Phosphorylation of the regulatory light chains of myosin affects Ca\(^{2+}\) sensitivity of skeletal muscle contraction

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The regulation of contraction in molluscan or vertebrate smooth muscles occurs via myosin, which either binds Ca\(^{2+}\) directly (molluscan) (16, 49) or undergoes a Ca\(^{2+}\)/calmodulin (CaM)-activated phosphorylation at the myosin regulatory light chains (RLC) in smooth muscle (1, 11, 39). Striated muscles are activated by the binding of Ca\(^{2+}\) to troponin C (TnC), which initiates a series of conformational changes within the proteins of the thin filaments and leads to muscle contraction (29, 51). Unlike molluscan or smooth muscles, the RLC of striated muscles do not play a primary regulatory role, and therefore it is of interest to understand their possible role in thin filament-regulated skeletal muscles. The crystal structure of skeletal myosin subfragment 1 (35) reveals that the RLC are located at the head–rod junction of the myosin molecule, implying their possible importance in cross-bridge cycling in contracting muscle. The NH\(_2\)-terminal domain of RLC contains a divalent cation-binding site that binds both Ca\(^{2+}\) and Mg\(^{2+}\). Under physiological conditions, in relaxed muscle, it is thought that this site is occupied by Mg\(^{2+}\) (14) and may become partially saturated with Ca\(^{2+}\), depending on the length of the Ca\(^{2+}\) concentration ([Ca\(^{2+}\)] transient (37). Analogous to smooth muscle myosin, the NH\(_2\)-terminal domain of RLC of skeletal myosin also contains two adjacent serine residues located in the proximity of the cation-binding site. During muscle contraction, the increase in [Ca\(^{2+}\)] activates the Ca\(^{2+}\)/CaM-dependent myosin light chain kinase (MLCK) and leads to phosphorylation of the RLC. In vivo phosphorylation of this kind correlates with potentiation of the rate of force development and maximal extent of isometric twitch tension (Ref. 43 and references within). In vitro, the rate of isometric force redevelopment of skinned muscle fibers was shown to increase with RLC phosphorylation that also caused an increase in the Ca\(^{2+}\) sensitivity of force (18–20, 24, 45). Although no effect on maximal steady-state force (developed at maximal Ca\(^{2+}\) activation) has been observed in skinned muscle fiber preparations under normal conditions, a small effect of RLC phosphorylation has been observed under fatigue conditions (8). The results presented in this paper show a ~15% increase in maximal force developed by RLC phosphorylated skinned fibers vs. those reconstituted with nonphosphorylated RLC. Unlike the correlation of RLC phosphorylation and force po-

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tention in intact muscle or the increase in the Ca\(^{2+}\) sensitivity of force development in skinned muscle fiber preparations, there is no clear understanding of the influence of RLC phosphorylation on the actin-activated myosin ATPase activity (28, 32, 41). The results presented here show that phosphorylation of the RLC has a dramatic effect on the Ca\(^{2+}\) sensitivity of the ATPase activity of reconstituted thin filaments. Myosin depleted of endogenous RLC and reconstituted with MLCK phosphorylated exogenous RLC increased the Ca\(^{2+}\) sensitivity of actin-tropomyosin (Tm)-tropinin (Tn) activity by a change of the -log of the [Ca\(^{2+}\)] that produced half-maximal activation (ApCa\(_{50}\)) of -0.25 vs. nonphosphorylated RLC. This is the first report of such an effect of RLC phosphorylation in the actomyosin ATPase system. It is worth mentioning that the magnitude of the effect of RLC phosphorylation on the Ca\(^{2+}\) sensitivity of force development observed in our skinned skeletal muscle fibers was also more extensive than previously reported. Because skinned fibers may contain different levels of RLC phosphorylation depending on how they are isolated, it will be critical in future experiments to measure the level of RLC phosphorylation to determine the contribution of this to any measured Ca\(^{2+}\) dependence. In summary, the role of RLC phosphorylation, as demonstrated here and earlier by others (Ref. 43 and references within) is more important in modulating skeletal muscle contraction than originally suspected or appreciated.

