Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1490–1494, February 1994
Biochemistry

Charge replacement near the phosphorylatable serine of the myosin regulatory light chain mimics aspects of phosphorylation

(smooth muscle/striated muscle/scallop/contraction/modulation of force)

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Communicated by C. Richard Taylor, September 30, 1993 (received for review July 15, 1993)

ABSTRACT **Phosphorylation of the myosin regulatory light chains (RLCs) activates contraction in smooth muscle and modulates force production in striated muscle. RLC phosphorylation changes the net charge in a critical region of the N terminus and thereby may alter interactions between the RLC and myosin heavy chain. A series of N-terminal charge mutations in the human smooth muscle RLC has been engineered, and the mutants have been evaluated for their ability to mimic the phosphorylated form of the RLC when reconstituted into scallop striated muscle bundles or into isolated smooth muscle myosin. Changing the net charge in the region from Arg-13 to Ser-19 potentiates force in scallop striated muscle and maintains smooth muscle myosin in an unfolded filamentous state without affecting ATPase activity or motility of smooth muscle myosin. Thus, the effect of RLC phosphorylation in striated muscle and its ability to regulate the folded-to-extended conformational transition in smooth muscle may be due to a simple reduction of net charge at the N terminus of the light chain. The ability of phosphorylation to regulate smooth muscle myosin's ATPase activity and motility involves a more complex mechanism.**

Phosphorylation of the regulatory light chain (RLC) of myosin is the primary regulator of muscle contraction in vertebrate smooth muscle (1). This phosphorylation also enhances the ATPase activity and movement of actin by smooth muscle myosin *in vitro* (2, 3). Moreover, RLC phosphorylation unfolds myosin from the bent monomeric conformation and thus favors myosin filament formation (4, 5). In invertebrate muscles, myosin RLC phosphorylation may dually regulate, or at least modulate, contractile activity (6, 7). In mammalian striated muscle, phosphorylation of the RLC increases the Ca^{2+} sensitivity of force production and increases the rate of force production (8–10). Thus, while phosphorylation/dephosphorylation of the RLC occurs in nearly all muscles, its functional importance varies. Nevertheless, it is interesting to speculate whether or not a common mechanism can explain the effects of RLC phosphorylation in all muscle types.

The N terminus of both the striated and smooth muscle isoforms of the myosin RLC contains a number of positively charged amino acids N-terminal to the phosphorylatable serine (Fig. 1). These amino acids have been shown to be important for catalytic activity of myosin light chain kinases (11). We considered that this highly charged N terminus might also be important in regulating interactions with myosin and that reduction of the net charge by phosphorylation would be the simplest mechanism to produce this effect. Accordingly, mutants of the human smooth muscle RLC (smRLC) were constructed and expressed in *Escherichia coli*. In some of the mutants, the positively charged amino acids closest to Ser-19 were singly replaced by alanine residues. A mutant in which both Arg-13 and Arg-16 were replaced by alanine residues was also produced, as were mutants where either Ser-19 or both Thr-18 and Ser-19 were replaced by glutamic residues. The sequences of wild-type and mutant RLCs are given in Fig. 1. The purpose of these mutations was to assess whether a reduction in the net charge near Ser-19 can mimic the effect of phosphorylation in terms of its ability to regulate both smooth and striated muscle.

A *Skeletal RLC* (Rabbit fast wild type)
P K K A K R R A A A E G G S *S* N V F S M....
Smooth RLC (Human wild type)
S S K R A K A K T T K K R - P Q R - A T *S* N V F A M....

B *Smooth RLC* (Human smooth muscle wild type)
S S K R A K A K T T K K R P Q R A T *S* N V F A M F....
SmRLC-K11A
S S K R A K A K T T **A** K R P Q R A T *S* N V F A M F....
SmRLC-K12A
S S K R A K A K T T K **A** R P Q R A T *S* N V F A M F....
SmRLC-R13A
S S K R A K A K T T K K **A** P Q R A T *S* N V F A M F....
SmRLC-R16A
S S K R A K A K T T K K R P Q **A** A T *S* N V F A M F....
SmRLC-R13A-R16A
S S K R A K A K T T K K **A** P Q **A** A T *S* N V F A M F....
SmRLC-S19E
S S K R A K A K T T K K R P Q R A T **E** N V F A M F....
SmRLC-T18E-S19E
S S K R A K A K T T K K R P Q R A **E E** N V F A M F....

