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Mechanism of phosphorylation of the regulatory light chain of myosin from tarantula striated muscle

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Abstract

Contraction is modulated in many striated muscles by Ca^{2+}-calmodulin dependent phosphorylation of the myosin regulatory light chain (RLC) by myosin light chain kinase. We have investigated the biochemical mechanism of RLC phosphorylation in tarantula muscle to better understand the basis of myosin-linked regulation. In an earlier study it was concluded that the RLC occurred as two species, both of which could be phosphorylated, potentiating contraction. Here we present evidence that only a single species exists, and that this can be phosphorylated at one or two sites. In relaxed muscle we find evidence for a substantial level of basal phosphorylation at the first site. This is augmented on activation, followed by partial phosphorylation of the second site. We find in addition that Ca^{2+} has a dual effect on light chain phosphorylation, depending on its concentration. At low concentration (relaxing conditions) only basal phosphorylation is observed, while at higher concentrations (activating conditions) RLC phosphorylation is stimulated. At still higher Ca^{2+} concentrations we find partial inhibition of RLC phosphorylation, suggesting an additional mechanism by which the muscle cell can fine tune contractile activity by controlling the level of free Ca^{2+}.

Introduction

The free Ca^{2+} concentration in the sarcoplasm regulates the interaction of the contractile proteins, actin and myosin, in muscle. Two basic calcium-dependent regulatory systems have been described. These are the thin filament or actin-linked regulatory system which involves the binding of Ca^{2+} to the regulatory protein troponin C (Ebashi and Endo, 1968; Weber and Murray, 1973), and the thick filament or myosin-linked system which involves the myosin molecule through Ca^{2+}-dependent phosphorylation of the regulatory light chain (RLC) of myosin (Perrie □□ □□., 1973) or direct binding of Ca^{2+} by the myosin head (Szent-Györgyi □□ □□., 1973; Chantler and Szent-Györgyi, 1980; Szent-Györgyi, 1996). Ca^{2+}-dependent phosphorylation of the RLC is observed in vertebrate striated muscles (Perrie □□ □□., 1973; Stull and High, 1977; Bárány and Bárány, 1979; Levine □□ □□., 1996), in invertebrate striated muscles (Kerrick and Bolles, 1981; Sellers, 1981; Craig □□ □□., 1987; Panté □□ □□., 1988; Levine □□ □□., 1991; Padrón □□ □□., 1991), and in vertebrate smooth muscles (Hartshorne, 1987; Sellers and Adelstein, 1987). Many muscles are regulated by both thin filaments and RLC phosphorylation (Lehman and Szent-Györgyi, 1975; Stull and High, 1977; Bárány and Bárány, 1979; Kerrick and Bolles, 1981; Sellers, 1981; Craig □□ □□., 1987; Panté □□ □□., 1988; Levine □□ □□., 1991, 1996; Padrón □□ □□., 1991).

RLC phosphorylation has been shown to be the primary mechanism for initiating contraction in smooth muscle and in non-muscle systems (Moussavi □□ □□., 1993). However, the effect of RLC phosphorylation in striated muscle is less clear. Biochemically it has been shown that RLC phosphorylation regulates (Sellers, 1981) or modulates (Craig □□ □□., 1987) actin activation of myosin MgATPase in □□□□□□□ and tarantula striated muscle, respectively. Physiologically it has been shown that RLC phosphorylation in striated muscle increases the rate of force development and potentiates isometric twitch tension in vertebrate fast-twitch skeletal muscle fibers (Manning and Stull, 1979, 1982; Klug □□ □□., 1982; Moore □□ □□., 1985), and potentiates force at low levels of sarcoplasmic calcium in skeletal and cardiac muscle (Sweeney and Stull, 1986; Levine □□ □□., 1998). The physiological effect of RLC phosphorylation in □□□□□□□ is less clear. It may be either required for (Wang □□ □□., 1993) or modulate (Ritter □□ □□., 1999) contraction.

To identify the structural basis of the observed physiological effects of RLC phosphorylation, thick

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filaments in relaxing medium (with non-phosphorylated regulatory light chains) and in activating medium (with phosphorylated regulatory light chains) have been examined by negative staining electron microscopy (Craig □□ □□., 1987; Levine □□ □□., 1996) and optical diffraction (Levine □□ □□., 1991, 1996). When relaxed, helically ordered thick filaments from tarantula, □□□□□ □□□, and vertebrate muscle are incubated in activating medium, the RLCs are rapidly phosphorylated and concomitantly the thick filaments equally rapidly lose their crossbridge order (Craig □□ □□., 1987; Levine □□ □□., 1991, 1996). It was suggested that this disordering was due to phosphorylation-dependent loosening of the myosin heads from the thick filament backbone, allowing the heads to extend from the filament surface, thereby promoting actin–myosin interaction. When myosin heads are already close to actin, phosphorylation has no additional effect on the calcium sensitivity of force generation or the rate of force production (Yang □□ □□., 1998). These structural effects of RLC phosphorylation are consistent with an increased mobility of myosin heads.

The aim of this report was to expand our knowledge of the biochemical mechanism of calcium-dependent regulation of RLC phosphorylation by myosin light chain kinase (MLCK) in a native striated muscle system. As experimental material we used filament suspensions of tarantula striated muscle. This is a well characterized system (Craig □□ □□., 1987) containing thick and thin filaments together with endogenous MLCK and calmodulin, whose activity can be experimentally controlled by alteration of Ca^{2+} concentration. We find that tarantula myosin contains a single phosphorylatable RLC species, and not two as previously reported (Craig □□ □□., 1987) and that this light chain can be phosphorylated at one or two sites. In relaxed muscle, there is a substantial level of basal phosphorylation at the first site, which is augmented on activation, followed by partial phosphorylation of the second site. We find in addition that Ca^{2+} stimulates or inhibits light chain phosphorylation, depending on its concentration. At low Ca^{2+} levels (relaxing conditions), RLC phosphorylation is low, while at higher levels (activating conditions) RLC phosphorylation is stimulated; at higher levels still, RLC phosphorylation is inhibited, suggesting an additional Ca^{2+}-dependent means of fine tuning contractile activity. To our knowledge this is the first report of inhibition of RLC phosphorylation by calcium in striated muscle.

Materials and methods

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Rigor solution contained 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 5 mM PIPES, 5 mM NaH_2PO_4, 1 mM NaN_3. Relaxing solution was prepared by adding 100 mM MgATP to rigor solution to a final 5 mM MgATP concentration. Activating solution was made by modifying relaxing solution to the desired free Ca^{2+} concentration by addition of calcium from a 100 mM $CaCl_2$ stock solution. Total and free ion concentrations were calculated using the computer program MaxChelator, Winmaxc v. 1.80, 1998 (Bers □□ □□., 1994). Permeabilizing solution was relaxing or rigor solution containing 0.1% (w/v) saponin. All solutions were adjusted to pH 7.0 with NaOH at room temperature.

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Pink-foot (□□□□□□□□ □□□□□□□□) and brown (family □□□□□□□□□□□□) tarantulas were obtained locally from San José de Guaribe, Guárico, Venezuela and from Carolina Biological Supply Co. (Burlington, NC, USA). Filament suspensions were prepared as previously described (Craig □□ □□., 1987) by briefly homogenizing saponin-permeabilized muscle in relaxing solution. The homogenate was briefly centrifuged to remove large debris and the supernatant containing the filament suspension was stored on ice and used the same day. The filament suspension was characterized by electron microscopy (negative staining with 1% uranyl acetate) and by SDS–PAGE. Myofibrils were prepared by permeabilizing and homogenizing muscles in rigor solution. Myofibril quality was evaluated by phase contrast light microscopy.

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RLC phosphorylation was carried out by the addition of calcium from a 100 mM $CaCl_2$ stock solution to the filament suspension in relaxing solution to achieve the desired free Ca^{2+} concentration, followed by incubation at room temperature for 10 min. The level of phosphorylation of the RLC in the filament suspension was monitored by the change in its mobility on urea–glycerol PAGE (Perrie and Perry, 1970; Craig □□ □□., 1987). The light chains are readily soluble in the urea–glycerol sample buffer whereas the myosin heavy chains are not. Gels were stained with 0.25% Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA, USA) in 50% methanol, 10% acetic acid, destained in a microwave oven (Hervieu, 1997), and analyzed by densitometry (Fluor-S MultiImager system, BioRad, Hercules, CA, USA).

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[γ-^{32}P]ATP labeling of the RLC was carried out as described (Craig □□ □□., 1987). SDS and urea–glycerol PAGE gels were stained with Coomassie brilliant blue G-250 and dried. Autoradiography was carried out by placing the dried gel in direct contact with X-ray film (Fuji RX) with intensifying screen (Kodak) at −70°C, for several days according to the activity.

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Myofibrils in rigor solution (absence of ATP) were incubated 10 min at room temperature with smooth muscle phosphatase (SMP IV), which can dephosphorylate intact RLC incorporated into the myosin molecule (Pato and Kerc, 1985).

Results

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Our goal in this paper was to study the mechanism of RLC phosphorylation in native tarantula myosin filaments. We therefore used a previously well characterized filament preparation from tarantula muscle (Craig □□ □□., 1987) and determined its properties by gel electrophoresis and electron microscopy to confirm its validity for our studies. On SDS–PAGE the filament suspension showed bands corresponding to the myosin heavy chain, actin and paramyosin (Levine □□ □□., 1983), the myosin regulatory (RLC) and essential (ELC) light chains (26 and 19 kDa respectively) (Craig □□ □□., 1987) and other bands corresponding to tropomyosin and troponin subunits (Figure 1A). The identity of the bands marked as myosin heavy and light chains and paramyosin was supported by SDS–PAGE of a purified preparation of native myosin filaments (from which thin filaments and soluble protein had been removed), which showed bands in identical positions (C. Hidalgo □□ □□., in preparation). Electron micrographs of negatively stained filament suspensions under relaxing conditions showed myosin filaments and actin filaments, consistent with the gel electrophoresis results (Figure 1G). The myosin filaments were helically ordered, as expected from X-ray diffraction and electron microscopy of relaxed muscle (Wray, 1982; Crowther □□ □□., 1985; Padrón □□ □□., 1992), supporting the preservation of their native structure.