MATERIALS AND METHODS

Rabbit Skeletal Phosphorylated and Nonphosphorylated RLC

RLC of myosin were isolated and purified as described by Wagner et al. (50). Briefly, myosin at a concentration of 12–15 mg/ml in 0.5 M KCl, 10 mM EDTA, and 10 mM phosphate buffer, pH 8.5, was incubated with 10 mM DTNB for 15 min on ice. Myosin was then precipitated by addition of 13 vol of 10 mM EDTA, pH 7.0, and centrifuged. The RLC-depleted myosin was then resuspended in 0.5 M KCl, 10 mM dithiothreitol (DTT), and 10 mM phosphate buffer, pH 7.0. This myosin was further redialyzed and used in the actin-activated ATPase assays as RLC-depleted myosin and used in the reconstitution experiments with nonphosphorylated and/or phosphorylated RLC. The supernatant containing the dissociated RLC was dialyzed against 2 M urea, 25 mM Tris-HCl (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride, 0.02% NaN3, and 1 mM DTT, and then it was applied to a Q-Sepharose column equilibrated with the same buffer. The RLC-depleted myosin was then eluted by using a salt gradient of 0–12 mM MgCl2, 50 mM MOPS, pH 7.0, 2 mM MgCl2, and 10 mM DTT, whereas RLC and P-RLC were dialyzed against the same buffer with the exception of the KCl being 0.1 M. Protein concentrations during dialysis were determined as stated earlier. RLC-depleted myosin and the P-RLC or RLC were then mixed in a 1:2 molar ratio and incubated on ice for 2 h to allow for reconstitution to take place. The P-RLC and/or RLC reconstituted myosins were then dialyzed against 0.4 M KCl, 50 mM MOPS (pH 7.0), and 10 mM DTT. To remove any excess RLC after dialysis, the reconstituted myosins were precipitated by the addition of 13 vol of cold H2O and collected by centrifugation. Pellets were resuspended into minimal volumes of myosin dialysis buffer and dialyzed for an additional 2 h. Protein concentrations were determined, and 0.1 mg/ml samples were used in the K+ EDTA and Ca\(^{2+}\) ATPases assays (22) to check the enzymatic activity of the native (undepleted), RLC-depleted, and P-RLC/RCL reconstituted myosins. Dialysis was continued until K+ EDTA and Ca\(^{2+}\) ATPase activity levels approached the published values (22).

Ca\(^{2+}\) Binding Studies

Fluorescence measurements. P-RLC and RLC were dialyzed against a solution of 90 mM KCl, 120 mM MOPS (pH 7.0), and 2 mM EGTA. For measurements in the presence of Mg\(^{2+}\), the dialysis buffer also contained 2 mM MgCl2. Measurements were performed by using SLM Spectrofluorometer model 8100 (SLM Instruments). The proteins (5 mM) were placed in a 2-ml quartz cuvette, and the tryptophan fluorescence of RLC was excited at 290 nm. Emission spectra were acquired in the range of 310 to 410 nm. All measurements were performed at room temperature (22°C). For Ca\(^{2+}\) titrations, peak fluorescence between 341 and 344 nm was acquired and averaged for each [Ca\(^{2+}\)]. Data were fitted to the Hill equation, analyzed, and plotted by using SigmaPlot 2000. The amount of Ca\(^{2+}\) added to achieve a desired free [Ca\(^{2+}\)] was calculated according to Robertson and Potter (36).

Flow dialysis. Flow dialysis was performed in a solution of 100 mM KCl and 20 mM imidazole, pH 7.0 (22°C). The proteins were equilibrated in this buffer before measurements. The flow-dialysis experiments were performed according to Colowick and Womack (4) with modifications. Briefly, the upper chamber of the apparatus containing the protein and the labeled substrate (50Ca\(^{2+}\)) was separated by a membrane from the lower chamber. The buffer was pumped through the lower chamber at a constant rate of 1.5 ml/30 s. The upper chamber was first equilibrated with 0.4 ml buffer for 15 min followed by the protein (0.4 ml) for 5 min. After 45Ca\(^{2+}\) was added, equilibrium was attained by flowing buffer through the lower chamber for 5 min. After steady state was reached, unlabeled substrate (Ca\(^{2+}\)) was added at regular intervals and in varying concentrations. Fractions were collected every 30 s, and the effluent was sampled for
measurement of radioactivity. The specific radioactivity of $^{45}$Ca$^{2+}$ used in the experiment was 12–16 mCi/mg (from NEN Life Science Products), and 2 μCi of $^{45}$Ca$^{2+}$ per experiment gave sufficient radioactivity in the dialysate for accurate measurements. Data were analyzed by using Scatchard analysis (34, 38)

$$[\text{Ca-bound}]/[\text{Ca-free}]/[\text{protein}] = -K_{ca} \times [\text{Ca-bound}]/[\text{protein}] + \eta K_{ca}$$

where [Ca-bound] and [Ca-free] represent the concentration of the bound and free metal, respectively, [protein] is the total number of Ca$^{2+}$ binding sites, and $K_{ca}$ is the Ca$^{2+}$ binding constant.

**Actin-Activated ATPase Assays**

Rabbit skeletal myosin was obtained as described earlier (26, 41). F-actin, Tn, and TnC were isolated and purified from rabbit skeletal muscle according to Strzelecka-Golaszewska et al. (42), Potter (35), and Smillie (40), respectively. Myosin was dialyzed to 0.4 M KCl, 50 mM MOPS (pH 7.0), and 1 mM DTT, whereas F-actin, Tn, and TnC were homogenized together in a ratio of 7:3:1, respectively, and dialyzed in the same buffer as the myosin but with 0.1 M KCl. Actin-activated ATPase assays were performed by using 1 μM myosin-4 μM F-actin-0.6 μM TnC-0.6 μM TnC in a solution containing 20 mM MOPS (pH 7.0), 35 mM KCl, 2 mM EGTA, 2.5 mM MgCl$_2$, and increasing [Ca$^{2+}$] from pCa 8 to 4 (36). The reaction was initiated with 2.5 mM ATP, and after 5 min incubation at 30°C terminated with 5% trichloroacetic acid. Inorganic phosphate was measured according to Fiske and SubbaRow (6).

