Myosin Regulatory Light Chain Modulates the Ca^{2+} Dependence of the Kinetics of Tension Development in Skeletal Muscle Fibers

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ABSTRACT   To determine the role of myosin regulatory light chain (RLC) in modulating contraction in skeletal muscle, we examined the rate of tension development in bundles of skinned skeletal muscle fibers as a function of the level of Ca^{2+} activation after UV flash-induced release of Ca^{2+} from the photosensitive Ca^{2+} chelator DM-nitrophen. In control fiber bundles, the rate of tension development was highly dependent on the concentration of activator Ca^{2+} after the flash. There was a greater than twofold increase in the rate of tension development when the post-flash [Ca^{2+}] was increased from the lowest level tested (which produced a steady tension that was 42% of maximum tension) to the highest level (producing 97% of maximum tension). However, when 40–70% of endogenous myosin RLC was extracted from the fiber bundles, tension developed at the maximum rate, regardless of the post-flash concentration of Ca^{2+}. Thus, the Ca^{2+} dependence of the rate of tension development was eliminated by partial extraction of myosin RLC, an effect that was partially reversed by recombination of RLC back into the fiber bundles. The elimination of the Ca^{2+} dependence of the kinetics of tension development was specific to the extraction of RLC rather than an artifact of the co-extraction of both RLC and Troponin C, because the rate of tension development was still Ca^{2+} dependent, even when nearly 50% of endogenous Troponin C was extracted from fiber bundles fully replete with RLC. Thus, myosin RLC appears to be a key component in modulating Ca^{2+} sensitive cross-bridge transitions that limit the rate of force development after photorelease of Ca^{2+} in skeletal muscle fibers.

INTRODUCTION

Myosin is a hexamer composed of two high-molecular-weight (~200,000) heavy chains and four low-molecular-weight (~20,000) light chain subunits, which include two alkali light chains (LC$_1$ and LC$_3$) and two so-called regulatory light chains (RLC). In vertebrate smooth muscles, some nonmuscle cells, and most invertebrate muscles, one or both types of myosin light chains appear to be primary molecular components of the Ca^{2+} regulation of contraction (Goodwin et al., 1990; Kwon et al., 1990, Kamm and Stull, 1985). However, our understanding of possible roles of the myosin light chains in vertebrate striated muscle is incomplete at present. Information from the crystal structure of the S1 subfragment of skeletal muscle myosin suggests that a probable role of the light chains may be to stabilize the α-helical neck region of the myosin head such that force resulting from conformational changes near the active site is transmitted to the rod region of the molecule (Rayment et al., 1993). Earlier experiments involving selective removal of the myosin RLC from skinned skeletal muscle fibers showed that removal of up to 50% of the endogenous RLC has little effect on either maximum Ca^{2+}-activated force or stiffness, but significantly increases force and stiffness at submaximum levels of Ca^{2+} (Hofmann et al., 1990). More recent in vitro force measurements have confirmed that

removal of RLC has little effect on maximum force (Van-Buren et al., 1994). Other studies involving partial extraction of RLC from skinned skeletal fibers indicated that the RLC may be involved in conferring Ca^{2+} sensitivity on cross-bridge transitions that limit the rate of force development in steadily Ca^{2+}-activated fibers (Metzger and Moss, 1992). In fibers containing a mutant myosin RLC having a defective divalent cation binding site, both maximum tension and stiffness were significantly reduced compared to control values (Diffee et al., 1995), suggesting that myosin heads containing RLC that is unable to bind Ca^{2+} or Mg^{2+} have a reduced ability to form strongly bound cross-bridges. These results suggest that rather than playing a strictly structural role such as stabilizing the structure of the myosin head, myosin RLC may also serve a regulatory role, such as modulating the availability of cross-bridges to bind to actin.

To investigate this possibility, in the present study we have examined the Ca^{2+} dependence of the rate of tension development as a function of RLC content in skinned skeletal muscle fibers. There are several lines of evidence suggesting that Ca^{2+} modulates the kinetics of cross-bridge interaction. Recent studies using flash photolysis of photolabile Ca^{2+} chelators (i.e., caged Ca^{2+}) have shown that the rate of tension development after photorelease of Ca^{2+} is highly sensitive to the concentration of Ca^{2+} released in frog skeletal muscle (Ashley et al., 1991; Lenart et al., 1993) and in both rabbit psoas fibers and rat cardiac myocytes (Araujo and Walker, 1994). These effects of Ca^{2+} are similar to results of earlier measurements of the Ca^{2+} dependence of the rate constant of tension redevelopment (k_{tr}) after a release and restretch maneuver applied to steadily activated skeletal muscle fibers (Brenner, 1988; Brenner

and Eisenberg, 1986; Metzger and Moss, 1992) and cardiac trabeculae (Wolff et al., 1995).

To assess the role of myosin RLC in modulating the kinetics of tension development, we measured the rates of tension development induced by flash photolysis of the Ca^{2+} chelator DM-nitrophen (Kaplan and Ellis-Davies, 1988) in bundles of skinned skeletal muscle fibers both before and after partial extraction of myosin RLC. A significant degree of Ca^{2+} dependence of the rate of tension development observed initially was eliminated by partial extraction of myosin RLC.

MATERIALS AND METHODS

Solutions

Skinning and storage solutions contained 2 mM EGTA, 1 mM $MgCl_2$, 10 mM imidazole, 4 mM ATP, and 100 mM KCl. Relaxing and activating solutions contained 5 mM EGTA, 0.1 mM or 1 mM free Mg^{2+}, 100 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethane sulfonic acid, pH 7.00), 14 mM total ATP (5 mM MgATP), 15 mM creatine phosphate, 5 mM DTT (dithiothreitol), free Ca^{2+} (either 10^{-9} M (relaxing solution) or $10^{-4.5}$ M (activating solution)), and sufficient K-propionate to adjust the ionic strength to 180 mM. The computer program of Fabiato (1988) was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex based on the stability constants listed by Godt and Lindley (1982). The apparent stability constants are 2.39×10^6 M^{-1} for Ca^{2+}-EGTA and 2×10^8 M^{-1} for Ca^{2+}-DM-nitrophen at an ionic strength of 180 mM, pH 7.0, and an experimental temperature of 15°C (Kaplan and Ellis-Davies, 1988). Caged Ca^{2+} was loaded into the fibers in a solution identical to relaxing solution, except that it contained either 1.0 mM or 0.1 mM free Mg^{2+}, 1 mM DM-nitrophen in place of EGTA, and $CaCl_2$ between 0.200 mM and 0.530 mM. A pre-activating solution used before loading was similar to relaxing solution, except that it contained 0.5 mM EGTA.

Tension measurements

Bundles of ~50 fibers were dissected from the psoas muscles of adult New Zealand rabbits. The bundles were tied to glass capillary tubes, kept in skinning solution containing 1% (v/v) Triton X-100 for 3 h, and then stored at −20°C for up to 21 days in storage solution containing 50% (v/v) glycerol. On the day of an experiment, a bundle was transferred to relaxing solution and split into smaller bundles of three or four fibers each. A control segment of the fiber bundle was saved for later protein analysis, and an experimental segment 2–3 mm in length was mounted on the experimental apparatus. One end of the bundle was attached to the arm of a motor positioner (model 350; Cambridge Technology, Cambridge, MA), and the other end was attached to a force transducer (model 403; Cambridge Technology). Complete details of the mounting procedure have been described previously (Moss et al., 1983). We used bundles of three or four fibers rather than single fibers to increase the signal-to-noise ratio during tension measurements after flash photolysis. All experiments were done with the resting sarcomere length set to 2.56 μm. Before each set of photolysis experiments, the fiber bundle was transferred into maximally activating solution (pCa 4.5). After developing steady tension the bundle was slackened to allow tension to fall to a zero baseline, and the fiber bundle was transferred back to relaxing solution. The difference between the baseline tension and the steady active tension was calculated as the maximum Ca^{2+}-activated tension (P_o), and this value was used to normalize tensions developed during photolysis experiments. Steady-state tension-pCa relationships were determined in single skinned fibers by first measuring tension in pCa 4.5 solution and then in randomly selected submaximum pCa solutions, with every fourth activation made in pCa 4.5 to assess any decline in fiber performance.

For the photolysis experiments, the fiber bundle was first transferred to pre-activating solution to reduce the EGTA content of fibers and then transferred to loading solution to allow the caged Ca^{2+} to diffuse evenly into the fibers. After 5 min in loading solution, the fiber bundle was transferred to an 80-μl quartz-walled photolysis chamber filled with silicone oil (Dow Corning 200 fluid, viscosity = 10 cs), where it was exposed to a flash of UV light (~360 nm) from a xenon flash lamp (Optoelektronic, Hamburg, Germany) directed to the quartz-walled trough. The level of post-flash activation was varied by adjusting the power supply to the UV flash lamp (HiTech Scientific, Salisbury, England) to one of three different levels (150 V, 200 V, or 375 V) to vary the extent of photolysis of DM-nitrophen, or, in some experiments, the free $[Ca^{2+}]$ in the loading solution was varied and the DM-nitrophen was photolyzed with a flash of the highest intensity. After the tension response was recorded, the fiber bundle was transferred back to relaxing solution and then subjected to either different loading conditions or different flash intensities for subsequent flashes. After the flash photolysis experiments, maximum tension at pCa 4.5 was again measured to determine whether significant fiber rundown had occurred.

Protein extraction

To assess the role of RLC in the kinetics of tension development, a set of photolysis experiments was carried out before and then after partial extraction of RLC from the fiber bundle. To extract RLC, fiber bundles were warmed to 38–40°C and transferred to extracting solution containing 25 mM KCl, 10 mM EDTA, and 10 mM imidazole (pH 7.0) for 2 h, a modification of the method described by Hofmann et al. (1990). The fibers quickly developed rigor tension in this solution and were manually slackened to minimize the deleterious effects of prolonged rigor tension at elevated temperature. After the extraction period, the fiber bundle was cooled to 15°C and reextended to the original sarcomere length. Because a small amount of Troponin C (TnC) was co-extracted with RLC as a result of this procedure, the fiber bundles were bathed in relaxing solution containing 0.5 mg/ml purified TnC, and tension was measured at pCa 4.5 to assess the extent of recombination of TnC. This cycle of TnC soak and maximum activation was repeated until maximum tension no longer increased. In some fiber bundles, tension measurements in RLC- extracted fibers were followed by the readdition of RLC to the fibers by soaking the fiber bundle in relaxing solution containing 1.0 mg RLC/ml for 1–2 h at 15°C. Rabbit native skeletal RLC was purified from rabbit fast-twitch muscle according to the method of Wagner (1982). Purified TnC was prepared from rabbit fast muscle by the method of Greaser and Gergely (1971). In some experiments the explicit effects due to partial TnC extraction alone were tested. TnC was partially extracted by bathing the fiber bundle in a solution of 10 mM imidazole, 5 mM EDTA, and 0.2 mM trifluoperazine for 1–2 min at 15°C. Tension measurements at pCa 4.5 showed that after this protocol the maximum Ca^{2+}-activated tension decreased to about 50% of pre-extraction values. To assess the effects of the RLC extraction protocol by itself, irrespective of changes in the amount of RLC present, in some fiber bundles the RLC extraction protocol was performed in the presence of rabbit skeletal RLC at a concentration of 6 mg/ml to prevent the loss of RLC.

The extent of RLC extraction and re-addition was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and an ultrasensitive silver stain technique (Hofmann et al., 1990). Gels were scanned with a laser scanning densitometer (model GS-670; Bio-Rad), and peak areas were analyzed with accompanying image analysis software. The RLC content was determined from the $RLC/(LC_1 + LC_3)$ ratio, and the extent of TnC extraction/recombination was assessed from the TnC/TnI ratio.

RESULTS AND DISCUSSION

Results of photolysis experiments at 1 mM free Mg^{2+}

The rate of tension development in skinned fiber bundles was determined in an initial series of experiments using

flash photolysis of DM-nitrophen in the presence of 1.0 mM free Mg^{2+}. Fig. 1, A and B, shows original tension records from a fiber bundle after the photorelease of different amounts of Ca^{2+}. Increases in the post-flash level of Ca^{2+} were achieved by increasing the extent of Ca^{2+} loading of DM-nitrophen, which increased the free $[Ca^{2+}]$ and thus the pre-flash tension level (Fig. 1 A). At the lowest level of activation (trace c), the loading solution had a calculated pCa of 6.2, which yielded a steady-state, pre-flash tension of $\sim5\%$ of P_o. Subsequent flashes were initiated after loading in solutions of pCa of 6.1 (trace b; pre-flash tension, 15% P_o) and pCa 6.0 (trace a; pre-flash tension, 48% P_o). In all fiber bundles, at all activation levels, the time course of tension development was well fit by a single exponential equation. As the level of Ca^{2+} activation was increased there was an increase in the rate of tension development. Fig. 1 B presents the same data normalized to the peak tension at each activation to illustrate the differences in the rate of tension rise. For this fiber bundle the rate constants of tension development were 9.4 s^{-1} for record a, 8.2 s^{-1} for b, and 5.6 s^{-1} for c.

Fig. 1, C and D, shows original tension records after partial extraction of myosin RLC from the same fiber bundle as shown in Fig. 1, A and B. The flash intensity used to photolyze DM-nitrophen and the loading conditions for each activation were identical to the corresponding controls, and the tension records were also well fit by single exponentials. After extraction of $\sim70\%$ of the endogenous RLC (see inset), flash photolysis of DM-nitrophen resulted in post-flash tension levels that were comparable to control

values. However, tension developed at a rate faster than that produced by the highest level of activation in control fibers, and this rate was virtually constant, regardless of the level of activator Ca^{2+}. For Fig. 1, C and D, rate constants of tension development were 15.1 s^{-1} for a, 14.7 s^{-1} for b, and 15.4 s^{-1} for c.

Fig. 2 A summarizes the mean ($\pm$ SEM) data for the experiments at 1 mM Mg^{2+}. We found that under conditions in which the post-flash tension was less than $\sim40\%$ of P_o, the tension traces were not reproducible, because of low overall force and consequently a decreased signal-to-noise ratio, and these records were therefore discarded. However, the range of activation levels presented in Fig. 2 likely includes the range of greatest Ca^{2+} sensitivity, because it has been observed previously that the greatest dependence of tension development kinetics on Ca^{2+} in skeletal muscle fibers occurs at $[Ca^{2+}]$ above the pCa_{50} (Metzger and Moss, 1992; Araujo and Walker, 1994). In control fiber bundles there was a significant difference between the rates of tension rise at all levels of activation tested, with a more than twofold difference in rate between the lowest and highest levels of activation. After RLC extraction, this activation dependence was lost, as there was no longer a significant difference in rates among any of the activation levels.

Fig. 2 A also reveals an apparent increase in the maximum rate of tension development in 1 mM Mg^{2+} after RLC extraction. The appearance of an increase in maximum rate is probably due to the lack of tension development data at maximum tension levels (100% of P_o) in control fibers at 1

FIGURE 1 Tension traces from flash photolysis experiments conducted at 1 mM free Mg^{2+}. (A) Tension, expressed as a fraction of maximum Ca^{2+}-activated tension (P_o), developed in a fiber bundle as a result of flash photorelease (F) of Ca^{2+} after loading with differing levels of Ca^{2+} (a, b, and c). (B) The same data normalized to the maximum tension at each activation (a, b, and c as above) to illustrate differences in the rate of tension rise. (C and D) Data from the same fiber bundle after partial extraction of RLC. The amount of Ca^{2+} in the loading solution was the same for a, b, and c as above. (Inset) SDS-PAGE analysis of RLC extraction. Lane 1 is a control segment of fiber bundle 06161 obtained before the RLC extraction procedure. Lane 2 is a segment from the same fiber bundle showing extraction of 67% of endogenous RLC. In seven experiments we extracted an average of 54% ($\pm12\%$) myosin RLC.
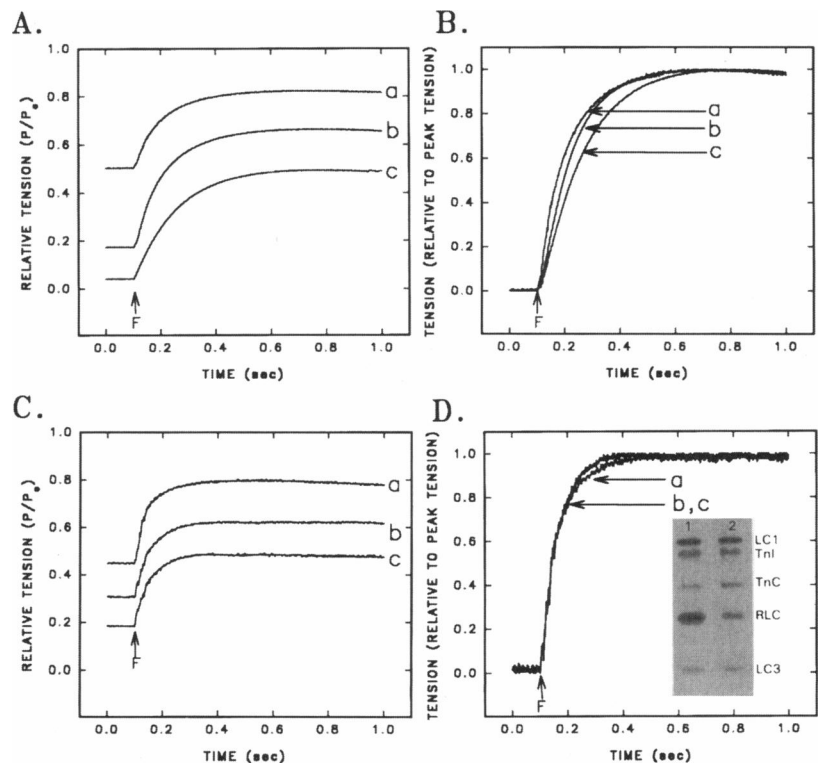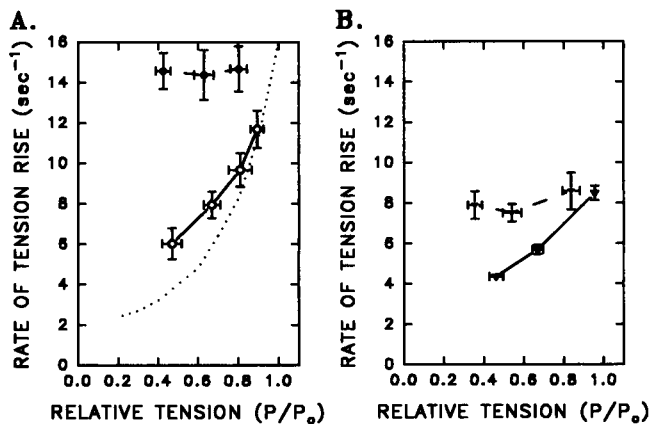
FIGURE 2 Summary of the effects of RLC extraction on the Ca^{2+} dependence of the kinetics of tension development. (A) Summary data from experiments at 1 mM free Mg^{2+}. Data are plotted as the rate of tension development at each activation versus the peak tension developed at that activation, as a fraction of P_o for that condition. Data are presented as means ± SEM for $n = 4$ experiments. Open circles are control fiber bundles, and the closed symbols are from the same bundles after partial extraction of RLC. The dotted line is unpublished data from Diffee et al., showing the dependence of k_{tr} upon the level of activation. (B) Summary data from experiments at 0.1 mM Mg^{2+}. Rates of tension development at each flash intensity are plotted versus the peak tension at that flash intensity. Data are presented as means ± SEM for $n = 6$ experiments. Open triangles are control fiber bundles, and closed triangles are from the same bundles after partial extraction of RLC.

mM Mg^{2+}. We were unable to achieve post-flash tensions of more than ~90% P_o in 1 mM Mg^{2+}, even when starting from a pre-flash tension of ~80% P_o, presumably because of the shallow slope of the tension-pCa relationship at these tension levels. It is likely that, given the steepness of the relationship between tension and rate of tension development at high levels of activation (see dotted line in Fig. 2 A, representing the activation dependence of k_{tr} in skeletal muscle fibers), the rate of tension development at P_o would approach the rates observed after RLC extraction. Thus, we conclude that RLC extraction does not result in a significant change in the maximum rate of tension development, but does eliminate the Ca^{2+} dependence of tension development rates.

Results of photolysis experiments at 0.1 mM free Mg^{2+}

In the experiments described above, partial extraction of RLC increased the level of pre-flash tension compared to controls when identical loading conditions were used. This observation is consistent with the leftward shift in the steady-state tension-pCa relationship that has been reported as a consequence of partial extraction of RLC (Hofmann et al., 1990; Metzger and Moss, 1992). Thus the increased rate of tension development after RLC extraction might be an indirect effect of the increased level of pre-flash activation rather than a direct effect of RLC extraction to modulate tension development kinetics. To resolve this issue we per-

formed a number of experiments in which the fiber bundles were initially in a relaxed state and were then activated to differing degrees by varying the extent of flash-induced release of Ca^{2+} from DM-nitrophen. To do this we reduced the free $[Mg^{2+}]$ to 0.1 mM in the experimental solutions. As a consequence of the lowered $[Mg^{2+}]$ both the Ca^{2+} sensitivity of tension and the amount of Ca^{2+} bound by DM-nitrophen increased. Thus, at 0.1 mM Mg^{2+} there were increases in both the amount of Ca^{2+} released by a given flash intensity and in the tension response to a given amount of Ca^{2+}, making it possible to study the rates of tension development between the relaxed state and a variety of activated states, including maximum activation.

Fig. 3, A and B, shows original tension records from a representative fiber bundle activated under low (0.1 mM) free Mg^{2+} conditions. In these experiments the fiber bundle was loaded with DM-nitrophen in a solution with a calculated pCa of 6.65, which yielded no measurable tension. The degree of Ca^{2+} release was varied by changing the intensity of the UV flash, which resulted in varying degrees of photolysis of DM-nitrophen. The rate of tension development showed a dependence on level of activation that was similar to that seen at 1 mM free Mg^{2+}. For the fiber bundle shown in Fig. 3, the rate constants of tension development were 8.8 s^{-1} for a, 6.0 s^{-1} for b, and 4.1 s^{-1} for c. Records in Fig. 3, C and D, show data from the same fiber bundle after partial extraction of RLC. The same three UV flash intensities were used as in the control condition. After RLC extraction rate constants in this fiber bundle were 8.9 s^{-1}, 7.6 s^{-1}, and 8.8 s^{-1} for a, b, and c, respectively. Thus, in 0.1 mM Mg^{2+}, there was no change in the maximum rate of tension rise after RLC extraction, but like the effects at 1 mM Mg^{2+}, tension developed at the maximum rate at all levels of activation.

The absolute rates of tension development under control conditions were higher in 1 mM Mg^{2+} (Fig. 2 A) than in 0.1 mM Mg^{2+} (Fig. 2 B). This difference might be explained by the existence of pre-flash tension in the 1 mM Mg^{2+} experiments compared to the relaxed pre-flash state in 0.1 mM Mg^{2+}. The partial activation of the thin filament before the flash may eliminate one or more steps that limit the rate of tension development after the flash. When tension is developed starting from the relaxed state, this step or steps would remain and may thus slow the rate of tension development, thereby explaining the decrease in overall rate when starting from a relaxed state in 0.1 mM Mg^{2+}.

Effects of RLC extraction on steady-state tension

At 0.1 mM free Mg^{2+}, partial extraction of RLC reduced peak tension at each flash intensity, which was opposite the effect observed at 1 mM Mg^{2+}. To examine the effects of altered $[Mg^{2+}]$ and RLC extraction on the Ca^{2+} sensitivity of tension, we characterized steady-state tension-pCa relationships before and after RLC extraction at both 1 mM and 0.1 mM Mg^{2+}. As shown in Fig. 4, at 1 mM Mg^{2+} we

FIGURE 3 Effect of RLC extraction on Ca^{2+} dependence of tension development kinetics at 0.1 mM free Mg^{2+}. (A) Tension, expressed as fraction of P_o, developed in a fiber bundle as a result of flash photorelease (F) of Ca^{2+} at high (a), intermediate (b), and low (c) flash intensities. (B) The same data normalized to the maximum tension at each flash intensity (a, b, and c as above) to illustrate differences in the rate of tension rise. (C and D) Data from the same fiber bundle after partial extraction of RLC. Flash intensities (a, b, and c) are as above.
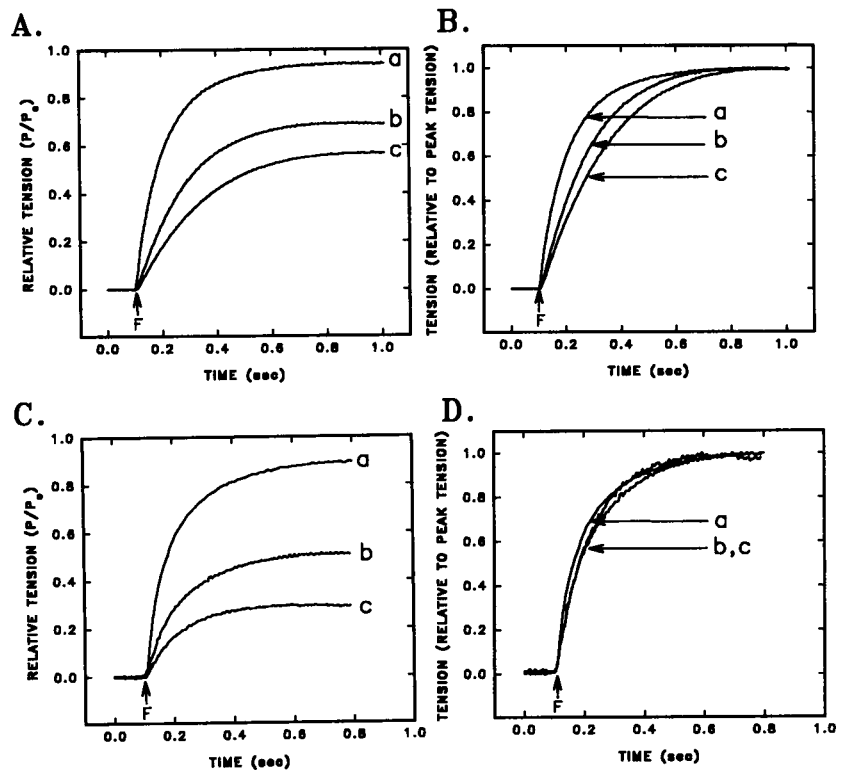
observed a left shift in the tension-pCa relationship as a result of partial extraction of RLC, which is consistent with effects reported previously (Hofmann et al., 1990; Metzger and Moss, 1992). At 0.1 mM Mg^{2+} we observed a significant leftward shift in the tension-pCa relationship in control fibers compared to 1 mM Mg^{2+}, but the effect of partial extraction RLC at low Mg^{2+} was to reduce tension at submaximum $[Ca^{2+}]$. That RLC extraction induced a right-
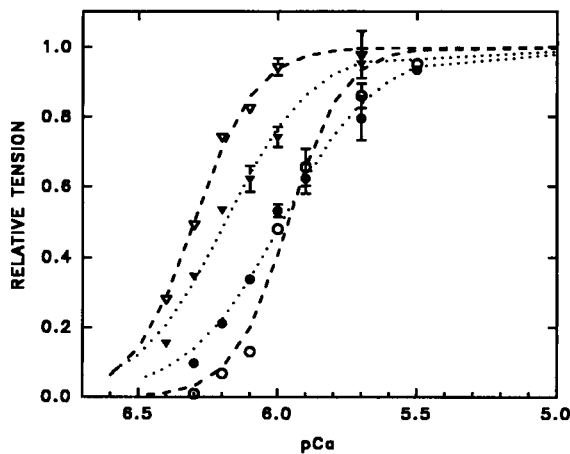

FIGURE 4 Steady-state tension-pCa relationships at 0.1 mM and 1 mM Mg^{2+}. Tension was measured as a function of pCa in 1 mM Mg^{2+} (○, ●) or in 0.1 mM Mg^{2+} (▽, ▼). Measurements were made on control fibers (○, ▽) and then on the same fibers after partial extraction of RLC (●, ▼). Using constants derived from the Hill equation, curves were drawn by computer using the equation $P/P_o = [Ca^{2+}]^n/(k^n+[Ca^{2+}]^n)$ (Hofmann et al., 1990). Data are means ± SEM of five experiments at each free $[Mg^{2+}]$.

ward shift of the tension-pCa relationship explains the decrease in post-flash tension that we observed during the photolysis experiments at 0.1 mM Mg^{2+}. The results presented in Fig. 4 suggest that the Ca^{2+} sensitivity of tension depends upon both RLC content and $[Mg^{2+}]$. It appears that the least Ca^{2+}-sensitive state results when RLC is present and occupied by Mg^{2+}, i.e., at 1.0 mM Mg^{2+}. The greatest Ca^{2+} sensitivity requires RLC to be present, but with presumably much less Mg^{2+} bound, i.e., at 0.1 mM Mg^{2+}. Intermediate Ca^{2+} sensitivity is obtained in RLC extracted fibers. These results imply that RLC with little or no Mg^{2+} bound has an activating effect on steady-state tension, because partial extraction of RLC under these conditions decreases tension at submaximum Ca^{2+}. In contrast, RLC with Mg^{2+} bound apparently has a repressive effect on tension, because RLC extraction at 1 mM Mg^{2+} increases submaximum tension. We are currently investigating the nature of the relationship between $[Mg^{2+}]$ and the effects of RLC extraction on steady-state tension. In any case, this differential effect of RLC extraction on steady-state tension at low and high Mg^{2+} is not manifested in the kinetics of tension development, because RLC extraction eliminates the Ca^{2+} dependence of the tension development rate at both 0.1 mM and 1 mM Mg^{2+}.

Some previous studies using this or similar RLC extraction protocols concluded that partial extraction of RLC had little effect on maximum tension (Hofmann et al., 1990; Metzger and Moss, 1992), but other studies have found wide variability in the effects of RLC extraction on maximum tension (Moss et al., 1982, 1983). In the present study,

we observed a decline in maximum tension at pCa 4.5 after RLC extraction to an average of 70% of pre-extraction tension, a value well within the range observed in some earlier studies (see, for example, figure 10 in Moss et al., 1983). The difference between the present results and earlier studies (Hofmann et al., 1990; Metzger and Moss, 1992) may be due to the fact that we extracted a somewhat higher percentage (up to 70%) of endogenous RLC in the present work.

Specificity of effects due to RLC extraction

Because previous studies have observed that this RLC extraction protocol also extracts some TnC (Moss et al., 1982, 1983; Hofmann et al., 1990), it was important to establish that the observed effect of RLC extraction to eliminate the Ca^{2+} dependence of the rate of tension development was specifically due to the changes in RLC content. To do this, we used SDS-PAGE to monitor the TnC content of fibers after extraction and found that addition of TnC after RLC extraction was sufficient to maintain a constant level of TnC in the fibers. As a further control we assessed the effects on kinetics due to extraction of up to ~50% of the TnC from fibers that were RLC replete. We found that the rate of tension development was still Ca^{2+} dependent after TnC extraction, consistent with the results of an earlier study (Metzger and Moss, 1991). Fig. 5 shows original tension records obtained at 0.1 mM Mg^{2+} before and after extraction of ~60% of endogenous TnC from the fiber bundle (see inset). In a number of TnC extraction experiments, the mean rate of tension rise was 4.55 ± 0.24 s^{-1} ($\pm$ SEM) at the lowest, 5.02 ± 0.29 s^{-1} at the intermediate, and 8.11 ± 0.42 s^{-1} at the highest levels of activation after TnC ex-
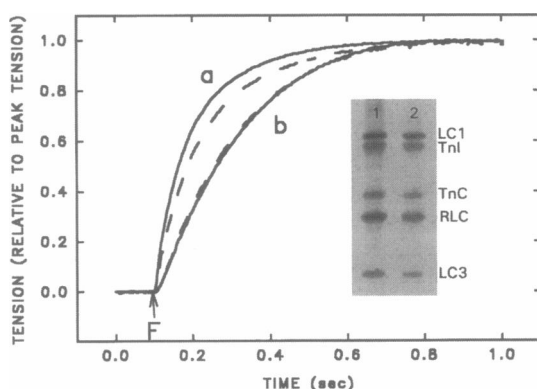

FIGURE 5 Effect of partial TnC extraction on tension development kinetics. Tension data from a representative fiber bundle before (solid lines) and after (dashed lines) TnC extraction. Tension is normalized to the peak tension achieved at either the highest (a) or the lowest (b) flash intensity. In this fiber bundle, tension development rates were 8.69 s^{-1} for curve a and 4.25 s^{-1} for curve b. After TnC extraction the rates were 8.03 s^{-1} for curve a and 4.63 s^{-1} for curve b. (Inset) SDS-PAGE analysis of TnC extraction from this fiber bundle. Lane 1 is the control segment and lane 2 is from the same fiber after extraction of ~60% of endogenous TnC. TnC/TnI ratios are 0.67 and 0.28 for lanes 1 and 2, respectively.

traction ($n = 5$). These values are similar to those observed in control fibers before extraction of TnC.

A second set of control experiments determined that the effects of RLC extraction could not be attributed to other nonspecific effects of the extraction protocol, because after simulated extractions there was no change in the rates of tension development as long as the RLC content of the fiber was maintained during the extraction procedure. Fig. 6 A shows that exposure to extraction conditions in the presence of excess RLC did not significantly affect the Ca^{2+} dependence of the rate of tension development. Furthermore, we found that the simulated extraction protocol and subsequent replacement of TnC had no effect on maximum steady-state tension or the peak tensions achieved after each flash. This result suggests that the decline in maximum tension after RLC extraction is specifically due to the loss of RLC. Finally, we observed that the effects of RLC extraction on tension development kinetics were partially reversed by the partial recombination of RLC into RLC-extracted fibers. Fig. 6 B shows an example of one such experiment in which partial reconstitution of an RLC-extracted fiber bundle partially restored the Ca^{2+} dependence of the rate of tension development. Earlier work from this laboratory (Hofmann et al., 1990; Metzger and Moss, 1992) has demonstrated that a substantial fraction of RLC is restored using this replacement protocol.

Basis for Ca^{2+} dependence of tension development kinetics

A number of earlier studies have also demonstrated that the Ca^{2+} dependence of tension development kinetics can be reduced or eliminated under certain conditions. For example, when the thin filament is activated in the absence of Ca^{2+} with either N-ethylmaleimide (NEM) modified myosin S1 (Swartz and Moss, 1992) or with a modified form of TnC (Chase et al., 1994), the Ca^{2+} dependence of k_{tr} is eliminated. In both of these cases it is likely that the experimental intervention increased thin filament activation to maximum levels, at least in terms of the kinetics of cross-bridge cycling. Thus, the elimination of the Ca^{2+} dependence of the rate of tension development by RLC extraction in the present study presumably involves an increase in the level of thin filament activation, possibly by increasing the number of strongly bound cross-bridges. The results of experiments at 1 mM Mg^{2+} are consistent with this hypothesis, because partial extraction of RLC increased both the rate of tension development and steady-state tension at submaximum Ca^{2+}. However, experiments at 0.1 mM Mg^{2+} indicate that under some conditions RLC extraction can increase the rate of tension development while also reducing steady-state tension levels and therefore the level of thin filament activation. Thus, the results of the present study indicate that the rate of tension development and the amount of steady-state tension can be differentially modulated, depending on the $[Mg^{2+}]$.
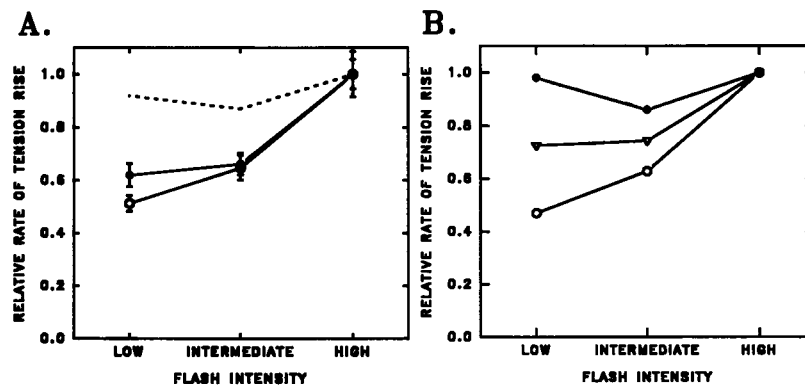
FIGURE 6   Experiments to control for nonspecific effects of RLC extraction protocol. (A) Effect of extraction protocol alone on the Ca^{2+} dependence of the rate of tension development. Fibers were subjected to the RLC extraction protocol in the presence of excess RLC to prevent extraction of RLC (see Materials and Methods). Data are means ± SEM from $n = 4$ experiments. Open circles show rates of tension development at the three flash intensities as a fraction of the rate at the highest level of activation in control fiber bundles. Closed circles are data from the same bundles after the simulated extraction protocol. The dotted line represents data from Fig. 2 B showing the effect of RLC extraction. (B) Reversibility of the effects due to RLC extraction. Data from a representative fiber bundle showing the effects of partial recombination of RLC into a RLC-depleted fiber. Open circles are data from the control fiber bundle and closed circles are data from the same fiber bundle after extraction of RLC. These data are also shown in Fig. 3. Open triangles are data from the same fiber bundle after partial readdition of RLC into the fiber bundle.

The effect of RLC extraction of eliminating the Ca^{2+} dependence of the rate of tension development is qualitatively similar to that seen in a previous study of the Ca^{2+} dependence of k_{tr} (Metzger and Moss, 1992). In that study, partial extraction of RLC had no effect on k_{tr} during maximum activation but increased k_{tr} at lower levels of Ca^{2+} activation, effectively reducing the Ca^{2+} dependence of k_{tr}. A striking difference between the earlier study on k_{tr} and the present study is the magnitude of the effect due to RLC extraction. In the earlier study, there remained a threefold difference between k_{tr} at lower levels of activation (40% of maximum) and k_{tr} at maximum activation after RLC extraction. Determination of k_{tr} involves the mechanical disruption of cross-bridges in steadily activated fibers using a release-restretch maneuver. The time course of tension redevelopment after this maneuver can then be determined with the thin filament already in an activated state. The similarity between k_{tr} and the rate of tension development after photorelease of Ca^{2+}, in terms of both absolute rates and Ca^{2+} sensitivity (Brenner, 1988; Brenner and Eisenberg, 1986; Metzger and Moss, 1992; Araujo and Walker, 1994), suggests that these two rates are controlled by similar processes. The results of the present study, however, in which a similar degree of RLC extraction (compared to Metzger and Moss, 1992) completely eliminated the Ca^{2+} dependence of the kinetics of tension development, suggest that the RLC may play a more substantial role in the processes that limit the rate of force production after photorelease of Ca^{2+} compared to those involved in tension redevelopment after mechanical detachment of cross-bridges in steadily activated fibers (k_{tr}).

Previous studies of the effects of RLC extraction on steady isometric tension and stiffness (Hofmann et al., 1990) and the kinetics of tension redevelopment (Metzger and Moss, 1992) had suggested the hypothesis that Ca^{2+}

binding to the Ca^{2+}/Mg^{2+} binding site on myosin RLC could increase both the rate of tension development and the amount of steady-state tension during submaximum activation. This hypothesis received further support from the finding that an intact divalent cation binding site on the RLC was necessary for normal tension-generating capability (Diffee et al., 1995). Whereas the results of the present study provide general support for the hypothesis that RLC represses the rate of tension development at submaximum Ca^{2+}, the available data regarding divalent cation binding to myosin RLC indicate that the process of Ca^{2+} replacement of Mg^{2+} at the Ca^{2+}/Mg^{2+} site is relatively slow (k_{off} for $Mg^{2+} = 0.057$ s^{-1}; Bagshaw and Reed, 1977; Holroyde et al., 1979). Thus it seems unlikely that significant Ca^{2+} could bind to the RLC during the time course of tension rise after flash photolysis of caged Ca^{2+} observed in this study, unless the kinetics of Ca^{2+} binding to RLC are significantly different in intact fibers compared to proteins in solution. Resolution of this question awaits measurement of Ca^{2+} binding to RLC in skinned fibers during the time course of activation. In any case, the results of the present study point to a substantial role for myosin RLC in conferring Ca^{2+} dependence on the rate of tension development in skeletal muscle.

REFERENCES

Araujo, A., and J. W. Walker. 1994. Kinetics of tension development in skinned cardiac myocytes measured by photorelease of Ca^{2+}. Am. J. Physiol. 267:H1643–H1653.

Ashley, C. C., I. P. Mulligan, and T. J. Lea. 1991. Ca^{2+} and activation mechanisms in skeletal muscle. Q. Rev. Biophys. 24:1–73.

Bagshaw, C. R., and G. H. Reed. 1977. The significance of the slow dissociation of divalent metal ions from myosin "regulatory" light chains. FEBS Lett. 81:386–390

Brenner, B. 1988. Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. Proc. Natl. Acad. Sci. USA. 85:3265–3269.

Brenner, B., and E. Eisenberg. 1986. Rate of force generation in muscle: correlation with actomyosin ATPase activity in solution. Proc. Natl. Acad. Sci. USA. 83:3542–3546.

Chase, P. B., D. A. Martyn, and J. D. Hannon. 1994. Isometric force redevelopment of skinned muscle fibers from rabbit activated with and without Ca^{2+}. Biophys. J. 67:1994–2001.

Diffee, G. M., M. L. Greaser, F. Reinach, and R. L. Moss. 1995. