The Regulatory Light Chains of Myosin Modulate Cross-bridge Cycling in Skeletal Muscle*

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We investigated the kinetics of Ca$^{2+}$-activation of skeletal muscle contraction elicited by the photolysis of caged Ca$^{2+}$. Previously we showed that partial extraction of the 18-kDa regulatory light chains (RLCs) of myosin decreased the rate of force development and was subsequently increased by ~20% following reconstitution with RLCs (Potter, J. D., Zhao, J. and Pan, B. S. (1992) FASEB J. 6, A1240). We extend here the RLC-extraction study to the complete removal of the RLCs. The complete removal of RLCs was achieved by a combination of 5,5'-dithiobis-(2-nitrobenzoic acid) and EDTA treatment followed by reduction of oxidized sulphydryl groups by dithiothreitol. Under these conditions the complete extraction of RLCs was accompanied by the extraction of endogenous troponin C, resulting in the loss of isometric tension. Steady state force was restored to 65-75% following troponin C reconstitution and this was increased to 75-85% as a result of RLC reorganization into the fibers. The rates of force transients generated by UV-flash photolysis of 1-(2-nitro-4,5-dimethoxyphenyl)-N,N,N',N'-tetraakis[(oxycarbonylmethyl)-1,2-ethanediamine] or nitrophenyl-EGTA, photoliberating bound Ca$^{2+}$, decreased 2-fold after RLC extraction and troponin C reconstitution and then increased to the values of intact fibers after RLC reconstitution. These results support our earlier findings that the regulatory light chains of myosin play an important role in the kinetics of cross-bridge cycling.

Vertebrate striated muscle contraction is triggered by the binding of Ca$^{2+}$ to troponin C (TnC), the Ca$^{2+}$ binding subunit of troponin, which together with tropomyosin forms the regulatory system of the contractile apparatus (for review see Refs. 2 and 3). Contraction of molluscan and vertebrate smooth muscles is regulated via myosin, which either binds Ca$^{2+}$-calmodulin (molluscan) (4-6) or undergoes a Ca$^{2+}$-bound Ca$^{2+}$-specific site, and this mutant was unable to regulate contraction of scallop muscle whose endogenous myosin RLC was replaced by this mutated one (19). In contrast to molluscan or smooth muscle myosin RLCs where their roles are quite well defined, their functional significance in skeletal muscle contraction is still not entirely clear. It has been shown that myosin RLCs may affect force development in skeletal muscle fibers (20, 21) or affect the conversion of chemical energy into movement in motility assays (22). It has been also shown that the cation binding site of RLC may have a modulatory role in skeletal muscle contraction (23).

The question of the physiological importance of the RLCs in skeletal muscle contraction is addressed in the present study. To test if RLCs affect cross-bridge cycling in force producing skeletal muscle fibers, we measured the Ca$^{2+}$-dependent activation elicited by the photolysis of caged Ca$^{2+}$ in the RLC-depleted fibers in comparison to RLC-reconstituted fibers. Our preliminary study (1) in agreement with the study of Hofmann et al. (20), have shown that partial extraction of RLCs from the fibers changes the rate of force development. In this study we demonstrate that the complete removal of RLCs from the fibers decreases the rate of force production by about 2-fold and that

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1. The abbreviations used are: TnC, troponin C; DM-nitrophen, 1-(2-nitro-4, 5-dimethoxyphenyl)-N,N,N',N'-tetraakis[(oxycarbonylmethyl)-1,2-ethanediamine]; DTNB, 5,5'-dithiobis(nitrobenzoic acid); RLC, regulatory light chain; ELC, essential light chain; NP, nitrophenyl; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]aminoethanesulfonic acid; HTDA, 1,6-diaminohexane-N,N,N',N'-tetraacetic acid.
reconstitution with rabbit skeletal myosin RLCs restored the rate of force development to the value of intact unextracted fibers.

MATERIALS AND METHODS

Preparations—Rabbit skeletal myosin was obtained as described in Stepkowski et al. (24). RLCs of myosin were isolated and purified according to Wagner (25). TnC was obtained as described earlier (26).

Skeletal muscle fibers isolated from rabbit psoas muscle were dissected into small bundles and chemically skinned with 50% glycerol, 1% Triton X-100 in the relaxing solution (the pCa 8 solution) containing 10−4 M Ca2+, 1 mM Mg2+, 7 mM EGTA, 5 mM MgATP2−, 20 mM imidazole, pH 7.0, 20 mM creatine phosphate, and 15 units/ml of creatine phosphokinase. Ionic strength = 150 mM, for 24 h at 4 °C. Fibers were then stored at −20 °C in the same solution without Triton X-100 for 2–4 weeks.

Extraction of Endogenous RLCs from the Skeletal Muscle Fibers—Extraction of myosin RLCs was initiated by incubation of the fibers in the pCa 8 solution containing 1% Triton X-100 for 10 min at room temperature, and then in the pCa 8 solution, pH 7.5, containing 10 mM DTNB for 5 min at 4 °C. The extraction of RLCs was completed in a solution containing 20 mM KCl, 10 mM EDTA, 10 mM imidazole, pH 7.0 for 15 min at room temperature and then in the same solution containing 2 mM DTT (for 30 min) followed by 20 mM DTT (for 45 min). The increased concentration of DTT was used to restore blocked sulfhydryl groups of cysteine residues on the muscle proteins. Under these conditions, total extraction of RLC was accompanied by the parallel extraction of endogenous TnC from the fibers.

In previous experiments (1) the RLCs were extracted from the fibers by incubation in the solution of 2 mM EDTA, pH 7.8. This procedure resulted in only partial (20–40%) extraction of RLCs from the fibers.

Reconstitution of the Fibers with TnC and RLCs—Reconstitution of TnC- and RLC-depleted fibers with TnC was performed by incubation in the pCa 8 solution containing 20 mM TnC for 30 min at room temperature. The level of reconstitution was tested by the steady state force measurements (see below) performed in the pCa 4 solution (the composition of this solution was the same as for the pCa 8 solution, except Ca2+ = 10−6 M).

Reconstitution of the TnC-replenished fibers with isolated rabbit skeletal RLCs was achieved by incubation of the fibers in a solution of 30 mM RLC (in pCa 8 buffer) for 1 h at room temperature. In the control experiments the TnC was present in the RLC’s reconstitution solution to prevent the possible extraction of TnC from the fibers during the RLC incorporation.

SDS-Gel Electrophoresis—Skeletal muscle fibers: control, extracted, and reconstituted with RLC were tested on 12% SDS-polyacrylamide gel electrophoresis according to Laemmli (27) with modifications described below. A cross-linking ratio of acrylamide to bis-acrylamide in the electrophoresis was performed on the 12% acrylamide gels with a cross-linking ratio of acrylamide to N,N’-methylene bis-acrylamide = 61:1. The pH of the resolving gel was 9.3. The fibers presented in this figure were the same as those used for the force measurements described in Fig. 3. A, actin; Tm, tropomyosin; TnT and TnC, troponin T and C; L1C1 and L1C3, essential light chains of myosin; RLC, regulatory light chain of myosin.

Produced and is the Hill coefficient.

Measurements of the Rate of Force Activation—For kinetics measurements the fibers were attached by tweezer clips to a force transducer. A square cuvette with an inner diameter of 1 mm was then positioned around the fibers such that the fibers could be bathed in different solutions without any movement. As in the steady state force experiments, the fibers were initially tested for force development in the pCa 4 solution and then relaxed in the pCa 8 solution. Then the cuvette was filled with the "caged Ca2+—solutions" containing either: 1) 2.5 mM 1-(2-nitro-4,5-dimethoxyphenyl)-N,N’-tetrazolyl(oxy carbonyl)benzyl)methyl]-1,2-ethanediamine (DM-nitrophen), 1.002 mM CaCl2, 100 mM TES, 1.2 mM MgCl2, 1.4 mM ATP, 10 mM glutathione, 29.4 mM HDTA, and 20 mM creatine phosphate, pH 7.1 (29); or 2) 2.1 mM NP-EGTA (nitrophospheryl-EGTA), 1.9 mM CaCl2, 1 mM Mg2+, 5 mM MgATP2−, 15 mM creatine phosphate, 20 mM imidazole, pH 7.0, ionic strength = 150 mM adjusted with KPr (30). Subsequent to irradiation by a 1-ms UV-light pulse from an xenon lamp (model XFL-35S-30171), both chelators were cleaved releasing free Ca2+. Their high affinity for Ca2+ before photolysis, Kp = 8.0 × 108 M−1 for NP-EGTA and Kp = 5.0 × 10−6 M for DM-nitrophen decreased to Kp = 10−3 M and Kp = 3.0 × 10−3 M, respectively following the UV-flash. As a result of the rapid Ca2+ release, the fibers developed isometric tension, characterized by a two exponential time course. The rate constants of activation for intact fibers, RLC-depleted (TnC-reconstituted) and RLC-reconstituted fibers, determined with both chelators, were calculated according to the equation: y = A (1 − e−k1t) + B (1 − e−k2t) + C, where k1 and k2 are the rate constants and A and B are the amplitudes of the two components of the force transient.

RESULTS

Extraction of Endogenous RLCs from Skinned Skeletal Muscle Fibers—Incubation of skinned skeletal muscle fibers for a short time (5 min at 4 °C) in the 10 mM DTNB solution, followed by the extraction of endogenous RLCs in the solution containing 10 mM EDTA and 2–30 mM DTT (see "Materials and Methods" for details) resulted in 90 ± 5% extraction of RLCs from the fibers (Fig. 1B). This method allows for the efficient extraction of the regulatory light chains of myosin without heating the fibers above room temperature, as employed in other techniques (21).

The reversible effect of the DTNB treatment on the fibers was tested using steady state force measurements (Fig. 2). Incubation of the fibers with the pCa 8 solution containing 10 mM DTNB (pH 7.5) under nonextracting conditions (no EDTA) resulted in the complete loss of isometric tension. About 90% of force was recovered by the subsequent exposure of the fibers to the solutions containing 2–30 mM DTT, as shown in Fig. 2. This reversible effect of DTNB with DTT treatment was also tested in the kinetic experiments where no difference in the time
course of force activation was seen after this treatment, as determined by the photoliberation of Ca\(^{2+}\) by DM-nitrophen (data not shown).

Restoration of Steady State Isometric Force following RLCs Extraction and Reconstitution into the Fibers—The RLC-depleted fibers (Fig. 1B) were fully reconstituted with myosin RLC following a 1-h incubation with a solution of 30 \(\mu\)M RLC in the pCa 8 buffer (Fig. 1C), as compared to intact fibers (Fig. 1A). Fig. 3 shows the protocol of steady state force measurements for intact fibers, following the extraction of endogenous RLCs (after TnC reconstitution) and after the RLCs reconstitution in the fibers.

As shown, extraction of the RLCs from the fibers was paralleled by the dissociation of TnC what subsequently resulted in the loss of force (data of 15–20 experiments). After readdition of TnC back to the fibers force was restored to 65–75% and increased to 80 ± 5% following full reconstitution of the fibers with TnC and RLCs.

The Effect of RLC Extraction and Reconstitution on the Ca\(^{2+}\) Dependence of Force Development—As shown in Fig. 4, the RLC-depleted fibers demonstrate a decreased Ca\(^{2+}\) sensitivity of force development. The force-pCa relationship shifted toward higher Ca\(^{2+}\) concentrations following TnC reconstitution of the RLC-depleted fibers. The pCa\(_{50}\) changed from 5.39 ± 0.03 for the untreated fibers to 4.80 ± 0.06 as a result of RLC extraction (TnC reconstituted). Interestingly, full reconstitution of the fibers with the RLCs only partially reversed this effect. The Ca\(^{2+}\) dependence of force development of TnC- and RLCs-reconstituted fibers had a pCa\(_{50}\) = 4.93 ± 0.04. In control experiments the force-pCa relationship was determined on fibers treated with DTNB and then DTT under nonextracting conditions. No shift in the Ca\(^{2+}\) dependence of force development was observed as compared to intact fibers (data not shown), indicating that this treatment did not change the Ca\(^{2+}\) sensitivity at force development.

The Rate of Force Activation—Table I and Fig. 5 illustrate the rates of Ca\(^{2+}\)-activated force development following photolysis of the caged Ca\(^{2+}\)-chelators and was obtained by fitting the experimental data (Fig. 5) to a biexponential function (see “Materials and Methods”). The faster rate constant (\(k_1\)) is directly related to the rate of muscle activation and varied depending on the presence of the RLCs within the muscle fibers. Extraction of the RLCs from the fibers resulted in a decrease of \(k_1\) by factor of 2.4 and 1.7 measured, respectively, with NP-EGTA and DM-nitrophen. Reconstitution of the fibers with myosin RLCs restored the fast rate of muscle activation to 80% of the values of intact nonextracted fibers. The slower rate constant (\(k_2\)) which had low amplitudes compared to \(k_1\), changed slightly, but their physiological significance is unknown and possibly diffusion related. Fig. 5 represents experimental curves of the force activation transient measured with DM-nitrophen (left panel) and NP-EGTA (right panel). The measurements were performed on the control fibers (A), RLC-depleted (TnC-reconstituted) fibers (B), and RLC- and TnC-reconstituted fibers (C). As shown, the transient force of RLC-depleted (TnC-reconstituted fibers) was about 10% of the transient force of nonextracted fibers and did not return to the initial values upon full reconstitution with RLCs and TnC. This observed decrease in the magnitude of the force transient is related to the decrease in the Ca\(^{2+}\) sensitivity of force development of the RLC-extracted (TnC-reconstituted) and the RLC reconstituted fibers (Fig. 4). Since the [Ca\(^{2+}\)] transient generated by the photolysis of the caged chelator is essentially the same in all of the experiments, it follows that the force attained, in response to a given [Ca\(^{2+}\)] transient, will be less in a fiber that has a lower Ca\(^{2+}\) sensitivity compared to control fibers with a higher Ca\(^{2+}\) sensitivity.
The rates of activation of RLC-depleted and RLC-reconstituted skinned skeletal muscle fibers measured with caged Ca\(^{2+}\) chelators, NP-EGTA, and DM-nitrophen

| Ca\(^{2+}\) caged chelator | Intact | RLC-depleted\(^a\) | RLC-reconstituted\(^b\) |
|---------------------------|--------|-------------------|-----------------------|
|                           | \(k_1\) (s\(^{-1}\)) | \(k_2\) (s\(^{-1}\)) | \(k_1\) (s\(^{-1}\)) | \(k_2\) (s\(^{-1}\)) | \(k_1\) (s\(^{-1}\)) | \(k_2\) (s\(^{-1}\)) |
| NP-EGTA                   | 32.9 ± 0.2 | 5.87 ± 0.02 | 13.9 ± 0.7 | 1.80 ± 0.11 | 26.5 ± 0.8 | 3.49 ± 0.1 |
| DM-nitrophen              | 26.6 ± 0.3 | 4.96 ± 0.04 | 16.0 ± 0.7 | 3.03 ± 0.08 | 21.4 ± 0.7 | 3.83 ± 0.1 |

\(^a\) RLC depleted (TnC-reconstituted).
\(^b\) RLC and TnC-reconstituted.

**DISCUSSION**

We have shown here that the regulatory light chains of myosin significantly affect the rate of activation of skeletal muscle fibers. The regulation of muscle contraction in vertebrate striated muscles occurs via Ca\(^{2+}\) binding to the thin filament regulatory protein TnC (2). The results presented here show additionally that RLCs play an important modulatory role in skeletal muscle contraction. The obvious question that arises from this is what is the mechanism of this RLC-induced modulation of skeletal muscle contraction? In the present study we investigated the structural role of the RLCs in skeletal muscle activation, utilizing skinned skeletal muscle fibers where steady state force development and the kinetics of muscle activation were readily determined. The method we have developed allows for the efficient extraction of RLCs from these skinned fibers and their subsequent reconstitution with isolated RLCs. This method resulted in the complete removal of RLCs (Fig. 1) at room temperature, conditions which did not require heating of the fibers to 30–37 °C as in previous studies (21, 31). Extraction of RLCs did not influence the maximal steady state force (Fig. 3) but significantly affected the rate constants of the force activation transient (Table I). The kinetics of muscle activation monitored with two different Ca\(^{2+}\) chelators (NP-EGTA and DM-nitrophen) showed the same decrease in the rate of force development in RLC-depleted fibers compared to nonextracted fibers. Further reconstitution of the fibers with RLCs restored the rate of force development to about 80% of the values of intact fibers. The decreased rates in the RLC-extracted (TnC-reconstituted) fibers were accompanied by a decrease in the Ca\(^{2+}\) sensitivity of steady state force development where the force-pCa relationship shifted toward higher Ca\(^{2+}\) concentrations, (\(\Delta p\text{Ca}_{50} = -0.59\)). Although the RLC-reconstituted fibers had essentially the same rates of activation as the intact fibers, the Ca\(^{2+}\) dependence of steady state force development did not return to that of the nonextracted fibers. This observed decrease in the Ca\(^{2+}\) sensitivity of force development affected the magnitude of the Ca\(^{2+}\) activated force transient (Fig. 5). Due to this rightward shift in the force-pCa relationship with higher Ca\(^{2+}\) concentrations, the amplitude of the force transients of the treated fibers dropped to ~10% of the value of untreated fibers exposed to the same [Ca\(^{2+}\)] transient. To exclude the possible effect of irreversible oxidation of sulphydryl groups on the force-pCa relationship, the Ca\(^{2+}\) dependence of force development was measured after a combined DTNB/DTT treatment under nonextracting conditions (absence of EDTA). No difference between untreated and DTNB/DTT-treated fibers was observed (data not shown). Thus the rightward shift toward higher [Ca\(^{2+}\)] observed for RLC-depleted (TnC-reconstituted) fibers is due to extraction of the RLCs from the fibers and is not due to the reversible modification of sulphydryl groups. This result of decreased Ca\(^{2+}\) sensitivity of force development is not consistent with the findings of Metzger and Moss (21), who utilized a different extraction procedure that removed 60% of the RLCs. In their experiments, they observed a leftward shift of the tension-pCa relationship to lower Ca\(^{2+}\) concentrations. Further studies will be necessary to explain these differences. Also, further experiments will be performed to determine why the force-pCa relationship does not return to the initial values upon full reconstitution of the fibers with RLCs and TnC.

The present work extends our previous findings that even partial extraction of RLCs from the fibers changes the kinetics of force activation (1). Previously, 35 ± 5% extraction of RLCs from the fibers was achieved with a solution containing 2 mM EDTA, pH 7.8, as compared to the 90 ± 5% extraction obtained in the present study. Moreover, in this work we employed the specific Ca\(^{2+}\) chelator, NP-EGTA which binds Mg\(^{2+}\) with a much lower affinity (K\(_d\) = 9 × 10\(^{-3}\) M) than DM-nitrophen (K\(_d\) = 2.5 × 10\(^{-16}\) M) (30) used in the earlier study. The reason for this was to test whether the observed differences in the rates of force development were exclusively due to the extraction of RLCs from the fibers and not to the changes in [Mg\(^{2+}\)] that, although minimized, occur with DM-nitrophen solution. The results demonstrate that both caged compounds gave similar
force transients. Thus, the different rates of activation seen for the native fibers, RLC-depleted (TnC-reconstituted) and RLC-reconstituted fibers are clearly related to the presence or absence of the myosin regulatory light chains.

In accord with our results, Lowey et al. (22), demonstrated that removal of the RLCs from skeletal muscle reduced the velocity of single actin filaments migrating on a myosin coated surface in an in vitro motility assay. The velocity of the actin movement was restored when myosin was reconstituted with RLCs. The effect was even larger when myosin was extracted and then reconstituted with both RLC and ELCs. Interestingly, the actin-activated ATPase activity of the light chain-depleted myosin did not follow the changes in the sliding velocity of single actin filaments. Based on these interesting results the authors concluded that even though the light chains were not important for the enzymatic activity of skeletal myosin, their removal decreased the maximal steady state isometric force development, they are important determinants of the kinetics of cross-bridge cycling in skeletal muscle fibers.

The location of the RLCs within the myosin head implies a possible significance of RLCs for the interaction of cross-bridges with actin during contraction (11–13). The crystal structure of skeletal myosin S1 (11) shows that RLCs are wrapped around a 10-nm long single α-helix of the heavy chain near the COOH-terminal region of the myosin head. It has been hypothesized that this part of the heavy chain acts like a lever arm of the working cross-bridge (34). Regarding the structural organization of the COOH-terminal region of the myosin head, it is possible that the RLCs located in this region may affect force-generating cross-bridges. However, the slower rate of the Ca\(^{2+}\)-dependent activation of skeletal muscle fibers after RLCs extraction (Table I) with little effect on maximal steady state force (Fig. 3) suggests that rather than the strength and stability of the myosin lever arms, RLCs affect their kinetics.

Further experiments will be necessary to investigate the role of the Ca\(^{2+}\)-Mg\(^{2+}\) binding site located in the NH\(_2\)-terminal region of RLC in force generation and the Ca\(^{2+}\)-dependent activation of muscle contraction. Interestingly, as was recently shown by Diffee et al. (23), an avian mutant of RLC having reduced affinity for Ca\(^{2+}\), did not restore maximal tension when exchanged with endogenous RLC in skeletal muscle fibers. We do not believe that Ca\(^{2+}\) binding to the RLCs influences the rate of force development in the photolysis, since the time course for the exchange of Mg\(^{2+}\) for Ca\(^{2+}\) would be very slow, primarily due to the slow dissociation of Mg\(^{2+}\) (17, 18, 35), compared to the time course of muscle activation that we measured. We can not make the same argument in the steady state force-Ca measurements in this case since there would be sufficient time for the exchange of Mg\(^{2+}\) for Ca\(^{2+}\) and it is possible that Ca\(^{2+}\) binding to the RLCs may affect this process. Further experiments with mutant light chains will hopefully answer this question.

It would also be interesting to know if phosphorylation of RLCs at the serine residues (Ser-14, Ser-15) affects the Ca\(^{2+}\)-dependence of force development and the rate of activation in skeletal muscle (36). Phosphorylation of RLCs plays a primary role in the Ca\(^{2+}\) regulation of smooth muscle contraction (7–9) and may somehow further modulate the activation of skeletal muscle contraction (37–39) possibly by influencing the interaction of skeletal myosin with actin (24, 40, 41). In summary, although the Ca\(^{2+}\)-control of skeletal muscle contraction is regulated by the thin filament proteins, troponin and tropomyosin, the regulatory light chains appear to modulate this process once activated.

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