**Skinned Fiber Preparation and Force Measurements**

Experiments were performed with glycerinated rabbit psoas muscle fibers dissected from rabbits and chemically skinned (as described in Refs. 17, 48). Fiber bundles of three to five single fibers were mounted on a force transducer [assembled according to Guth and Potter (10)]. The fibers were then treated with the pCa 8 relaxing solution, containing 1% Triton X-100, for 15 min. The composition of the pCa 8 solution was as follows: 10$^{-8}$ M [Ca$^{2+}$], 1 mM Mg$^{2+}$, 7 mM EGTA, 5 mM MgATP$^{2-}$, 20 mM imidazole (pH 7.0), 20 mM creatine phosphate, and 15 units/ml of creatine phosphokinase (ionic strength = 150 mM) (5). To judge the quality of the fibers, they were contracted (in the pCa 4 solution containing the same composition as the pCa 8 solution except [Ca$^{2+}$] = 10$^{-4}$ M) and relaxed several times to obtain stable force values. Sarcomere length was adjusted to 2.4 μm. Sarcomere length control was not available. The maximal force per cross-sectional area, calculated for all newly mounted fibers before the reconstitution experiments, was 301 ± 29 kN/m$^2$. To further evaluate the fibers suitability for further testing, the Ca$^{2+}$ dependence of force development was measured twice to make certain that it was consistent with our established Ca$^{2+}$ dependence for these solutions and stable. All measurements were performed at room temperature (22°C). Once the fibers passed these initial tests, they were then used for RLC treatment (see below). Fibers not passing these initial tests were rejected and new ones were tested.

**Preparation of Skinned Fibers Containing Fully Dephosphorylated RLC**

The protocol described earlier (17, 48) for skeletal muscle fiber preparation resulted in various levels of RLC phosphorylation. To obtain fully dephosphorylated fibers, small strips of psoas muscle (~2 mm in diameter) were dissected from rabbit psoas muscle and incubated in (in mM) 60 KPr, 2 MgCl$_2$, 5 EGTA, 25 MOPS (pH 7.0), 1 Na$_2$, and 25% glycerol for 1 h at 4°C. The fibers were then transferred to a fresh solution for an additional 24 h, followed by incubation in the same solution containing 50% glycerol. The latter solution was changed every 12 h for 48 h, after which the fibers were transferred to this solution and stored at −20°C for no longer than 6 wk.

**Phosphorylation of Endogenous RLC in Skinned Fibers With Ca$^{2+}$/CaM-Activated MLCK**

Phosphorylation of RLC in the skeletal muscle fibers was performed in the pCa 6 solution (the composition of this solution was the same as the pCa 8 buffer except [Ca$^{2+}$] = 10$^{-6}$ M, plus 5 μM bovine CaM and 0.5 μM MLCK. The same catalytically active truncated fragment of the rabbit skeletal muscle MLCK (12) was used to phosphorylate the RLC in the fibers or in the isolated state (7). After 30 min of phosphorylation, the fibers were washed with the pCa 8 solution and subjected to force measurements.

**Reconstitution of RLC-Depleted Skinned Fibers With Exogenous RLC**

After initial force measurements on the control, untreated skinned skeletal muscle fibers, the endogenous RLC were extracted according to the protocol described in Szczesna et al. (48). Because of the partial extraction of TnC, the RLC-depleted fibers were first incubated with 20 μM rabbit skeletal TnC in the pCa 8 solution for 30 min at room temperature (22°C), rinsed in the pCa solution, and tested for Ca$^{2+}$-dependent force development. RLC-depleted (TnC-reconstituted) fibers were then incubated with 30 μM RLC dissolved in the pCa 8 buffer, rinsed with the same buffer without protein added, and tested for force development. P-RLC or nonphosphorylated RLC were used in the reconstitution experiments; when the nonphosphorylated RLC were utilized, the fibers were further treated with Ca$^{2+}$/CaM-activated MLCK to phosphorylate the reconstituted RLC.

**Steady-State Force Measurements**

As described above, bundles of three to five single fibers were mounted on a force transducer with stainless steel clips and incubated for 15 min at room temperature (22°C) in the pCa 8 solution, containing 1% Triton X-100. Maximal force was measured in the pCa 4 solution, and the fibers were then relaxed in the pCa 8 solution. The effect of RLC phosphorylation was tested in a series of parallel experiments performed on fibers reconstituted with various RLC. These measurements were performed on the control, RLC-, and TnC-depleted fibers, RLC-depleted and TnC-reconstituted fibers, and finally on the RLC- and TnC-reconstituted fibers. Fibers were reconstituted with either P-RLC or nonphosphorylated RLC that were further phosphorylated in the fibers with Ca$^{2+}$/CaM-activated MLCK (for 30 min at room temperature). Ca$^{2+}$ dependence of force development was measured in solutions of increasing [Ca$^{2+}$] (from pCa 8 to 4). The data were fitted to the following equation

$$Y_H = 100 \times \frac{[\text{Ca}^{2+}]_{0.1} ([\text{Ca}^{2+}]_{0.1} + [\text{Ca}^{2+}]_{150} \text{[n]}))}{[\text{Ca}^{2+}]_{0.1} ([\text{Ca}^{2+}]_{150} + [\text{Ca}^{2+}]_{150} \text{[n]})}$$

where $Y_H$ is the relative force change expressed as a percentage of the maximal change, [Ca$^{2+}$]$_{150}$ is the [Ca$^{2+}$] that produces 50% change in force, and n$_H$ is the Hill coefficient.
Measurements of the Rate of Force Development

