of these heads thus acts like a biochemical memory⁴⁸ that strengthens contraction upon subsequent stimulation. Upon prolonged relaxation, MLCK would become inactive, allowing MLCP to dephosphorylate Ser45 (Fig. 9d, a), so that subsequent twitches would be of normal size.

Possible implications for other striated muscles

We have proposed a simple structural explanation for RLC phosphorylation (Fig. 8) and its relation to activation and potentiation in tarantula. Is this model related to phosphorylation in other species? All reported RLC sequences have either short or long NTFs. For striated muscles, those with long NTFs are all arthropods (Supplementary Table 1), like tarantula. These have pairs of non-contiguous phosphorylatable serines (“S...S”) located in sequences that would be targets for PKC and MLCK homologous to Ser35/Ser45 in tarantula. Thus this mechanism or a variation of it may be common in Arthropod striated muscles. A similar head arrangement to tarantula is present in other arthropods, including *Limulus*⁴⁹ and scorpion (Pinto, Sanchez, Alamo & Padrón, unpublished data), both with two phosphorylatable serines on their RLCs^{2,26}. Thus, of the arthropods studied so far, both Arachnida (tarantula, scorpion) and Merostomata (*Limulus*) have the same head organization as well as dual PKC/MLCK phosphorylation sites, consistent with a common mechanism of regulation. If other arthropods also have such head arrangements, similar kinase accessibility constraints would suggest a similar structural basis for regulation in these muscles. A comparison with RLCs having short NTFs (see Supplementary Discussion) suggests that the model we are proposing may be limited to the arthropods, which have a clear constitutive/potentiating phosphorylatable serine pair. However, even though constitutive and potentiating phosphorylation may not be the same with short NTFs, steric constraints may still play a role in determining which heads are phosphorylated in which order. For example, vertebrate striated muscle appears to have a blocked/free head organization similar to tarantula at two out of every three levels of heads (although the heads interact more weakly)³¹. Thus, initial phosphorylation may occur on the free head, with the blocked head playing a potentiating role.

Our model for tarantula striated muscle supports the general concepts for phosphorylation-based potentiation both during and after a tetanus advanced previously for fast-twitch vertebrate muscle^{4,48}. This mechanism hypothesized that light chain phosphorylation moved the average position of myosin heads away from the thick filament backbone^{3,11}, increasing the rate at which they can attach to actin, thus explaining potentiation both during and after a tetanus. Our model (Fig. 9) proposes a specific physical mechanism for some of these effects in tarantula, based on structural constraints in the filament (Fig. 8). It also takes into account the dual RLC phosphorylation characteristic in this muscle, suggesting specific roles for constitutive (Ser35) and potentiating (Ser45) phosphorylation. While the overall function of phosphorylation in arthropod and vertebrate skeletal muscles 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 mono-phosphorylated free heads (via PKC and its partner phosphatase). Potentiation both during and after a tetanus in tarantula muscle could thus be considered as a way to increase force when needed, while saving ATP when not needed. This would be energetically favorable in an animal that spends large amounts of time immobile, with only occasional bursts of energy required for capturing food or avoiding predators.

Materials and Methods

Native thick and thin filament suspension

Filament suspensions containing thick and thin filaments plus endogenous phosphatases, kinases and CaM, whose activity can be controlled by Ca²⁺ concentration, were prepared from tarantula (pink-foot, *Avicularia avicularia*) striated (leg) muscle by skinning muscles in relaxing solution (100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 5 mM PIPES, 3 mM NaN₃, 5 mM Mg.ATP, pH 7.0) with 0.2% saponin, then homogenizing^{3,8}. The homogenate was centrifuged for 20 min at 14,000 RPM and the supernatant (filament

suspension) kept on ice until used for *in vitro* motility assay experiments, electrophoresis and mass spectrometry analysis. To test for the presence of phosphorylation *in vivo*, the tarantula was first rested for several hours at room temperature, then cold-anesthetized/immobilized by placing at 4°C for 1 hr (as for the skinned preparations). It was then dropped suddenly into liquid nitrogen to rapidly arrest all muscle enzymatic activity while in the normal resting state. Following dissection under liquid nitrogen, muscles were placed quickly in ice-cold relaxing solution, immediately homogenized for two 3-second bursts, and the homogenate immediately precipitated with ice-cold TCA (Fig. 1b, homogenate). Placing in ATP-free (rigor) solution instead of relaxing solution, or directly in TCA for homogenization (to even more rapidly arrest kinase activity) produced similar results. One aliquot was additionally centrifuged at 14,000 RPM for 3 min at 4°C (Fig. 1b, supernatant) before treatment with TCA (see below).

Gel electrophoresis

Activated thick filaments were prepared by adding CaCl₂ to the relaxed homogenate to pCa 6.0 (1 μM) for 30 min. at 22°C. WimMax32 software (<http://www.stanford.edu/~cpatton/maxc.html>) was used to calculate pCa. Non-activated samples were treated with relaxing solution for the same period of time. Samples were precipitated with ice-cold TCA and washed with ice-cold acetone⁸. Light chains were extracted in urea-glycerol buffer (8M urea, 122 mM glycine, 200 mM Tris, 5 mM DTT) and subjected to 200 V urea-glycerol gel electrophoresis^{20,3} and stained with 0.25% Coomassie Brilliant Blue G-250 (BioRad).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The thick filament suspension was diluted in an equal volume of relaxing solution (relaxing conditions) or incubated in activating solution at pCa 6.0 for 30 minutes at room temperature (activating conditions) followed by precipitation with 3% TCA. These samples were analyzed by urea-glycerol gel electrophoresis. Gels were handled with gloves in sterile conditions, and the Coomassie Blue G-250 stained RLC bands were excised and kept at -20°C. Bands were reduced with 0.01 M DTT and alkylated with 0.015M iodoacetamide. Samples were washed with 0.05M Tris pH 8.5/acetonitrile (50:50) and vacuum dehydrated. Bands were rehydrated, and digested with trypsin (0.08 μg/ml) (Roche Biochemicals) overnight at 32°C. Tryptic digested peptides were extracted with 50% acetonitrile, 2% TFA in water and analyzed on an electro-spray quadrupole-time of flight Q-TOF liquid chromatography (LC) LC/MS/MS mass spectrometer with an in-line nanoflow LC (Micromass Q-ToFTM, Waters Corporation, USA). Resulting MS and MS/MS data were processed and viewed using MassLynx 4.0 software.

In vitro motility assay experiments

Solutions—Rigor solution was 25 mM KCl, 3 mM MgCl₂, 1 mM EGTA, 5 mM PIPES, 3 mM NaN₃, 1mM DTT, pH 7.5. Washing relaxing solution was prepared by adding 5 mM Mg.ATP, 25 mM imidazole, 3 mg/ml BSA to rigor solution and adjusting to pH 7.5. Activating solution was prepared by adding 0.95 mM CaCl₂ to relaxing solution to pCa 6.0 and adjusting pH to 7.5. Aliquots from the samples used for *in vitro* motility assay experiments were analyzed by urea-glycerol gel electrophoresis to determine the phosphorylation status. Tarantula thin filaments (see above) or rabbit purified F-actin prepared according to⁵⁰ were labeled with 0.3 μg/ml of Rhodamine/Phalloidin solution (100 μg/ml Rhodamine/Phalloidin (Sigma P-1951) in methanol⁵¹, kept at -20°C).

Myosin-actin interaction was quantified by measuring the sliding velocity of fluorescently Rhodamine/Phalloidin-labeled rabbit F-actin or tarantula thin filaments over tarantula thick filaments adhering to a cover slip flow cell⁵². Movement was tracked by video-enhanced

microscopy using an epi-fluorescent/DIC Nikon E-600 light microscope with a digital camera (Hamamatsu model ORCA-12ERG; Hamamatsu Photonic Systems, U.S.A.) connected to a PC running Metamorph software (Universal Imaging Corporation, U.S.A.). This enabled real time image acquisition and analysis, speed calculations and statistical analysis of tracked filaments. For activating experiments, 15 μ l of a thick filament suspension were introduced into the flow chamber, filaments allowed to adhere to the cover slip, and then the coverslip was washed with relaxing solution. 15 μ l of fluorescently labeled F-actin or thin filaments were then introduced, followed immediately by activating solution and recording of filament motility. Experiments were performed at room temperature (21–25°C) and 38–65% relative humidity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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We dedicate this paper to the memory of Prof. Samuel Perry.

Abbreviations

aa	amino acids
CaM	calmodulin
ELC	essential light chain
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
NTF	N-terminal fragment
RLC	regulatory light chain
S2	subfragment 2
PKC	protein kinase C

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Figure 1.