FIG. 1. (*A*) Comparison of the N termini of the human smRLCs and the rabbit fast skeletal muscle RLCs. Positively charged amino acids are shown in boldface letters, and the phosphorylatable serines are in italic letters. (*B*) Comparison of amino acid sequences of the N termini of the human smRLC and mutant RLCs used in this investigation. The positively charged amino acids that were the focus of mutagenesis are at the N terminus of the RLC sequence and are shown in boldface letters. The phosphorylatable serine (Ser-19) is italicized. Replacement amino acids are indicated by underlined boldface letters.

To assay the effects of the mutations on the properties of smooth muscle myosin, RLC mutants were exchanged for native RLCs in purified turkey gizzard smooth muscle myosin (12). The myosin was then assayed for its ability to assume the folded monomeric conformation, ATPase activity, and *in vitro* motility.

The force-potentiating effect of RLC phosphorylation in striated muscle can only be assessed with a permeabilized muscle preparation. Permeabilized scallop striated muscle provides a useful assay system for RLC mutants, since the wild-type scallop RLC is easily removed (13). The primary regulation of force generation in scallop muscle is due to Ca^{2+}

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Abbreviations: RLC, regulatory light chain; smRLC, smooth muscle RLC.

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binding to the myosin head at a site that may be comprised of elements of the essential light chain, the C terminus of the RLC, and the myosin heavy chain (14). Vertebrate smRLCs can be substituted for scallop RLCs and restore Ca^{2+}-dependent regulation (13) as well as provide a phosphorylatable N terminus. Taking advantage of the ease of RLC removal in scallop (15), we have assayed both wild-type and mutant smRLCs in permeabilized bundles of scallop striated muscle fibers. The effect of smRLC phosphorylation in scallop striated muscle was qualitatively identical to the effect of RLC phosphorylation in mammalian striated muscle.

The results of this study suggest that the ability of RLC phosphorylation to convert the folded form (10S) of smooth muscle myosin to the unfolded form (6S) may be mechanistically related to the phosphorylation effect on force potentiation in striated muscle. Both may result from a simple charge reduction at the N terminus, which is accomplished physiologically by RLC phosphorylation. This scheme is depicted in Fig. 2. The data also show that the mechanism by which RLC phosphorylation regulates ATPase activity and motility of smooth muscle myosin does not depend simply on alteration of net charge at the N terminus. This regulation must involve structural alterations that have both charge and spatial requirements that are satisfied by a phosphate moiety.

MATERIALS AND METHODS

Construction and Expression of smRLCs. The cDNA for the human smRLC (16) was subcloned into the expression vector pET3a and expressed in HMS 174 (DE3) cells. Site-directed mutagenesis was carried out as described (17).

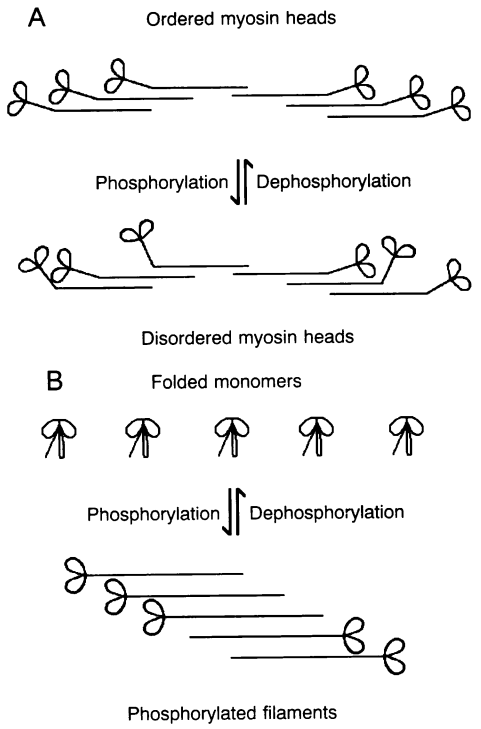

FIG. 2. Schematic depicting the putative common effect of myosin RLC phosphorylation in striated and smooth muscle. This study suggests that the ability of RLC phosphorylation to potentiate force and force development in striated muscle thick filaments (*A*) and its ability to maintain smooth muscle myosin in an extended filament-forming conformation (*B*) involve electrostatic interactions between the phosphate moiety and adjacent positively charged amino acids. In the absence of RLC phosphorylation, these positively charged amino acids may constrain the myosin structure via electrostatic interactions with an undetermined region of the myosin heavy chain. Note that in the case of smooth muscle myosin (*B*), the electrostatic charge-induced unfolding of the myosin does not activate the enzymatic activity of the myosin.