Before the kinetics measurements, the fibers were contracted (pCa 4) and relaxed (pCa 8) several times to reach a stable force level, and their sarcomere length was reset to 2.4 μm. Then they were treated with the pCa 8 solution containing 1% of Triton X-100 for 15 min at room temperature (22°C), contracted in the pCa 4 solution, and incubated with a “low EGTA” pCa 8 solution (same as pCa 8 solution except containing 0.5 mM instead of 7 mM EGTA). Fibers were then exposed to (in mM) 2.5 DM-nitrophen, 1.002 CaCl₂, 100 TES, 1.2 MgCl₂, 1.4 ATP, 10 glutathione, 29.4 1,6-hexamethylenediamine-2-aminooethane sulfonic acid, and 20 creatine phosphokinase (pH 7.1, ionic strength 150 mM adjusted with potassium propionate) (9). After that, the entire fibers were illuminated in air with an ultraviolet (UV) light pulse xenon flash lamp (model XPL-355-3017, Advanced Radiation). Total air exposure was ~3 s. The duration of the UV pulse from the xenon lamp was ~2 ms with the total UV energy (~400 nm) equal to ~43 mJ. Average energy density exposure of the fiber was ~3.4 mJ/mm² (light spot diameter = 4 mm). To minimize the period of high-tension development, fibers were transferred to relaxing solution (pCa 8) within 1.5 s of the initial flash. As a result of the rapid Ca²⁺ release, fibers developed isometric tension, characterized by a double-exponential time course (48). The rate constants of activation developed isometric tension, characterized by a double-exponential time course (48). The rate constants of activation were calculated according to the equation: \( y = A(1 - e^{-kt_1}) + B(1 - e^{-kt_2}) + C \), where \( k_1 \) and \( k_2 \) are the rate constants, \( A \) and \( B \) are the amplitudes of the force transient, \( C \) is a constant, and \( t \) is time of force transient. The rates of force activation were measured before and after phosphorylation of the fibers with Ca²⁺/CaM-activated MLCK.

SDS-Urea Gel Electrophoresis

The level of RLC phosphorylation in skinned skeletal muscle fibers was tested by using 11% polyacrylamide-SDS gels containing 6 M urea and 0.08 M Tris-glycine buffer, pH 8.6. Control (not treated) fibers as well as the MLCK-treated fibers were sonicated in a solution of 8 M urea for 25 min at room temperature and loaded onto mini-slab gels. The electrode buffer contained 0.08 M Tris-glycine, pH 8.6. The gels were silver stained (23) to increase the visibility of the protein bands, and the level of RLC-phosphorylation was quantified by densitometry of the stained gels.

Statistical Analysis

Differences between the measurements of the Ca²⁺ sensitivity of the thin filament ATPase activity, force development, and kinetics of force activation of nonphosphorylated RLC vs. P-RLC were determined by using an unpaired Student’s t-test (Sigma Plot 2000), with significance defined as \( P < 0.05 \).

RESULTS

Ca²⁺ Binding to Isolated RLC

The binding of Ca²⁺ to P-RLC or nonphosphorylated RLC and the effect of Mg²⁺ on this binding were studied with the fluorescence method, in which the Trp fluorescence of the single Trp residue of the RLC was monitored. The Ca²⁺ affinity to the single Ca²⁺-Mg²⁺ binding site of the RLC was confirmed with the flow-dialysis method. In agreement with Alexis and Gratzer (2), the \( K_{Ca} \) for nonphosphorylated rabbit skeletal RLC was ~2.26 × 10⁵ M⁻¹ (reported 2.5 × 10⁵ M⁻¹), as determined by the fluorescence method. Phosphorylation of the RLC with Ca²⁺/CaM-activated MLCK only slightly decreased its Ca²⁺ affinity (\( K_{Ca} \sim 1.83 \times 10⁵ \) M⁻¹). In the presence of 2 mM Mg²⁺, the apparent equilibrium constant of Ca (\( K_{Ca} \)) decreased to 1.28 × 10⁵ M⁻¹ and 3.89 × 10⁴ M⁻¹ for nonphosphorylated RLC and P-RLC, respectively. Similar \( K_{Ca} \) and \( K'_Ca \) values were obtained with the flow-dialysis method. \( K_{Ca} \) was ~1.50 × 10⁵ M⁻¹ for nonphosphorylated RLC and ~1.04 × 10⁵ M⁻¹ for P-RLC in the absence of Mg²⁺. \( K_{Ca} \) was ~6.02 × 10⁴ M⁻¹ for nonphosphorylated RLC and ~5.19 × 10⁴ M⁻¹ for P-RLC in the presence of 2 mM Mg²⁺. Low \( K_{Ca} \) values monitored either by the flow-dialysis or fluorescence method and the low sensitivity to Mg²⁺ suggest that the binding of Ca²⁺ to the isolated RLC may not reflect the physiological situation observed in muscle. The Ca²⁺ affinity to the RLC bound to myosin has been reported to be 100-fold higher than to isolated RLC (14).