Urea-glycerol gels showing myosin RLC bands in: (a) tarantula demembranated muscle homogenate in relaxing and activating conditions (bands used for the LC-MS/MS analysis, Fig. 3–5); (b) quick-frozen relaxed live tarantula muscle (left: homogenate, right: supernatant; see Materials and Methods). Arrows point to non-phosphorylated bands (Non-P: A, A'), mono-phosphorylated bands (Mono-P: B, B') and to bi-phosphorylated band (Bi-P: C'); ' indicates the activated state.

Figure 2.
Amino acid sequence of *Avicularia avicularia* tarantula myosin RLC⁹ (GenBank EU090070, UniProtKB/TrEMBL B4XT43). The 196 aa sequence contains 10 serines (red), only two (Ser35 and Ser45, boxed) determined by MS (Fig. 3) to be phosphorylatable, belonging to peptides 1 (Ser35-containing peptide) and 2 (Ser45-containing peptide) underlined in blue. Consensus sequences for PKC (magenta) and smMLCK (green) phosphorylation are shown (K/R-XXSX-K/R⁵⁴ and XXRXSXBB⁵⁵ respectively; determined using PhosphoMotif finder, Human Protein Reference Database⁵⁶). “X” is any aa and “B” is any hydrophobic aa (A, V, F, I, or L).

Figure 3.

Collision-induced fragmentation spectra of RLC phosphorylatable peptides 1 and 2 (b). Both MS/MS spectra show relative abundance of ions. Peptide 1 (Ser35-containing peptide) and 2 (Ser45-containing peptide) spectra show phosphorylation at Ser35 or Ser45 respectively. “b” and “y” designate the N- and C-terminal peptide fragments produced by peptide cleavage. Sub-index numbers on “b” and “y” represent residue position from either N or C terminus respectively. Daughter ion peaks with H_3PO_4 loss of 98 Da, characteristic of a phosphorylated peptide, are indicated as -98. $M-H^+$ = molecular ion + H^+ , i.e. the intact peptide plus one H^+ . Mass in Da. “m” mass and “z” charge (+3).

Figure 4. Combined LC/MS scans of the 20–40 min region of the chromatogram where phosphorylated and non-phosphorylated forms of tryptic peptide 1 (Ser35-containing peptide) elute. (a) Relaxation: bands A and B. The 3+ ion peak at m/z 682 in both band A and band B corresponds to the non-phosphorylated peptide (2043.0) and the 3+ ion peak at m/z 708.7 in band B is the phosphorylated form of the peptide (mass 2123.0). (b) Activation: bands A', B', and C'. The phosphorylated forms of peptide 1 are present in B' and C', but are not in A'. The non-phosphorylated component of B' (which we know is a mono-phosphorylated band) must correspond to Ser45 mono-phosphorylation, seen in Fig 5bB'. In relaxation the non-phosphorylated peak at m/z 682 in Fig. 4aB may come from a

small amount of mono-phosphorylation on Ser45 (Fig. 5aB), from possible phosphate loss in MS/MS, or from smearing of band A (MS analysis of multiple bands from SDS-PAGE gels commonly reveals much more overlap in proteins than expected from the staining pattern). The latter may also explain the non-phosphorylated peak in Fig. 4bC'.

Figure 5. Combined LC/MS scans of the 87–115 min region of the chromatogram where phosphorylated and non-phosphorylated forms of tryptic peptide 2 (Ser45-containing peptide) elute. (a) Relaxation: bands A and B. The 3+ ion peak at m/z 695.7 in both band A and band B corresponds to the non-phosphorylated peptide (mass 2084.1) and the ion at m/z 701 is the peptide oxidized at Met-50 (an artifact from MS preparation procedure). The ion at m/z 721.7 in band A is a trypsin autodigestion product. (b) Activation: bands A', B', and C'. The 3+ ion peak at m/z 722.4 in B' and C' corresponds to the phosphorylated peptide (mass 2164.2), and the ion at m/z 727.7 is the peptide oxidized at Met-50. Low recovery of the peptide in band A' makes comparison difficult, although the non-phosphorylated peptide is clearly present, whereas in B' and C' the phosphorylated form predominates. Similarly to

Fig. 4, the non-phosphorylated component of B' (which we know is mono-phosphorylated) would correspond to Ser35 mono-phosphorylation, seen in Fig 4bB'.

Figure 6.

In vitro motility assay of mono-phosphorylated tarantula thick filaments. (a) Differential interference contrast micrograph of thick filaments. Long straight ~5 μm features resembling thick filaments are indicated by white arrows. (b) Rhodamine/Phalloidin fluorescently labeled ~1.6 μm long rabbit purified F-actin filaments (arrows, Supplementary Movies 1, 3) (c) Rhodamine/Phalloidin labeled ~1.9 μm long tarantula thin filaments (arrows, Supplementary Movies 2, 4). (d) Twelve sequential frames (30.3 ms each) from a movie of Rhodamine/Phalloidin labeled tarantula thin filaments sliding along a thick filament under activating conditions (Supplementary Movie 4). The frames show one thin filament (white) that starts sliding (frame 3, yellow arrow) along a thick filament. The thin filament moves at 11.7 μm/s along a ~5 μm straight path (depicted in green) for 8 frames, at which point it stops (frame 10, red arrow) (e) Average sliding speed of rabbit F-actin (left) or tarantula thin filaments (right) sliding over single tarantula thick filaments as measured on *in vitro* motility assay experiments. F-actin filaments slides at 3.55±1.29 μm/s (n=321) in the presence (red) or 5.35±2.05 μm/s (n=574) in the absence (green) of Ca²⁺. Tarantula thin filaments (right) move only in the presence of Ca²⁺ (red) at 9.78±1.92 μm/s (n=130).

Figure 7. Thick filament and RLC atomic models. (a) Surface representation of the front view of the atomic model (PDB 3DTP) of tarantula myosin interacting-heads⁹ with blocked (green) and free (blue) heads docked on the 3D filament map (EMDB 1535). The myosin heavy chain, S2, ELC and RLC are depicted as graded green (blocked head) or blue (free head) tones. The RLC NTFs are shown in the S2-RLCs junction in orange (blocked head) or yellow (free head). Ser35 and Ser45 of the free head (FH) NTF are shown as enlarged red spheres. (b) Side-view of filament model shown in (a) after 90°-rotation to the left. The Ser35 and Ser45 are separated by ~1.6 and 2.5 nm in the blocked (BH) and free heads respectively. A scale-model smMLCK catalytic core⁵⁷ (left), with its catalytic domain (beige), active site (pink), ATP site (light blue), and CaM (light green) along the kinase NTF (yellow), approaches the blocked and free head RLCs. The kinase can readily access Ser35 and Ser45 located on the free head surface, but not the blocked head Ser35 and Ser45, located between the blocked head RLC and the backbone. The filament map⁹ (gray) shows subfilaments running vertically; bare zone is at the top. (c) RLC NTF region: stereo-pair of the atomic model of

blocked and free head tarantula RLCs⁹ in the relaxed filament, oriented as in (a) with their NTFs in orange and yellow respectively. The free head NTF is on top of the blocked head RLC domain 1, which is on top of the blocked head NTF. Ser35/Ser45 (red, enlarged spheres) of the free head RLC NTF are on its surface, while Ser35/45 of the blocked head RLC NTF are below the blocked head domain RLC 1 and cannot be seen from this view. (d) Electrostatic surface charges (blue positive, red negative) of both RLCs (same orientation as in (a) with the free head NTF (see Fig. 8) detached and rotated to the right from the RLC blocked head to show charge complementarity. To aid in tracking this charge complementarity, the free head NTF is shown as a yellow ribbon on both sides (see Supplementary Movie 9).

Figure 8. Structural mechanism of phosphorylation-activation of tarantula thick filaments. Atomic model of two adjacent myosin interacting-heads of relaxed tarantula thick filament (a) with heads in, and (b–d) during activation, with heads protruding (bare zone is at the top). The orientation and colors are the same as in Fig. 7a. In (a) free heads have Ser35 constitutively mono-phosphorylated, with double arrows indicating swaying in and out (Supplementary Movie 10). The non-phosphorylated Ser45s of the blocked heads are below their RLC domain 1s (Fig. 7c, d), which are themselves covered by free head NTFs; these Ser45s are therefore not readily accessible to kinase (Fig. 7; Supplementary Fig. 2). The blocked heads cannot be released as partner free head NTFs are on top of their RLCs for most of the time. On activation (b), when Ca^{2+} concentration is high for long enough to activate MLCK, the bottom swaying head is permanently released (arrow) by bi-phosphorylation of its NTF (yellow) which elongates and becomes rigid. This outward movement of the free head opens the space it previously occupied (circle), allowing access of activated MLCK (see Supplementary Fig. 2c) to phosphorylate the NTF (brown) of the blocked head above, allowing this head to sway (c). This Ser45 phosphorylation makes the blocked head NTF less compact (c, brown), hindering docking back of its partner swaying free head, which thus also becomes mobile, even without bi-phosphorylation (d).

Figure 9. Model for activation, potentiation and post-tetanic potentiation in tarantula striated muscle. (a) One helix of thick filament in relaxed state showing three adjacent interacting-head motifs (bare zone is at the top): most free heads (blue) are Ser35 constitutively mono-phosphorylated (yellow spheres) and interact with their partner blocked heads (green). These heads have relatively weak contacts with the thick filament and are therefore in equilibrium with a detached state that occasionally moves out (double arrows; “swaying” heads; light/dark blue). In a twitch, when Ca²⁺ concentration increases, detached swaying heads can attach to thin filaments producing single or summated twitch force (b), which reverses when Ca²⁺ concentration is lowered (a). If Ca²⁺ concentration remains high (c), free head Ser45

can become phosphorylated (red triangles) so that these heads are permanently released. Blocked head Ser45 can now be phosphorylated (red triangles) and released for actin interaction, along with their partner free heads (see text). These released blocked and free heads lead to force potentiation, the level depending on the number of heads released (2 levels of potentiation ratios, i.e. the actual number vs. the initial number of heads producing force, are shown 4:3 and 6:3). If Ca^{2+} concentration decreases, relaxation is achieved (d) by switching off of the thin filaments. If Ca^{2+} concentration increases again without a long delay (e), the recruited blocked heads, as well as swaying heads, will be immediately available to produce high twitch force (post-tetanic potentiation). When Ca^{2+} concentration remains low long enough to switch off MLCK, MLCP would dephosphorylate Ser45-phosphorylated heads, returning to relaxation (a).

Table 1

Tryptic digested non-phosphorylated and phosphorylated peptides 1 (Ser35-containing peptide) and 2 (Ser45-containing peptide) from tarantula myosin RLC as detected by liquid chromatography-tandem mass spectrometry.

Peptide	Sequence	m/z	m
Peptide 1	17-AEEEGGDAPAAPPAPKPPSQK-37	682.0	2043.0
Peptide 1 phosphorylated	17-AEEEGGDAPAAPPAPKPPpSQK-37	708.7	2123.1
Peptide 2	43-SGSNVFAMFTQHQQVEFK-60	695.7	2084.1
Peptide 2 phosphorylated	43-SGpSNVFAMFTQHQQVEFK-60	722.4	2164.2

“m” molecular mass (Da) calculated from the MS spectra, “m/z” measured mass/charge ratios. The charge z in all cases is +3. Peptide 1 and 2 are phosphorylated on Ser35 or Ser45 respectively (fig. 3). The mass difference between the phosphorylated and non-phosphorylated peptides 1 and 2 is 80.1 Da, corresponding to additional phosphate group. The m/z value of a protonated molecular ion is calculated according to the formula: $m/z = (M + n \cdot H)/n$ or $m/z = M/z + 1$ where M is the molecular mass of the molecule, H is the mass of proton and n is the number of attached protons. The mass values are obtained as follows: the m/z value for a 3+ ion is used to determine the m of the peptide by multiplying times 3. This is a protonated mass, however, and the mass of three protons has to be subtracted, so the precursor peptide mass of m/z 682 is: $682 \cdot (3) - 3 = 2046 - 3 = 2043$, the expected value.

Table 2

Sliding speed ($\mu\text{m/s}$) of rabbit F-actin filaments or tarantula native thin filaments sliding over mono-phosphorylated (native) or bi-phosphorylated tarantula thick filaments in the presence or absence of Ca^{2+} as measured by *in vitro* motility assay.

	Mono-phosphorylated thick filaments	Bi-phosphorylated thick filaments
Rabbit F-actin		
No Ca^{2+}	$5.35 \pm 2.05 \mu\text{m/s}$, n =574	$6.01 \pm 1.91 \mu\text{m/s}$ (n=78)
With Ca^{2+}	$3.55 \pm 1.29 \mu\text{m/s}$, n =321	$3.89 \pm 1.51 \mu\text{m/s}$ (n=74)
Tarantula thin filaments		
No Ca^{2+}	no movement	no movement
With Ca^{2+}	$9.78 \pm 1.92 \mu\text{m/s}$, n =130	$10.08 \pm 2.51 \mu\text{m/s}$ (n=105)