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

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Phosphorylation of the regulatory light chains of myosin affects Ca\(^{2+}\) sensitivity of skeletal muscle contraction. J Appl Physiol 92: 1661–1670, 2002. First published December 21, 2001; 10.1152/japplphysiol.00858.2001.—The role of phosphorylation of the myosin regulatory light chains (RLC) is well established in smooth muscle contraction, but in striated (skeletal and cardiac) muscle its role is still controversial. We have studied the effects of RLC phosphorylation in reconstituted myosin and in skinned skeletal muscle fibers where Ca\(^{2+}\) sensitivity and the kinetics of steady-state force development were measured. Skeletal muscle myosin reconstituted with phosphorylated RLC produced a much higher Ca\(^{2+}\) sensitivity of thin filament-regulated ATPase activity than nonphosphorylated RLC (change in \(-\log\) of the Ca\(^{2+}\) concentration producing half-maximal activation \(\approx -0.25\)). The same was true for the Ca\(^{2+}\) sensitivity of force in skinned skeletal muscle fibers, which increased on reconstitution of the fibers with the phosphorylated RLC. In addition, we have shown that the level of endogenous RLC phosphorylation is a crucial determinant of the Ca\(^{2+}\) sensitivity of force development. Studies of the effects of RLC phosphorylation on the kinetics of force activation with the caged Ca\(^{2+}\), DM-nitrophen, showed a slight increase in the rates of force development with low statistical significance. However, an increase from 69 to 84% of the initial steady-state force was observed when nonphosphorylated RLC-reconstituted fibers were subsequently phosphorylated with exogenous myosin light chain kinase. In conclusion, our results suggest that, although Ca\(^{2+}\) binding to the troponin-tropomyosin complex is the primary regulator of skeletal muscle contraction, RLC play an important modulatory role in this process.

steady-state force; calcium regulation; regulatory light chain depletion; myosin light chain kinase

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 analogous to smooth muscle myosin, 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 kinase 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 \(\sim 15\%\) 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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tentation 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 $[\text{Ca}^{2+}]$ producing half-maximal activation ($\Delta$pCa$_{50}$) 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% NaN$_3$, and 1 mM DTT, and then it was applied to a Q-Sepharose column equilibrated with the same buffer. The RLC was eluted by using a salt gradient of 0.45 M KCl in the above buffer. Fractions containing the purified protein (96–98% purity, as determined by SDS-PAGE) were pooled and stored at $-80^\circ$C. The concentrations of the proteins were determined by using the Coomassie Plus Assay (Pierce).

**Phosphorylation of the Nonphosphorylated RLC with Ca$^{2+}$/Ca$^+$-Activated MLCK**

RLC (100–160 $\mu$M) was dialyzed against 20 mM phosphate buffer (pH 8.0), and 30 mM KCl. Phosphorylation of the protein was generated by the addition of 0.1 mM CaCl$_2$, 12 mM MgCl$_2$, 5 mM ATP, 5 $\mu$M bovine testicular CaM, and 0.5 $\mu$M MLCK. A catalytically active truncated fragment of the rabbit skeletal muscle MLCK was used in this study (12). The MLCK, missing the first 256 amino acids, was expressed in Sf9 cells infected with a recombinant virus (7). After 2 h of room temperature incubation, the level of RLC-phosphorylation was checked by 12.5% urea-SDS-PAGE. These conditions were shown to achieve 100% RLC phosphorylation. Phosphorylated RLC (P-RLC) was further purified on a Q-Sepharose column, pooled, and stored at $-80^\circ$C (as previously described).

**Reconstitution of RLC-depleted Myosin With Nonphosphorylated and/or Phosphorylated RLC**

RLC-depleted myosin was dialyzed into a buffer containing 0.4 M KCl, 50 mM MOPS, pH 7.0, 2 mM MgCl$_2$, and 10 mM DT, whereas RLC and P-RLC were dialyzed against the same buffer with the exception of the KCl being 0.1 M. Protein concentrations after 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 DT. To remove any excess RLC after dialysis, the reconstituted myosins were precipitated by the addition of 13 vol of cold H$_2$O 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/RIC 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 MgCl$_2$. Measurements were performed by using SLM Spectrofluorometer model 8100 (SLM Instruments). The proteins (5 $\mu$M) 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 a Coldovick and Womack (4) with modifications. Briefly, the upper chamber of the apparatus containing the protein and the labeled substrate ($^{45}$Ca$^{2+}$) 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 $^{45}$Ca$^{2+}$ 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 \(^{45}\text{Ca}^{2+}\) used in the experiment was 12–16 mCi/mg (from NEN Life Science Products), and 2 µCi of \(^{45}\text{Ca}^{2+}\) 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 concentration of the protein, \(\eta\) is the total number of \(\text{Ca}^{2+}\) binding sites, and \(K_{ca}\) is the \(\text{Ca}^{2+}\) binding constant.