Regulation of Actin-Tm-Tn-Activated Myosin ATPase Activity by RLC Phosphorylation

DTNB treatment of skeletal muscle myosin resulted in ~70% RLC-deficient myosin (data not shown). This RLC-depleted myosin bound nonphosphorylated RLC and/or P-RLC with the same stoichiometry of intact untreated myosin. Figure 1 demonstrates the effect of the RLC phosphorylation on actin-Tm-Tn-activated myosin ATPase activity. Ca²⁺ regulation of the ATPase activity was determined for the control rabbit skeletal myosin (nonphosphorylated), RLC-depleted myosin, and myosin reconstituted with either P-RLC or non-
phosphorylated RLC. Ca\(^{2+}\) sensitivity of actin-activated ATPase activity of myosin depleted of the decreased RLC by a ΔpCa\(_{50}\) of approximately \(-0.15 \pm 0.02\) compared with untreated, nonphosphorylated myosin. Reconstitution of the RLC-depleted myosin with nonphosphorylated RLC did not significantly change the pCa\(_{50}\) value (pCa\(_{50}\) = \(-6.55 \pm 0.02; n = 3, P > 0.1\)); however, P-RLC-reconstituted myosin dramatically increased Ca\(^{2+}\) sensitivity of the actin-Tm-Tn ATPase activity. The difference expressed in the pCa\(_{50}\) units between P-RLC-reconstituted and RLC-depleted myosin was a ΔpCa\(_{50}\) of \(-0.29 (n = 3, P < 0.01)\), and between P-RLC and the myosin reconstituted with nonphosphorylated RLC was a ΔpCa\(_{50}\) of \(-0.25 (n = 3, P < 0.01);\) Fig. 1).

**Skinned Fiber Studies**

*Steady-state maximal force.* To study the effect of phosphorylation of RLC, we have performed a series of experiments by measuring the steady-state force development in skinned skeletal muscle fibers with the use of the following conditions (Table 1). 1) Nonextracted fibers were phosphorylated with Ca\(^{2+}\)/CaM-activated MLCK; 2) RLC and TnC were extracted from the fibers according to our previously published procedure (48), and the fibers were reconstituted with TnC; 3) after TnC reconstitution, the fibers were reconstituted with nonphosphorylated RLC; 4) fibers were phosphorylated with Ca\(^{2+}\)/CaM-activated MLCK; or 5) alternatively the fibers were reconstituted with prephosphorylated P-RLC. The experiments described here, including the RLC extraction, reconstitution with exogenous TnC and RLC, and then phosphorylation with Ca\(^{2+}\)/CaM-activated MLCK, were time consuming (3-4 h), and fiber rundown was observed. Therefore, the level of maximal force after protein reconstitution was \(-20\)–\(-30\)% lower compared with control fibers. All measurements were performed at room temperature. As shown in Table 1, treatment of the control, nonextracted skinned fibers with Ca\(^{2+}\)/CaM-activated MLCK slightly increased maximal steady-state force to 105 \pm 4% (n = 10). Reconstitution of the RLC-depleted fibers with nonphosphorylated RLC (and TnC) resulted in 68.9 \pm 8.9% force recovery compared with control fibers. Subsequent incubation of these reconstituted fibers with Ca\(^{2+}\)/CaM-activated MLCK resulted in an additional 15.4% increase in force (n = 7; Table 1). The observed increase in maximal force (to 84.3 \pm 10.1%, P < 0.02) was clearly a result of the RLC phosphorylation. Alternatively, when RLC-depleted fibers were reconstituted with prephosphorylated RLC, the force recovery was higher than for nonphosphorylated RLC (80 \pm 6.9 vs. 68.9 \pm 8.9%, 0.02 < P < 0.05; Table 1).

### Ca\(^{2+}\) Sensitivity of Force Development

Figure 2A demonstrates the effect of Ca\(^{2+}\)/CaM-activated MLCK in skinned (not RLC extracted) skeletal muscle fibers on force-pCa dependence. In this example, a leftward shift, toward a lower [Ca\(^{2+}\)], of ΔpCa\(_{50}\) \(-0.14\), was observed. No shift (ΔpCa\(_{50}\) of approximately \(-0.06 \pm 0.05\), data not shown) was observed when the fibers were treated with MLCK buffer alone (minus MLCK). Because control fibers used in the experiments presented in Fig. 2A were partially phosphorylated, this yielded a significant variation (standard deviation) in the pCa\(_{50}\) values (5.58 \pm 0.08). Figure 2B summarizes the effect of phosphorylation on the force-pCa relationship for fibers 1) depleted of RLC, 2) reconstituted with nonphosphorylated rabbit skeletal RLC (and TnC), and 3) were then phosphorylated with the Ca\(^{2+}\)/CaM MLCK. Extraction of the RLC led to a rightward shift in the force-pCa relationship, as shown previously (48). When nonphosphorylated RLC was reincorporated into the fibers, there was no further change in the force-pCa dependence; however, when the reconstituted fibers were exposed to Ca\(^{2+}\)/CaM-activated MLCK, the force-pCa relationship matched that of the unextracted skinned fibers. Again, this suggests that the control fibers used in this experiment were partially phosphorylated. This result accounts for our original results (48), where we were unable to fully restore the Ca\(^{2+}\) dependence of the extracted fibers to the control values with rabbit nonphosphorylated RLC. Similarly, when the RLC-extracted fibers were reconstituted with rabbit P-RLC, the original Ca\(^{2+}\) dependence of force was restored (Fig. 2C). Interestingly, the fibers chosen for the experiments shown in Fig. 2C, where P-RLC was reconstituted into RLC-depleted fibers, were phosphorylated during skinnning procedure with endogenous MLCK. The pCa\(_{50}\) = 5.67 of these control fibers was then different from these from Fig. 2, A and B (pCa\(_{50}\) = 5.58).

### Effect of Phosphorylation of Endogenous RLC on the Ca\(^{2+}\) Sensitivity of Force Development in Untreated Skinned Fibers

A very strong correlation exists between the level of endogenous phosphorylation of the RLC in muscle fibers and the force-pCa relationship. SDS-urea gel electrophoresis was used to measure the level of RLC phosphorylation in the control skinned skeletal muscle.

<table>
<thead>
<tr>
<th>Skinned Skeletal Muscle Fibers</th>
<th>Relative Force, % of Control</th>
<th>Number of Experiments</th>
</tr>
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<tbody>
<tr>
<td>Incubated with Ca(^{2+})/CaM-activated MLCK</td>
<td>105 \pm 4</td>
<td>10</td>
</tr>
<tr>
<td>RLC and TnC depleted</td>
<td>6.1 \pm 2.8</td>
<td>14</td>
</tr>
<tr>
<td>TnC reconstituted and RLC depleted</td>
<td>55.3 \pm 9.6</td>
<td>14</td>
</tr>
<tr>
<td>Nonphosphorylated RLC reconstituted and incubated with Ca(^{2+})/CaM-MLCK</td>
<td>68.9 \pm 8.9*</td>
<td>7</td>
</tr>
<tr>
<td>Nonphosphorylated RLC reconstituted and incubated with Ca(^{2+})/CaM-MLCK</td>
<td>84.3 \pm 10.1</td>
<td>7</td>
</tr>
<tr>
<td>Phosphorylated RLC reconstituted</td>
<td>80 \pm 6.9*</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are means \pm SD. RLC, regulatory light chains; CaM, calmodulin; MLCK, myosin light chain kinase; TnC, troponin C.

*Nonphosphorylated and phosphorylated RLC-reconstituted fibers: 0.02 < P < 0.05.
fibers. As shown in Fig. 3, the fibers used in steady-state measurements had different levels of RLC phosphorylation. Fresh fibers usually demonstrated a high level of RLC phosphorylation, which decreased gradually as the fibers were stored over time in 50% glycerol at −20°C. Figure 3A demonstrates an example of the fully phosphorylated (lane 5) or fully dephosphorylated (lane 4) fibers. Figure 3B is an example of 60% phosphorylated fibers (lane 2). The force-pCa measurements clearly indicated that as the level of phosphorylation of the RLC was increased, so was the Ca\(^{2+}\) sensitivity of force development and vice versa. This was confirmed in experiments with fully phosphorylated fibers.

**Fig. 2.** Effect of RLC phosphorylation on the Ca\(^{2+}\) sensitivity of steady-state force development. A: control fibers (○; pCa\(_{50}\) = 5.58 ± 0.08, n = 7) and fibers incubated with myosin light chain kinase (MLCK; Δ; pCa\(_{50}\) = 5.72 ± 0.02, n = 7). Fibers incubated with phosphorylation buffer with no MLCK demonstrated pCa\(_{50}\) = 5.52 ± 0.05, n = 4 (data not shown). Data are means ± SD of n experiments. B: effect of RLC extraction, and reconstitution and phosphorylation of RLC in the fibers after reconstitution. Control fibers (○; pCa\(_{50}\) = 5.58 ± 0.05, n = 7) were treated with the DTNB-dithiothreitol (DTT) method to extract RLC (48). (1), RLC-depleted fibers were reconstituted with TnC (Δ; pCa\(_{50}\) = 5.39 ± 0.04, n = 6); (2), TnC-reconstituted fibers were subsequently reconstituted with nonphosphorylated RLC (□; pCa\(_{50}\) = 5.47 ± 0.08, n = 6); (3), fibers were then treated with MLCK (■; pCa\(_{50}\) = 5.58 ± 0.06, n = 3). In each case, the force-Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) relationship was determined. Experimental points (pCa\(_{50}\) values) are means ± SD of n experiments. C: effect of reconstitution of RLC-depleted fibers with P-RLC. Control fibers (○; pCa\(_{50}\) = 5.67 ± 0.05) were endogenously phosphorylated. RLC-extracted (TnC-reconstituted) fibers (■) had pCa\(_{50}\) = 5.47 ± 0.04, n = 6. Fibers reconstituted with P-RLC (□) had pCa\(_{50}\) = 5.63 ± 0.8, n = 3.

**Fig. 3.** SDS/urea-PAGE of skinned skeletal muscle fibers containing various levels of RLC phosphorylation. A: Tm (lane 1); nonphosphorylated skeletal muscle myosin (lane 2); alkali light chains of myosin (LC1 and LC3; lane 3); fully RLC-dephosphorylated fibers (lane 4); P-RLC fibers (lane 5). B: nonphosphorylated myosin (lane 1); 60% phosphorylated fibers (lane 2); RLC-depleted fibers (lane 3).
DISCUSSION

RLC of myosin are a member of the Ca$^{2+}$-binding “EF-hand” protein family like TnC, CaM, parvalbumin, or the alkali light chains of myosin (3, 21). The divalent cation binding site of RLC is located in the first α-helix-loop-α-helix motif in the NH$_2$-terminal domain of RLC. In agreement with previous studies (2), we have shown that this site in isolated RLC binds either Ca$^{2+}$ or Mg$^{2+}$. The low affinity for Ca$^{2+}$ (in the range of 10$^5$ M$^{-1}$) slightly decreased on the phosphorylation of the RLC. The $K_{ca}$ of phosphorylated RLC was ~1.4-fold lower than that of nonphosphorylated RLC. It has been shown that the affinity of RLC for both cations increases by a factor of 100 in skeletal myosin (14). Because RLC binds Ca$^{2+}$ and Mg$^{2+}$ in a competitive way, it was expected that the $K_{ca}^\prime$ determined in the presence of Mg$^{2+}$ was smaller for both P-RLC and nonphosphorylated RLC. However, the extent of the Mg$^{2+}$-induced change in the Ca$^{2+}$ affinity of isolated RLC was not large (2- to 4.7-fold). This suggests that the RLC specificity for Ca$^{2+}$ may change depending on the complexity of the system (isolated state, bound to myosin, bound to myosin in muscle). Likewise, the effect of RLC phosphorylation could be different in the isolated state and when bound to myosin in the muscle cell.

The structural significance of RLC in skeletal muscle contraction has been addressed in previous studies (48) and has been studied intensively by others using various extraction/reconstitution methods applied to skinned muscle fibers. The studies of Moss et al. (30), Hofmann et al. (13), Metzger and Moss (25), and Patel et al. (31) have shown that partial extraction of RLC from skeletal muscle fibers increased the rate of tension redevelopment at submaximal [Ca$^{2+}$]. Our laboratory’s studies (48) have revealed that removal of RLC decreased the rate of force development by a factor of two and that this could be restored by reincorporation of RLC in the fibers. In the present work, we have investigated the effect of RLC phosphorylation and Ca$^{2+}$ binding to RLC on the regulation of skeletal muscle contraction (46). Although regulation in vertebrate striated muscles occurs via Ca$^{2+}$ binding to the thin filament proteins, the binding of Ca$^{2+}$ to RLC and phosphorylation of RLC with Ca$^{2+}$/CaM-activated MLCK seem to play a role in these regulatory processes.

Table 2. Effect of phosphorylation of the RLC in skinned skeletal muscle fibers on rates of force development and Ca$^{2+}$ sensitivity of contraction

<table>
<thead>
<tr>
<th>Skinned Skeletal Muscle Fibers</th>
<th>$k_1$, s$^{-1}$</th>
<th>$k_2$, s$^{-1}$</th>
<th>pCa50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.5 ± 3.3†</td>
<td>5.3 ± 1.6†</td>
<td>5.58 ± 0.08‡</td>
</tr>
<tr>
<td>Incubated with Ca$^{2+}$/CaM MLCK</td>
<td>27.6 ± 3.2*</td>
<td>6.8 ± 2.6†</td>
<td>5.72 ± 0.02‡</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 7). $k_1$, Constant for rapid activation rate of contraction; $k_2$, slow component constant; pCa50, −log of the Ca$^{2+}$ concentration producing half-maximal activation. * 0.05 < P < 0.1; † P > 0.1; ‡ P < 0.01.
Consistent with Metzger et al. (24) and Sweeney et al. (43), we have shown that the force-pCa relationship was shifted toward lower concentrations of Ca^{2+} as a result of RLC phosphorylation, although the effect seen by these authors was much smaller than the one observed in this study. Likewise, our laboratory's preliminary work (46, 47) and this study demonstrate that phosphorylation of the RLC not only increases the Ca^{2+} sensitivity of force development (24, 43, 44). On the basis of this information, it was proposed that RLC phosphorylation causes potentiation of isometric twitch tension by increasing the sensitivity of the contractile proteins to Ca^{2+}.