Analysis by urea–glycerol PAGE of filament suspensions in relaxing solution showed three major protein bands, a doublet labeled RLC and RLC-PI (see later for explanation of labeling), and a second band labeled ELC (Figure 1C, Lane 1). Comparison of this gel pattern with that coming from a purified preparation of tarantula myosin showed previously (Craig □□ □□., 1987) that these three bands correspond to the □□□ myosin light chains (regulatory and essential) seen by SDS–PAGE. It was suggested in this earlier work that the doublet (in which the bands are usually approximately equal in intensity) corresponded to two species of RLC having the same molecular weight (26 kDa) but differing slightly in net charge.

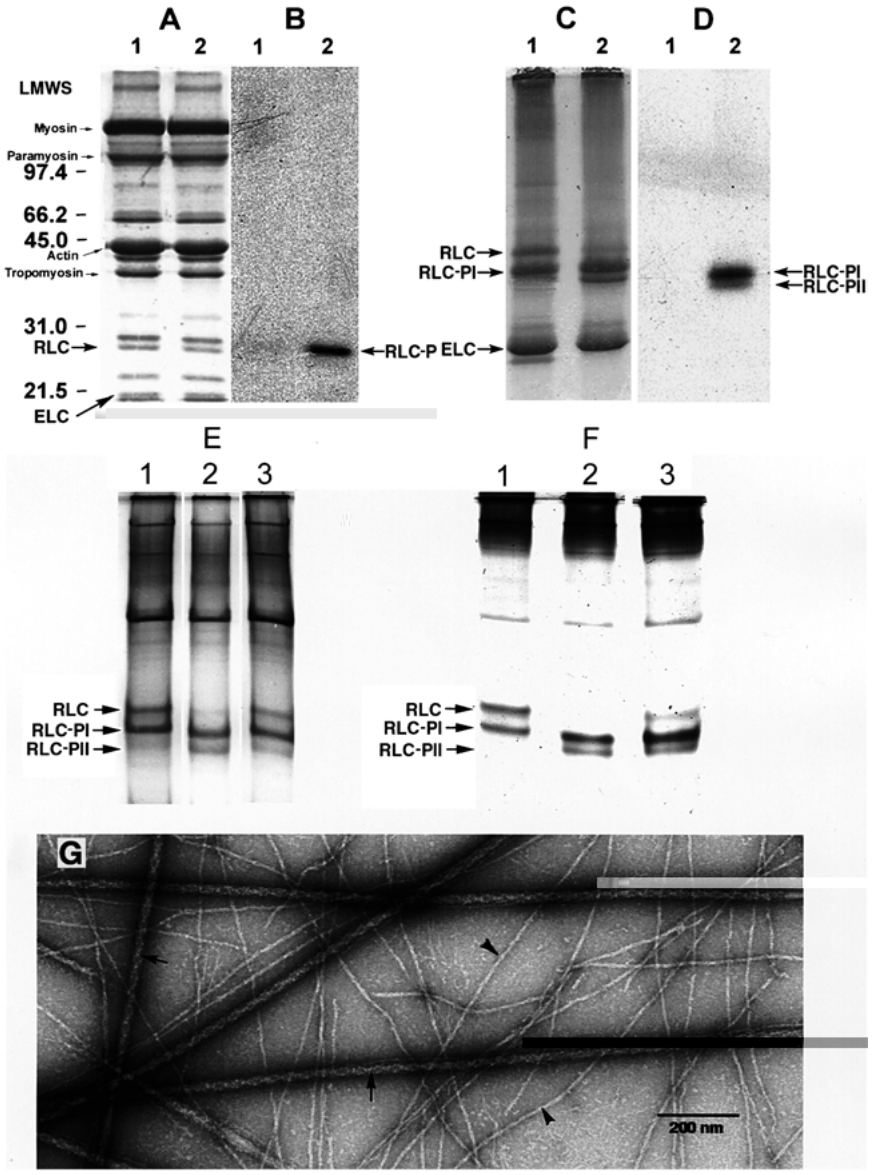
Analysis by SDS–PAGE of filament suspensions to which Ca^{2+} had been added (pCa 6.0) showed that the RLC, but no other proteins, incorporated ^{32}P from [γ-^{32}P]ATP (Figure 1A, B), as found previously (Craig □□ □□., 1987). By urea–glycerol PAGE, the upper band of the doublet (RLC) became weaker, while the lower band (RLC-PI) became stronger, and a new band appeared with higher mobility labeled RLC-PII (Figure 1C, E, F). Densitometric analysis (Figure 2C) showed that the intensity of the RLC-PI band was stronger than the RLC-PII band, which never exceeded ~20–30% of the entire RLC species, comparable to the situation in smooth muscle (Sobieszeck, 1990). The RLC-PI and RLC-PII bands both incorporated ^{32}P, implying that they are both phosphorylated light chains (Figure 1C, D).