A colony containing a RLC construct in pET3a was grown overnight at 37°C in 5 ml of NZCYM medium (GIBCO/BRL). The overnight cell culture was inoculated with 500 ml of NZCYM at 37°C for 90–120 min and then induced with 1 mM isopropyl β-D-thiogalactoside and grown for 90–120 min. The cells were harvested at 8000 × *g* for 10 min, and the pellet was resuspended with 7 M guanidine·HCl (stirred at 4°C for 1–2 hr to lyse cells). The lysate was centrifuged at 18,000 × *g* for 10 min, and the supernatant fraction was dialyzed overnight against 20 mM Mops/1 mM dithiothreitol/1 mM EDTA/1 mM EGTA, pH 7.2. The dialyzed solution was centrifuged at 18,000 × *g* for 10 min, and the pellet was dissolved in 6 M urea/50 mM Tris/0.5 mM $MgCl_2$, pH 7.5. The protein solution was applied to a DEAE-Sephacel column equilibrated in the Mops buffer and eluted in the same buffer with a gradient of 0–400 mM NaCl. The light chain fraction (analyzed by SDS/PAGE) was pooled, concentrated, and dialyzed against 10 mM Mops/100 mM NaCl/1 mM dithiothreitol/1 mM EDTA/1 mM EGTA, pH 7.2. The light chains were stored at −20°C.

Permeabilized Scallop Fiber Preparation. Bundles of permeabilized striated muscle fibers from sea scallops (*Placopecten magellanicus*) were prepared as described by Simmons and Szent-Gyorgyi (18) with several modifications. The striated adductor muscle, while still attached to the two halves of shell, was stored in ice-cold fresh sea water and kept in a refrigerator at 4°C. Small strips of muscle were separated from the main body by a glass rod with a blunt bent end, severed at both ends, and transferred into cold Ringer solution. Muscle strips were then permeabilized in pCa 8.0 (relaxing solution) containing 1% Triton X-100 for 20–30 min. All procedures were conducted at 4°C. Muscle strips were further separated into small fiber bundles about 100 μm in diameter in relaxing solution (pCa 8.0). The fiber bundles were then attached to the mechanics apparatus.

In each experiment, the permeabilized fiber bundle was first maximally activated to evaluate force production (at 15°C), then washed with a Mg^{2+}-free rigor solution, and finally placed into RLC-extracting solution at 25°C. This procedure completely removes RLCs without denaturing myosin (18).

RLC-depleted fiber bundles were reconstituted with wild-type or mutant smRLC by incubation with Mg^{2+}-containing rigor solution at 15°C for 3–5 hr. Reconstitution was confirmed by restoration of Ca^{2+}-sensitive force production, with no tension development in relaxing solution. Phosphorylation of the RLC was achieved by incubating the muscle bundle with rabbit skeletal muscle myosin light chain kinase at 0.13 mg/ml and 2 μM calmodulin in a pCa 5.5 contractile solution for 30 sec.

Force–pCa relationships were determined at 15°C by using a series 400A force transducer system (Cambridge Technology, Cambridge, MA). The temperature was controlled by a thermocontroller (University of Pennsylvania Biomedical Instrument Group), which drove a Peltier device that was coupled to the experimental chamber. Before each activation, the muscle fiber bundle was briefly bathed in preactivating solution. At the end of the mechanical experiments, the fiber bundle was immersed in 5% trichloroacetic acid and stored at −20°C for gel electrophoretic analysis.

Solutions for Scallop Experiments. The following solutions were used: Ringer solution (440 mM NaCl/10 mM KCl/14 mM $MgSO_4$/30 mM $MgCl_2$/10 mM $CaCl_2$/10 mM imidazole, pH 7.7), RLC extracting solution (40 mM KCl/10 mM EDTA/20 mM imidazole, pH 7.0), Mg^{2+}-free rigor solution (165 mM KCl/20 mM imidazole/2.5 mM EGTA/2.5 mM EDTA, pH 7.0), Mg^{2+}-containing rigor solution (165 mM KCl/20 mM imidazole/2.5 mM EGTA/5 mM $MgCl_2$, pH

7.0), and preactivating solution (50 mM KCl/5 mM $MgCl_2$/0.1 mM EGTA/5 mM Na_2ATP/20 mM imidazole, pH 7.0).

Ca^{2+}-containing experimental solutions for force measurements ranged in Ca^{2+} concentration from 10 μM (pCa 8.0; relaxing solution) to 10 μM (pCa 5.0). All contained 4 mM EGTA, 5 mM MgATP, 15 mM creatine phosphate, 200 units of creatine kinase per ml, 1 mM inorganic phosphate, 3 mM free Mg^{2+}, 100 mM free K^+, 10 mM free Na^+, various levels of free Ca^{2+}, >70 mM methane sulfonate, and sufficient imidazole (>30 mM) to bring the ionic strength to 0.2 M at pH 7.1.

Smooth Muscle Myosin Preparation and RLC Exchange. Dephosphorylated smooth muscle myosin was prepared from turkey gizzards (19). Wild-type RLC and the RLC carrying the Ser-19 → Glu mutation were phosphorylated with smooth muscle light chain kinase, calmodulin, Ca^{2+}, and MgATP or Mg adenosine 5'-[γ-thio]triphosphate (5). Skeletal muscle light chain kinase was used to phosphorylate the Arg-13 → Ala/Arg-16 → Ala mutant. Mutant and wild-type RLCs in either the dephosphorylated or phosphorylated state were exchanged into dephosphorylated gizzard myosin to >90% at elevated temperature as described (12).

Assay for Folded Smooth Muscle Myosin. A pelleting assay was used to quantitate the amount of filamentous and soluble folded myosin formed in the presence of nucleotide. MgATP (1 mM) was added to exchanged myosin (10 mM imidazole, pH 7/0.15 M KCl/5 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol), and the mixture was spun at 23 psi (1 psi = 6.89 kPa) for 15 min in a Beckman Airfuge. The percent soluble myosin was quantitated either by gel densitometry (EC densitometer) or by a colorimetric protein assay (20). Gel filtration chromatography confirmed that the soluble myosin was in the folded monomeric conformation (21).

ATPase Assay and *in Vitro* Motility Assays. Actin-activated ATPase assays of the hybrid myosins were done at 37°C in the presence of 10 μM skeletal muscle actin and 2.5 μM gizzard tropomyosin as described (12). *In vitro* motility assays were performed at 30°C as described in detail (12).

RESULTS AND DISCUSSION

Force Potentiation in Scallop Fibers. The extraction/replacement protocol in scallop striated bundles resulted in complete replacement of wild-type scallop RLC with wild-type or mutant human smRLC (data not shown), as reported for other vertebrate RLCs (13). The maximal Ca^{2+}-activated force (pCa 5.0) following smRLC replacement was 78.7 ± 2.3% of pre-RLC extraction values for the scallop striated muscle bundles used in this study. RLC phosphorylation levels exceeded 80% and were not increased by the inclusion of okadaic acid in experimental solutions. Similar values of force potentiation at selected Ca^{2+} levels were obtained with either inclusion of a Ca^{2+}-independent [via proteolytic cleavage (22)] skeletal muscle myosin light chain kinase (data not shown) or prephosphorylation (at pCa 5.0) by using calmodulin and skeletal muscle myosin light chain kinase (Fig. 3).

Fig. 3 depicts force–pCa relationships of permeabilized scallop striated muscle bundles after replacement of scallop RLCs with wild-type (both dephosphorylated and phosphorylated) and mutant human smRLCs. Phosphorylation of the wild-type smRLC results in a leftward shift of the force–pCa relationship. Thus, phosphorylation of the smRLC increases force production at submaximal Ca^{2+} concentrations in scallop striated muscle. This same effect of RLC phosphorylation has been reported in mammalian striated muscle (8–10, 23).