Fig. 2. Experimental protocol for the measurements of steady-state force and the kinetics of force development. The results presented in this paper have shed light on this initial observation. As shown in Fig. 2, B and C, only P-RLC was able to restore the force-pCa dependence to the level of not extracted fibers when reconstituted in RLC-depleted myosin. Compared with the myosin reconstituted with nonphosphorylated RLC, ΔpCa_{50} was ~0.25. The initial maximal level of the ATPase activity determined for untreated nonphosphorylated myosin was decreased after RLC depletion and fully recovered after RLC reconstitution. To our knowledge, this is the first report of such a significant effect of RLC phosphorylation on the Ca^{2+} sensitivity of the Tm/Tn-regulated actomyosin ATPase activity. An important factor that may influence the effect of RLC phosphorylation on force/ATPase measurements is the initial level of RLC phosphorylation without MLCK added.
phorylated RLC and allowed us to investigate the effect of RLC phosphorylation in nonextracted skinned fibers. Figure 4 demonstrates the force-pCa relationship for two types of skinned skeletal fibers (obtained with two different methods) containing either fully phosphorylated or fully dephosphorylated RLC. Both types of fibers were prepared under conditions that either activated or deactivated endogenous MLCK with no exogenous enzymes added. As expected, the fibers containing dephosphorylated RLC were less sensitive to Ca$^{2+}$ than the phosphorylated ones, by $\Delta pCa_{50} \sim 0.15 \pm 0.02$. In summary, as the level of phosphorylation of the RLC was increased, so was the Ca$^{2+}$ sensitivity of force development and vice versa. Thus, depending on the level of endogenous RLC phosphorylation, the rightward shift in Ca$^{2+}$ dependence after RLC extraction could vary. Perhaps various phosphorylation-dependent changes in the Ca$^{2+}$ sensitivity of force development presented by other laboratories resulted from different levels of initial phosphorylation of the RLC. This seems to be a crucial issue in the proper determination of the maximal effect of RLC phosphorylation on the Ca$^{2+}$ sensitivity of force development in skinned skeletal muscle fibers. As we have shown, endogenous RLC phosphorylation (in the control fibers) can vary, and this effect may attenuate the difference in pCa$_{50}$ between different fibers containing either P-RLC or nonphosphorylated RLC. Therefore, the initial level of the RLC phosphorylation should be controlled or at least known in all studies involving measurements of the Ca$^{2+}$ sensitivity of force development in skinned muscle fibers. Interestingly, consistent with previous reports (24, 45), phosphorylation of the RLC in skinned skeletal muscle fibers only slightly increased the kinetics of force activation; however, this change was of low significance ($0.05 < P < 0.1$; Table 2). We are planning more detailed future experiments to assess the effect of RLC phosphorylation on the kinetics of force development in this skeletal muscle system. The question remaining is: How does phosphorylation of the RLC modulate skeletal muscle contraction? Does the phosphorylation of the RLC directly affect the interaction of myosin and actin or is it an indirect allosteric effect of the RLC on the Ca$^{2+}$ binding to TnC in the thin filaments of skeletal muscle? Additionally, the relationship between phosphorylation of RLC and metal binding to the single Ca$^{2+}$ and Mg$^{2+}$ binding site on the RLC needs to be established. It is possible that these two important regions of RLC, the Ca$^{2+}$-binding and the phosphorylation sites, are communicating with each other and that the binding of Ca$^{2+}$ to this site is required to see the phosphorylation-dependent effects in muscle contraction. Although the RLC phosphorylation-dephosphorylation process is too slow to be an obligatory mechanism for skeletal muscle contraction, it plays a role in maintaining a specific level of force at a lower [Ca$^{2+}$] and could be important for working muscle (e.g., improving performance, etc.). The phosphorylation-dependent enhancement of muscle function through increases in actomyosin ATPase activity and tension as the free [Ca$^{2+}$] progressively rises are especially important under kinetic conditions in which the [Ca$^{2+}$] in the muscle cell does not saturate the thin filament regulatory system. At the molecular level, the phosphorylation-dependent force potentiation may simply result from the recruitment of more strongly bound cross bridges as the phosphorylation of the RLC causes cross bridges to move away from the thick filament backbone and become more accessible to actin (43).

In summary, phosphorylation of the RLC had a dramatic effect on the Ca$^{2+}$ sensitivity of the ATPase activity of reconstituted thin filaments ($\Delta pCa_{50} \sim 0.25$). Likewise, the Ca$^{2+}$ sensitivity of force development also increased on RLC phosphorylation ($\Delta pCa_{50} \sim 0.15$). In addition, maximal steady-state force of the P-RLC fibers was $\sim 15\%$ higher than fibers reconstituted with nonphosphorylated RLC. These results suggest that although the thin filament proteins Tn and Tm mediate the regulation of skeletal muscle contraction, the role of RLC in these processes cannot be ignored and need to be further explored.

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