Actin-Activated ATPase Assays

Rabbit skeletal myosin was obtained as described earlier (26, 41). F-actin, Tm, and Tn were isolated and purified from rabbit skeletal muscle according to Stryer (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, Tm, and Tn 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 Tm-0.6 µM Tn in a solution containing 20 mM MOPS (pH 7.0), 35 mM KCl, 2 mM EGTA, 2.5 mM MgCl\(_2\), and increasing [\(\text{Ca}^{2+}\)] 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 [\(\text{Ca}^{2+}\)], 1 mM Mg\(^{2+}\), 7 mM EGTA, 5 mM MgATP\(^{2-}\), 20 mM imidazole (pH 7.0), 20 mM creatinine phosphate, and 15 units/ml of creatine phosphokinase (ionic strength = 150 mM) (5). To judge the quality of the fibers, fibers were contracted (in the pCa 8 solution) and incubated for 15 min at room temperature (22°C), rinsed in the pCa solution, and tested for \(\text{Ca}^{2+}\)-dependent force development. RLC-depleted (TnC-reconstituted) fibers were then incubated with 30 µM 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 \(\text{Ca}^{2+}\)/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 \(\text{Ca}^{2+}\)/CaM-activated MLCK (30 min at room temperature). \(\text{Ca}^{2+}\) dependence of force development was measured in solutions of increasing \([\text{Ca}^{2+}]\) (from pCa 8 to 4). The data were fitted to the following equation

\[
Y_H = 100 \times \frac{[\text{Ca}^{2+}]^n}{([\text{Ca}^{2+}]^n + [\text{Ca}^{2+}]_0^n)}
\]

where \(Y_H\) is the relative force change expressed as a percentage of the maximal change, \([\text{Ca}^{2+}]_0\) is the \([\text{Ca}^{2+}]\) 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 \mu m. 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, 1.0 CaCl₂, 100 TES, 1.2 MgCl₂, 1.4 ATP, 10 glutathione, 29.4 1,6-hexamethylenediamine-2-aminooethane sulfonic acid, and 20 creatine monohydrate. The Ca²⁺ fluorescence of the single Trp residue of the RLC was monitored. The Ca²⁺ fluorescence of the single Trp residue of the RLC was fluorescence method. Phosphorylation of the RLC with Ca²⁺/CaM-activated MLCK only slightly decreased its Ca²⁺ affinity (K_Ca = 1.83 \times 10^6 M⁻¹). In the presence of 2 mM Mg²⁺, the apparent equilibrium constant of Ca (K_Ca) decreased to 1.28 \times 10^5 M⁻¹ and 3.89 \times 10^4 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 0.50 \times 10^6 M⁻¹ for nonphosphorylated RLC and and −1.04 \times 10^5 M⁻¹ for P-RLC in the absence of Mg²⁺. K_Ca was the same as K_Ca for nonphosphorylated RLC and −5.19 \times 10^4 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-

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 \times 10^5 M⁻¹ (reported 2.5 \times 10^5 M⁻¹), as
phosphorylated RLC. Ca$^{2+}$ sensitivity of actin-activated ATPase activity of myosin depleted of the decreased RLC by a $\Delta$Pc50 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 pC50 value ($pC50 = -6.55 \pm 0.02, n = 3, P > 0.1$); however, P-RLC-reconstituted myosin dramatically increased Ca$^{2+}$ sensitivity of the actin-Tm-Tn ATPase activity. The difference expressed in the pC50 units between P-RLC-reconstituted and RLC-depleted myosin was a $\Delta$Pc50 of $-0.29 (n = 3, P < 0.01)$, and between P-RLC and the myosin reconstituted with nonphosphorylated RLC was a $\Delta$Pc50 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 $\sim 20–30$% 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 $\Delta$pC50 $\sim 0.14$, was observed. No shift ($\Delta$pC50 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 pC50 values (5.58 $\pm 0.08$). Figure 2B summarizes the effect of phosphorylation on the force-pC50 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-pC50 relationship, as shown previously (48). When nonphosphorylated RLC was reincorporated into the fibers, there was no further change in the force-pC0 dependence; however, when the reconstituted fibers were exposed to Ca$^{2+}$/CaM-activated MLCK, the force-pC0 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 skinning procedure with endogenous MLCK. The pC50 = 5.67 of these control fibers was then different from these from Fig. 2, A and B (pC50 = 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-pC0 relationship. SDS-urea gel electrophoresis was used to measure the level of RLC phosphorylation in the control skinned skeletal muscle fibers...
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 (lane 1).
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 \(K'_{Ca}\) 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.

**DISCUSSION**

Interestingly, studies of the effect of RLC phosphorylation on the kinetics of force activation with the caged Ca\(^{2+}\), DM-nitrophen, showed no significant change in the rates of force development (Table 2). The protocol for these experiments (Ca\(^{2+}\) dependence of steady-state force and the kinetics of force activation on phosphorylation of the RLC) is presented in Fig. 5. It demonstrates a typical flash photolysis experiment that was reproduced several times (\(n = 7\); Table 2), with slight variations between experiments. As shown, the transient force was lower after the second UV exposure. This is probably due to either fiber rundown or to the incomplete equilibration of the caged chelator before the first flash. After the first flash, the subsequent flashes yielded the same force transient amplitude. Table 2 summarizes the activation constants \((k_1\) and \(k_2\)) calculated by using a two-exponential fit analysis of the experimental data. The \(k_1\) constant expresses a rapid activation rate of contraction, whereas the \(k_2\) constant is a slow component, possibly due to diffusion processes related to reequilibration of the fiber with the bulk solution after the flash. Even though there was a significant change in the force-pCa relationship with RLC phosphorylation \((P < 0.01)\), only a slight phosphorylation-dependent increase of low statistical significance \((0.05 < P < 0.1)\) in the rates of force activation was observed (Table 2).

**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>pCa(_{50})</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>24.5 ± 3.3(\dagger)</td>
<td>5.3 ± 1.6(\dagger)</td>
<td>5.58 ± 0.08(\ddagger)</td>
</tr>
<tr>
<td>Incubated with Ca(^{2+})/CaM MLCK</td>
<td>27.6 ± 3.2(\dagger)</td>
<td>6.8 ± 2.6(\dagger)</td>
<td>5.72 ± 0.02(\ddagger)</td>
</tr>
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Values are means ± SD \((n = 7)\). \(k_1\), Constant for rapid activation rate of contraction; \(k_2\), slow component constant; pCa\(_{50}\), -log of the Ca\(^{2+}\) concentration producing half-maximal activation. \(\dagger\) 0.05 < \(P < 0.1\); \(\dagger\) \(P > 0.1\); \(\ddagger\) \(P < 0.01\).
Fig. 5. Experimental protocol for the measurements of steady-state force and the kinetics of force activation. Force-pCa relationship and the rates of force transient were determined in skinned skeletal fibers before and after treatment with Ca\(^{2+}\)/CaM-activated MLCK. Data are presented in Tables 1 and 2. Steady-state force measurements at different [Ca\(^{2+}\)] \((\text{force-pCa dependence})\) were performed with a minute time scale. A force plateau at a given [Ca\(^{2+}\)] was achieved in ~2–3 min. Time scale of the flash photolysis experiment is indicated. RT, room temperature; UV, ultraviolet.

(Ref. 43 and references within). Numerous in vivo studies, which have utilized intact skeletal muscles, have demonstrated that the level of myosin phosphorylation significantly increases after tetanic stimulation (15, 19, 27) or a low-frequency repetitive stimulus train (staircase potentiation) (43). Studies on skinned skeletal muscle fibers have shown that phosphorylation of RLC slightly increased the Ca\(^{2+}\) sensitivity of isometric tension and the rate 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\(^{2+}\).

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 (\(\Delta\text{pCa}_{50} \sim 0.15\)) but also raises the maximal steady-state force (Table 1). Godt and Nosek (8) also reported phosphorylation-dependent increases in maximal force in frog muscle fibers under fatigue conditions. It is worth mentioning that the RLC phosphorylation-dependent changes in the contractility of skinned skeletal muscle fibers assessed in this study were much larger than had been reported by others. The effect of RLC phosphorylation was even more pronounced in the ATPase activity assays performed on reconstituted thin filaments with the use of the RLC (with or without phosphorylation)-reconstituted myosin (Fig. 1). P-RLC induced a large shift toward lower [Ca\(^{2+}\)] in the actin-Tm-Tn-activated myosin ATPase activity. Compared with the myosin reconstituted with nonphosphorylated RLC, \(\Delta\text{pCa}_{50}\) 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. Our skinned fiber results suggest that the endogenous level of RLC phosphorylation is crucial for determining the level of the Ca\(^{2+}\) sensitivity of force and important for the proper evaluation of the effects of the RLC extraction/reconstitution on the Ca\(^{2+}\)-sensitivity of the force. As demonstrated in our laboratory’s previous study (48), depletion of the RLC from skinned fibers resulted in a decrease in the Ca\(^{2+}\) sensitivity of force development. Our laboratory also demonstrated that reconstitution of RLC-depleted fibers with nonphosphorylated RLC did not restore the Ca\(^{2+}\) sensitivity 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 fibers. We also found that the protocol for the preparation of skinned fibers utilized previously (48) resulted in various levels of RLC phosphorylation (usually >50%), whereas the protein used for reconstitution was always nonphosphorylated. The protocol for the preparation of fully dephosphorylated skinned fibers used in this study (see MATERIALS AND METHODS) resulted in dephos-
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 $\Delta pC_{a_{50}} \sim 0.15 \pm 0.02$. 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 ($\Delta pC_{a_{50}} \sim 0.25$). Likewise, the Ca$^{2+}$ sensitivity of force development also increased on RLC phosphorylation ($\Delta pC_{a_{50}} \sim 0.15$). In addition, maximal steady-state force of the P-RLC fibers was 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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