Examination of the force–pCa relationships in Fig. 3 reveals that replacement of either Lys-11 or Lys-12 with alanine results in a smRLC that is indistinguishable from wild-type smRLC. However, replacement of either Arg-13 or Arg-16 with alanine shifts the force–pCa curve toward that of phosphorylated wild type (the Arg-16 curve shifted more than the Arg-13 curve). The replacement of both Arg-13 and Arg-16 with alanine residues resulted in a shift in the force–pCa curve that was slightly greater (i.e., greater force potentiation) than was obtained with phosphorylated wild-type smRLC. Replacement of Ser-19 with a glutamic residue gave a leftward shift similar to the Arg-16 mutant, while the Thr-18 → Glu/Ser-19 → Glu double mutation produced a shift nearly identical to the phosphorylated wild-type curve. Preliminary

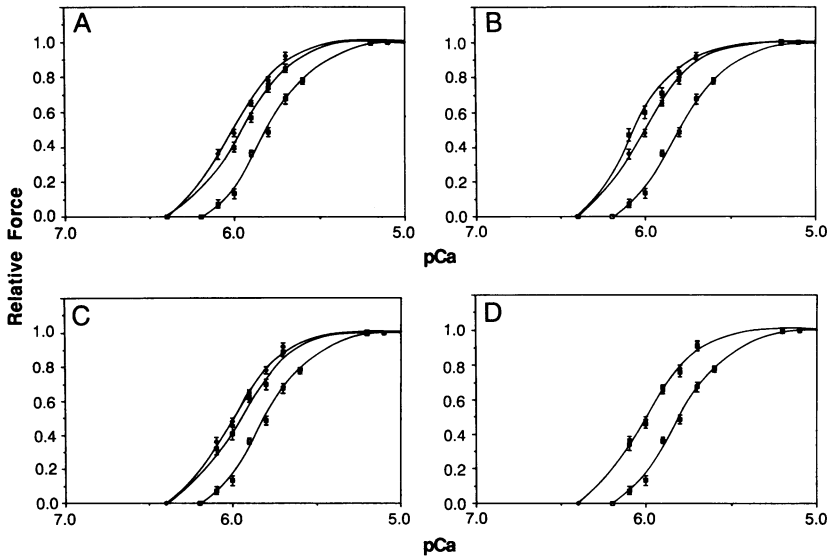

FIG. 3. Force–pCa relationship of scallop striated muscle bundles in which scallop RLCs have been replaced by nonphosphorylated mutant smRLCs. Mutant RLCs were not phosphorylated. The force–pCa relationships obtained with phosphorylated wild-type RLCs and dephosphorylated wild-type RLCs are plotted on each graph for comparison. Force was measured at 15°C. ⊡, Average values ± SE (n = 13) for force values (normalized to dephosphorylated wild type at pCa 5.0) of scallop bundles after reconstitution with wild-type smRLCs; ●, average values ± SE (n = 13) for force values (normalized to dephosphorylated wild type at pCa 5.0) of scallop bundles after phosphorylation of reconstituted wild-type smRLCs; ■, values for force–pCa curves obtained after reconstitution with mutant smRLCs. In the case of the mutant RLCs, n = 8 in each case. (*A*) Replacement of Arg-16 with alanine (R16A). (*B*) Replacement of Arg-13 and Arg-16 with alanines (R13A, R16A). (*C*) Replacement of Ser-19 with glutamate (S19E). (*D*) Replacement of Thr-18 and Ser-19 with glutamates (T18E, S19E).