The above characterization of the filament suspension from tarantula muscle is similar to that established previously (Crowther □□ □□., 1985; Craig □□ □□., 1987), demonstrating that we are studying intact filaments in close to their native state (cf. Crowther □□ □□., 1985) and that the components responsible for phosphorylation are present and active in the filament suspension (Craig □□ □□., 1987). Having validated the use of this preparation for our experiments, we characterized it further by (1) showing that the phosphorylation process is reversible, (2) obtaining additional evidence that it involves a specific myosin light chain kinase □□ □□□□, and (3) showing that all muscles in the tarantula leg behave similarly.

To check the reversibility of the phosphorylation observed in the preparation, we studied the effect of decreasing the free Ca^{2+} concentration after RLC phosphorylation had taken place. Filament suspensions were incubated for 2 min at room temperature in relaxing solution or (in duplicate) in activating solution (pCa 6.0). Following this incubation, EGTA was added to one of the activated samples to produce final pCa's in the range 6.2–7.0. Samples were then incubated for 10 min at room temperature. With addition of EGTA to pCa ≥6.5, there was an increase in the intensity of the RLC band (Figure 1F), suggesting that some dephosphorylation of phosphorylated RLC had taken place due to a lowering of free Ca^{2+}. The simplest interpretation is that the preparation contains an endogenous myosin light chain phosphatase (MLCP), whose activity becomes apparent when the endogenous MLCK becomes inactivated by lowering of the free Ca^{2+} concentration.

To confirm that light chain phosphorylation was due to a specific myosin light chain kinase, filament suspensions were incubated in activating solution (pCa 5.2) with 500 nM ML-7 (Calbiochem-Novachem Corp. La Jolla, CA, USA), a potent and selective inhibitor of MLCK ($□_I = 300$ nM) (Saitoh □□ □□., 1987). In the presence of ML-7 we detected an increase of the density of the RLC band and a decrease of the RLC-PII band (Figure 1E). This result suggests that RLC phosphorylation is inhibited by ML-7, consistent with the view that RLC phosphorylation is mediated by endogenous MLCK.

To test the possibility that tarantula legs may have different muscle types (cf. Levine □□ □□., 1989) that are regulated differently we analyzed RLC phosphorylation



□□□□ □□ Characterization of tarantula striated muscle filament suspension. A–F: SDS–PAGE and urea–glycerol PAGE analysis of filament suspensions. (A) SDS–PAGE of filament suspensions incubated for 10 min at room temperature in relaxing solution and 1 μCi [γ-^{32}P]ATP (lane 1) or in activating solution (pCa 6.0) with 1 μCi [γ-^{32}P]ATP (lane 2). LMWS: dashes show positions at which low molecular weight standards run. RLC, regulatory light chain, ELC, essential light chain. (B) Autoradiography of gel A. RLC-P, RLC phosphorylated. (C) Urea–glycerol PAGE of filament suspensions incubated for 10 min at room temperature as in (A): relaxing solution (lane 1) and activating solution (pCa 6.0) (lane 2). RLC-PI, RLC mono-phosphorylated. (D) Autoradiography of gel C. RLC-PII, RLC bi-phosphorylated. (E) Effect of the MLCK inhibitor ML-7 on RLC phosphorylation monitored by urea–glycerol PAGE. Filament suspensions were incubated for 10 min at room temperature in relaxing solution (lane 1), in activating solution (pCa 5.2) (lane 2), or in activating solution (pCa 5.2) plus 500 nM ML-7 (lane 3). (F) Effect of EGTA on RLC phosphorylation monitored by urea–glycerol PAGE. Filament suspensions were incubated for 2 min at room temperature in relaxing solution (lane 1) or in activating solution (pCa 6.0) (lane 2). Then EGTA was added to a final pCa of 7.0 (lane 3) and all specimens further incubated for 10 min at room temperature. The increase in the RLC band in lane 3 implies reversal of phosphorylation, as full RLC phosphorylation is attained in < 20 s ($t_{1/2} = 3.6$ s; data not shown). (G) Electron micrograph of filament suspension negatively stained with 1% uranyl acetate, showing thin filaments (arrowheads) and helically ordered myosin filaments (arrows).

in individual muscles of the tarantula leg. Filament suspensions from each muscle (the femur muscles, □□□□□ □□□□□□□□ □□□□□□□□□ and □□□□□ □□□□□□□□ □□□□□□ (Ellis, 1949), and a third distinct muscle, that we have identified morphologically, and the tibia muscles □□□□□ □□□□□□□□□ □□□□□□□□□ and □□□□□ □□□□□□□□□ □□□□□□ (Ellis, 1949)) were incubated in relaxing solution or activating solution (pCa 6.0). Urea–glycerol PAGE of all five individual muscles gave results identical to those from the mixed filament suspension (data not shown). The results showed that the RLCs in all muscles of the femur and tibia segments of tarantula legs were phosphorylatable.

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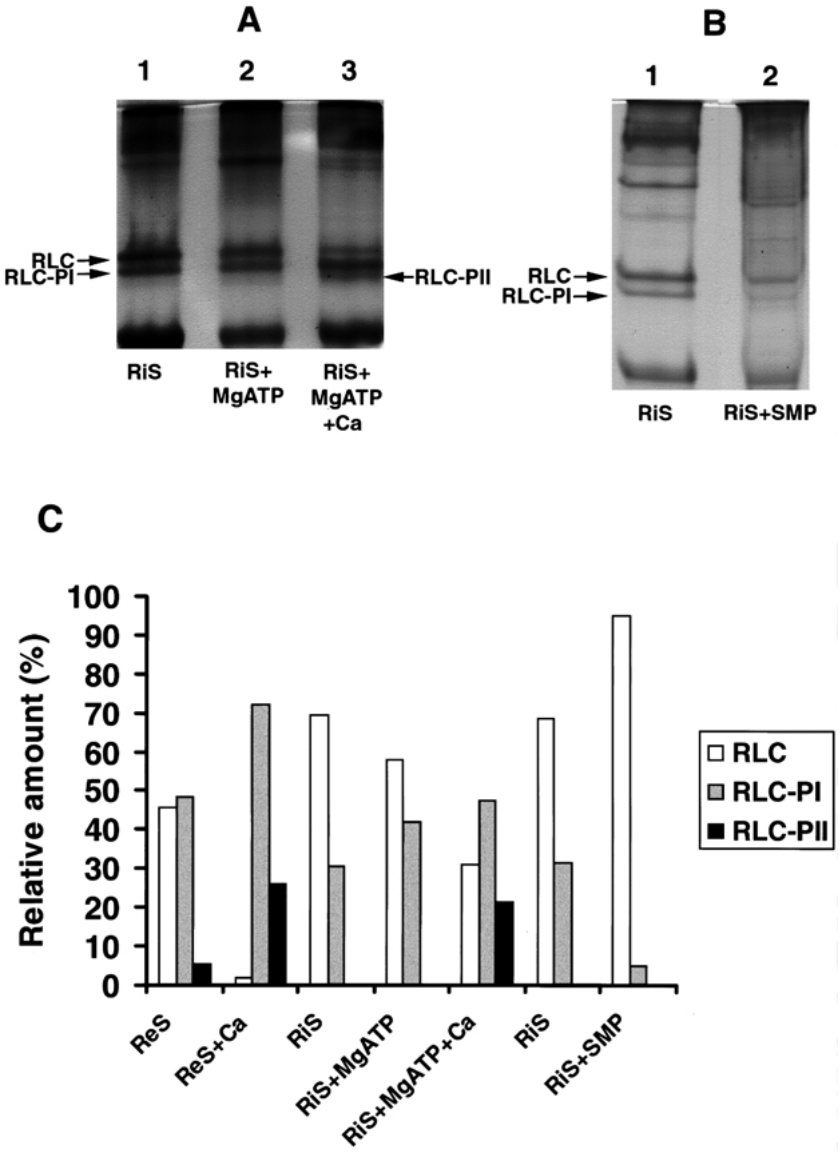
In the earlier study of this system (Craig □□ □□., 1987), it was concluded that the 26 kDa RLC seen by



□□□□ □□ Effect of MgATP and phosphatase on RLC phosphorylation. A, B. Urea–glycerol PAGE analysis of myofibrils from tarantula striated muscle. (A) Myofibrils in rigor solution (RiS) (lane 1), in RiS plus 5 mM MgATP (lane 2), and in RiS plus 5 mM MgATP plus 1 mM $CaCl_2$ (lane 3). (B) Myofibrils in RiS (lane 1), and in RiS plus smooth muscle phosphatase (SMP IV) (lane 2). (C) Densitometry of RLC, RLC-PI and RLC-PII bands on urea–glycerol gels. ReS (relaxing solution) and ReS + Ca represent typical densitometry of relaxed and activated preparations (e.g. Figures 1C, E, F, Figure 3). The other five conditions represent densitometry of gels A and B of this figure.

SDS–PAGE (Figure 1A) occurred in relaxing solution as two species with slightly different charges, producing the doublet seen by urea–glycerol PAGE (Figure 1C). Phosphorylation of these two species was thought to increase the mobility of both bands by a similar amount, producing a new doublet, here labeled RLC-PI and -PII. An alternative interpretation, suggested by studies of the RLC of vertebrate smooth muscle (Sobieszeck, 1990) and of □□□□□□□ muscle (Ritter □□ □□., 1999), is that the initial doublet represents a non-phosphorylated and mono-phosphorylated form of a □□□□□□ RLC species (the upper and lower bands of the doublet respectively), and that the third band reflects phosphorylation at a second site on this light chain. The doublet under relaxing conditions would result from basal mono-phosphorylation of the RLC. We carried out experiments to test this hypothesis.

A possible reason for the appearance of the lower band of the doublet in relaxed muscle, if it represents basal, mono-phosphorylated RLC, is that there is some activity of endogenous MLCK even in relaxed conditions and/or insufficient activity of its antagonist, endogenous MLCP that would otherwise dephosphorylate the RLC. We tested this possibility by preparing homogenates in the absence of MgATP and phosphate ions, which are both inhibitors of MLCP (Sato and Ogawa, 1999); in addition to increasing phosphatase activity, removal of MgATP would also prevent kinase activity. Urea–glycerol PAGE analysis of myofibrils in rigor solution (absence of MgATP) still showed a doublet, but in this case the upper band was stronger than the lower, while in preparations made conventionally in relaxing solution, the two bands are similar in intensity (Figure 2A, lane 1; Figure 2C). Addition of

MgATP to such a rigor preparation, effectively producing relaxing conditions (Ca^{2+} still low), caused an increase of the lower band and sometimes weak appearance of the third band (Figure 2A, lane 2; Figure 2C). These observations are inconsistent with the hypothesis that the RLC is present as two species, and suggest instead that the lower band represents basal monophosphorylation of the RLC even in relaxing solution. To further test this hypothesis, myofibrils from tarantula striated muscle in rigor solution were incubated with smooth muscle phosphatase IV (SMP IV; Pato and Kerc, 1985). Urea–glycerol PAGE showed a strong RLC band and a very weak RLC-PI band after phosphatase treatment (Figure 2B, lane 2 cf. lane 1; Figure 2C). Removal of the lower band by SMP IV implies that it had been phosphorylated. We conclude, therefore, that the RLC is present as a single species in tarantula striated muscle and that the three bands seen under different conditions by urea–glycerol PAGE represent the same light chain in different phosphorylation states. The simplest interpretation is that the three bands represent non-, mono-, and bi-phosphorylated RLC (cf. Ritter □□ □□., 1999) and we have accordingly labeled them RLC, RLC-PI, and RLC-PII, respectively in Figures 1–3.

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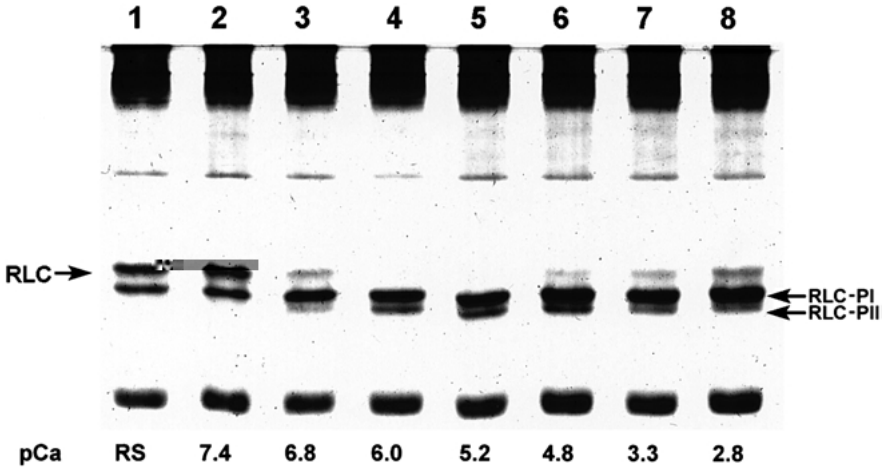
Phosphorylation of the regulatory light chains in vertebrate smooth muscle occurs at a pCa of 6.6, but is inhibited at higher Ca^{2+} levels (pCa 6.3), apparently by reduction in activity of MLCK brought about by its own phosphorylation at high free [Ca^{2+}] (Ikebe and Reardon, 1990; Tansey □□ □□., 1992, 1994; Stull □□ □□., 1993; Gallagher □□ □□., 1997). We have explored whether light chain phosphorylation in tarantula striated muscle is similarly modulated by Ca^{2+}. Filament suspensions were incubated for 10 min at room temperature in relaxing solution or in activating solution with increasing calcium concentrations. Urea–glycerol PAGE showed that RLC phosphorylation was detectable (above basal levels) at pCa 6.8, and was maximal in the range pCa 6.0–5.2 (Figure 3, lanes 3–5). However, with higher levels of free Ca^{2+} (pCa ≤4.8) the electrophoretic pattern reverted to one similar to that obtained at pCa 6.8 (Figure 3, lanes 6–8). Densitometric analysis showed that the density of the RLC-PII band decreased and the RLC band increased. These results are consistent with Ca^{2+}-dependent partial inhibition of RLC phosphorylation at Ca^{2+} concentrations higher than or equal to pCa 4.8.

Discussion

Phosphorylation of the myosin RLC has been shown to regulate or modulate contractile activity in a variety of smooth and striated muscles (Kamm and Stull, 1985; Sweeney and Stull, 1986; Hartshorne, 1987). In tarantula striated muscle it was shown that phosphorylation □□□□□□□□□ contraction, which is primarily regulated by a troponin-tropomyosin switch on the thin filaments (Craig □□ □□., 1987). Light chain phosphorylation in tarantula muscle is accompanied by a disordering of the myosin heads on the surface of the myosin filament, suggesting a loosening of their relaxed interactions with each other or with the filament backbone (Craig □□ □□., 1987). Similar results have been obtained with striated muscles from mammalian (Levine □□ □□., 1996, 1998; Yang □□ □□., 1998) and other invertebrate muscles (Levine □□ □□., 1991). In each of these studies it was concluded that this phosphorylation-induced loosening might be the basis of the observed enhanced interaction of myosin heads with actin. Because of the importance of phosphorylation in modulating contraction in many striated muscles (as well as in smooth muscle), we investigated this process further here, using a well characterized fresh filament suspension of tarantula



□□□□ □□ Effect of calcium concentration on RLC phosphorylation monitored by urea–glycerol PAGE. Filament suspensions were incubated for 10 min at room temperature in relaxing solution (lane 1), and in activating solution with increasing free calcium concentrations (pCa 7.4, 6.8, 6.0, 5.2, 4.8, 3.3 and 2.8, respectively) (lanes 2–8).

muscle (Craig □□ □□., 1987) in order to understand more fully its cellular mechanism.

Analysis of the properties of the preparation showed that it behaved exactly as was observed previously (Craig □□ □□., 1987), demonstrating its validity for use in our studies. From this biochemical and structural characterization (Figure 1), we conclude that thick and thin filaments are present in the preparation in close to their native state, that RLC phosphorylation occurs via a Ca^{2+}-calmodulin activated myosin light chain kinase which is present endogenously, and that phosphorylation is reversed at low Ca^{2+} by endogenous phosphatase.

Our further analysis provides new insights into this mechanism and shows that one aspect of the previous work was incorrectly interpreted. Contrary to the earlier conclusion that the RLC occurred as two species of the same molecular weight (26 kDa) but with different charge, we have shown here that there is only one species of regulatory light chain (Figure 2). Our results suggest that the faster moving band of the doublet seen on urea–glycerol PAGE in relaxing conditions is actually a phosphorylated form of the slower band (Figure 2): because it can be almost entirely removed by phosphatase treatment, moving to the position of the slower moving band, the faster band is not a second species. This interpretation is supported by the observation that filament suspensions prepared in rigor solution (in which endogenous MLCK activity is low and endogenous phosphatase activity is expected to be higher) show a stronger slow band (RLC non-phosphorylated) and a weaker fast band (RLC mono-phosphorylated). The RLC mono-phosphorylated band (RLC-PI) increases when MgATP is added, producing relaxing conditions (low Ca^{2+}), suggesting that the preparation contains some Ca^{2+}-insensitive MLCK. Filament suspensions prepared in relaxing solution thus contain light chain that already exhibits a significant amount of basal phosphorylation (typically 35–50%; Figures 1–3), creating the faster running band of the RLC doublet of relaxed preparations. Comparable basal RLC phosphorylation has been found in myosin from rabbit striated muscle (Stepkowski □□ □□., 1985; Adhikari □□ □□., 1999), in □□□□□□□ skeletal muscle (Wang □□ □□., 1993; Ritter □□ □□., 1999) and in vertebrate smooth muscle (Silver and Stull, 1982). Removal of basal phosphorylation of tarantula filaments by phosphatase treatment could prove useful for improving filament helical order and thus resolution of filament structures used in atomic modeling (Padrón □□ □□., 1998; Offer □□ □□., 2000).

Addition of Ca^{2+} to the relaxed filament preparation causes almost complete disappearance of the RLC band (e.g. Figure 1C, E, F lane 2; Figure 2C; Figure 3, lanes 4–5). This presumably involves endogenous, Ca^{2+}-sensitive MLCK. The RLC-PI band becomes stronger, and the RLC-PII band appears. Both bands incorporate ^{32}P, implying that both are phosphorylated, suggesting different phosphorylation states of the RLC, presumably mono- and bi-phosphorylated (RLC-PI and RLC-PII; cf. Ritter □□ □□., 1999). This appears to be similar to the situation in vertebrate smooth muscle. Turkey and chicken gizzard RLC can be mono- or bi-phosphorylated by MLCK isolated from turkey gizzard, such that a single non-phosphorylated RLC band on urea–glycerol PAGE is converted by endogenous MLCK to two additional bands, mono- and bi-phosphorylated, with electrophoretic mobilities higher than the non-phosphorylated band (Sobieszeck, 1990). The first site, at serine 19, is phosphorylated preferentially, followed more slowly, and less completely, by the second site at threonine 18 (Ikebe and Hartshorne, 1985). The results are also similar to those obtained with striated muscle from the horseshoe crab, □□□□□□□ (another chelicerate) and from scorpion (Linarez □□ □□., in preparation), except that □□□□□□□ has two regulatory light chains of different molecular weight, both of which can be phosphorylated (Sellers, 1981; Levine □□ □□., 1991; Wang □□ □□., 1993) at one or two sites (Ritter □□ □□., 1999).

Our experiments also demonstrate that not only does an initial increase in Ca^{2+} concentration from relaxed levels induce phosphorylation, but higher levels still inhibit this phosphorylation. Thus Ca^{2+} appears to perform two opposing regulatory functions, depending on its concentration. This is the first report of such a finding in striated muscle. A similar response has been seen in vertebrate smooth muscle, where Ca^{2+} concentration required for half-maximal RLC phosphorylation is pCa 6.6, while at pCa 6.3 there is a Ca^{2+} desensitization (i.e. inhibition) of RLC phosphorylation. This desensitization mechanism apparently involves MLCK phosphorylation by CaM protein kinase II at site A of MLCK, adjacent to the calmodulin binding site (Ikebe and Reardon, 1990; Tansey □□ □□., 1992, 1994; Tang □□ □□., 1992; Stull □□ □□., 1993; Gallagher □□ □□., 1997). Phosphorylation of MLCK increases the concentration of Ca^{2+}/calmodulin required for activation and hence increases the Ca^{2+} concentration required for MLCK activity in cells. Thus Ca^{2+} desensitization due to MLCK phosphorylation would occur physiologically under situations where cytosolic Ca^{2+} concentrations rise to high levels, greater than those necessary to initiate RLC phosphorylation. The Ca^{2+}-dependent inhibition of RLC phosphorylation observed in tarantula also occurs in the physiological range of Ca^{2+} concentrations, although at higher levels than those in smooth muscle. Inhibition is observed at free Ca^{2+} concentrations (Figure 3, lane 6) comparable to those reached during maximal activity of contracting skeletal muscle (Miledi □□ □□., 1982; Hollingworth □□ □□., 1996). There are several possible explanations for the decrease in RLC phosphorylation at high Ca^{2+}. MLCK activity may be inhibited at high Ca^{2+}, as in the case of smooth muscle (Ikebe and Reardon, 1990; Tansey □□ □□., 1992, 1994; Tang □□ □□., 1992; Stull □□ □□., 1993; Gallagher □□ □□., 1997). However, the Ca^{2+} concentration required for inhibition is higher than that needed for the activation of CaM protein kinase II that is responsible

for Ca^{2+} desensitization in smooth muscle. Alternatively, Ca^{2+}-activated phosphatases (e.g. type IIB; unpublished observations) might be present in the preparation, decreasing the RLC phosphorylation level. However, type IIB phosphatase cannot dephosphorylate intact myosin, so it is less likely that this system is responsible for the decrease in RLC phosphorylation at high Ca^{2+}. Identification of the factors responsible for the inhibition of phosphorylation requires further study. These differences in Ca^{2+} dependence of phosphorylation may represent a key feature of the regulation of RLC phosphorylation in tarantula (and possibly other) striated muscles.

The phosphorylation of the RLC that we have studied biochemically correlates structurally with a disordering of the helical organization of the myosin heads that characterizes relaxed (non-phosphorylated) filaments (Craig □□ □□., 1987; Levine □□ □□., 1991, 1996, 1998). MLCK phosphorylation of the RLC, located in the neck of the myosin head, near its junction with the tail, loosens the connection of the head with other heads or with the filament backbone, leading to disorder. This loosening may account for the ability of phosphorylated heads to interact more productively with thin filaments, increasing actin-activated ATPase of myosin filaments (Craig □□ □□., 1987; Sweeney □□ □□., 1993). The increased interaction observed in solution may explain the physiological observation that RLC phosphorylation leads to an increase in the rate and extent of force development in intact muscles (Sweeney and Stull, 1986; Sweeney □□ □□., 1993; Levine □□ □□., 1998). Fine tuning of this modulatory effect could be provided by the partial inhibition of phosphorylation that we have observed at high levels of Ca^{2+}. At the end of contractile activity, when Ca^{2+} concentration has returned to relaxed levels, phosphatase would predominate over MLCK activity and dephosphorylated heads would return to their helically defined positions close to the filament backbone, where their interaction with thin filaments is minimized.

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