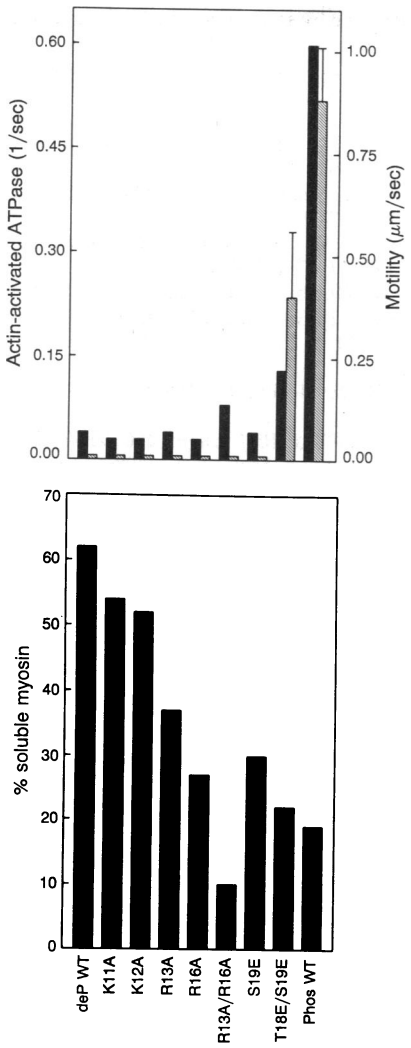

FIG. 4. (*Lower*) Assessment of the effect of mutant RLC exchange on folding of gizzard smooth muscle myosin. Mutant RLCs were not phosphorylated. The results were obtained by using a sedimentation assay. Soluble myosin is in the folded form. The dephosphorylated wild-type RLC is labeled deP WT, while Phos WT represents the results obtained with the phosphorylated wild-type RLC. (*Upper*) Assessment of the effect of mutant RLC exchange on actin-activated ATPase activity (37°C) and *in vitro* motility (30°C) of gizzard smooth muscle myosin. Mutant RLCs were not phosphorylated. The dephosphorylated wild-type RLC is labeled deP WT, while Phos WT represents the results obtained with the phosphorylated wild-type RLC.

experiments involving exchange of these mutant RLCs into rabbit psoas muscle fibers (data not shown) have yielded data that are in agreement with the results in the scallop fiber bundles.

Assembly and Motility Properties of Hybrid Smooth Muscle Myosins. Mutant RLCs were exchanged to >90% into turkey gizzard smooth muscle myosin (12). The ATPase activity and *in vitro* motility of myosin in which the phosphorylated human RLC was exchanged for the native RLC were both >80% of the values obtained with native phosphorylated (no exchange) gizzard smooth muscle myosin.

The ability of the mutant RLCs to favor the unfolded, filamentous state of myosin (Fig. 4 *Lower*) closely paralleled the shifts in the force–pCa curves obtained with scallop. Mutations that mimicked the ability of phosphorylation to potentiate force (leftward shift of force–pCa curve) in scallop striated muscle promoted unfolding of smooth muscle myosin. As revealed in the normalized data shown in Table 1, the

Table 1. Summary of results from functional tests of smRLC mutations

		Relative smooth myosin results		
	Relative force potentiation at pCa 6.0 in scallop muscle	Amount of unfolded myosin	Actin-activated ATPase activity	Average motility
smRLC-wt				
Dephosphorylated	0.00	0.00	0.00	0.00
Phosphorylated	1.00	1.00	1.00	1.00
smRLC-K11A	0.00	0.08	0.00	0.00
smRLC-K12A	0.00	0.06	0.00	0.00
smRLC-R13A	0.58	0.51	0.00	0.00
smRLC-R16A	0.83	0.75	0.00	0.00
smRLC-R13A/R16A				
Dephosphorylated	1.25	1.20	0.07	0.00
Phosphorylated	—	—	—	1.39
smRLC-S19E				
Dephosphorylated	0.85	0.84	0.00	0.00
Phosphorylated*	—	—	—	0.92
smRLC-T18E/S19E	0.96	0.93	0.16	0.45
smRLC-T18D/S19D	—	—	—	0.59

Results are expressed relative to phosphorylated wild-type smRLC (smRLC-wt). Mutations are shown in single-letter code. *(T18) smRLC-S19E.

effects were not only qualitatively similar but also the magnitude of each mutant's ability to unfold myosin was similar to the magnitude of the potentiation of force in scallop striated muscle at pCa 6.0.

The data shown in Fig. 4 *Upper* reveal that the only dephosphorylated mutant that demonstrated some activation of ATPase activity and *in vitro* motility was the mutant carrying the double replacement of Thr-18 and Ser-19 with glutamic residues. All other mutants were indistinguishable from dephosphorylated wild-type human smRLC. However, this was not due to a major disruption of the N terminus accompanying the mutations because phosphorylation of either the smRLC mutant carrying Arg-13 → Ala and Arg-16 → Ala changes or the mutant carrying the Ser-19 → Glu change (phosphorylated at Thr-18) activates motility of gizzard smooth muscle myosin (Table 1).

Comparative Effects of Phosphorylation. Normalized data from both scallop striated muscle and isolated smooth muscle myosin experiments are summarized in Table 1 to facilitate comparisons. The results show that decreasing the net charge in the region from Arg-13 to Ser-19 can mimic the ability of RLC phosphorylation to potentiate force in scallop striated muscle and to unfold smooth muscle myosin. However, simple charge replacement does not activate ATPase activity or motility of smooth muscle myosin. Only in the case of the replacement of both Thr-18 and Ser-19 with glutamic residues is there a slight activation of ATPase activity and moderate activation of motility. Based on this, we also created a mutant RLC with aspartate residues substituted for Thr-18 and Ser-19. This mutant was evaluated only in terms of its activation of motility of smooth muscle myosin, and the results are listed in Table 1. In the case of the motility assays involving both the Thr-18 → Glu/Ser-19 → Glu and the Thr-18 → Asp/Ser-19 → Asp mutants, those actin filaments that moved had velocities approximately half of that obtained with phosphorylated myosin (see Table 1) but significantly fewer actin filaments in the field moved (29% for the Thr-18 → Glu/Ser-19 → Glu double mutant and 34% for the Thr-18 → Asp/Ser-19 → Asp double mutant, compared with >95% of the field moving with phosphorylated myosin). These results suggest that two adjacent negatively charged residues

mimic (albeit poorly) both the spatial and charge contributions of a phosphate group.

When substituted for the native scallop striated muscle RLC, the smRLC restores Ca^{2+}-dependent regulation via direct Ca^{2+} binding to myosin (13). Phosphorylation of the smRLC does not regulate scallop striated muscle in a Ca^{2+}-independent manner but instead potentiates force in a manner similar to that observed in mammalian striated muscle (8–10) (Fig. 3). As summarized in Table 1, this effect of phosphorylation is duplicated by simply reducing the net charge near the site of phosphorylation (Arg-13 to Ser-19).

It has been suggested that the effect of myosin RLC phosphorylation in striated muscle can be explained either by movement of myosin heads away from the thick filament backbone (9, 24, 25) or by a disordering of the heads, which may indicate increased mobility (26, 27). The results of this study suggest that if this is the physical explanation for the phosphorylation effect, then the restricted mobility may be due to electrostatic interactions between the N terminus of the RLC and some yet-to-be-determined structure (possibly the thick filament backbone). This electrostatic interaction at the N terminus of the RLC arises from the positively charged amino acids near the phosphorylatable serine and can be reversed by phosphorylation of the serine.

A similar electrostatic mechanism may account for the ability of RLC phosphorylation to unfold the bent smooth muscle myosin monomer to the extended conformation. A simple charge reduction at the N terminus (provided that the rest of the RLC is native), which is accomplished physiologically by phosphorylation, can account for myosin's inability to form a stable folded monomer. Phosphorylation-dependent unfolding of the rod, a process that also causes heads that were bent toward the rod to be released away from the rod (28), thus becomes the mechanistic equivalent of the phosphorylation effect in striated muscle.

These data also show that the interactions that regulate the folded-to-extended conformational transition in smooth muscle myosin are distinct from those that control activity. Myosin that is kept unfolded and assembled into filaments due to charge substitutions in the RLC is inactive, unless the mutant RLC chain is phosphorylated (see Table 1). This is consistent with previous studies that showed phosphorylation-dependent activation of myosin that was locked in the filamentous state by virtue of monoclonal antibodies (29) or incorporation of a chimeric N-skeletal/C-smooth RLC (12). Thus, phosphorylation must exert a direct effect on the active site of the molecule, which is not tightly coupled to myosin's ability to undergo the folded-to-extended transition, as was originally proposed based on studies with monomeric myosin (30). This activation of ATPase activity and motility, unlike folding, does not depend solely on alteration of net charge at the N terminus but also has spatial constraints that are satisfied only by a phosphate moiety. Recent work suggests that this likely involves a complex structural rearrangement that minimally involves the phosphorylation site and a C-terminal region of the RLC (12).

We thank Dr. Chandra Kumar for the human smRLC DNA. This work was supported by grants from the National Institutes of Health (AR 35661, HL06296, HL 26043, and HL 38113) and the Bashour Research Fund. H.L.S. and K.M.T. are Established Investigators of the American Heart Association.

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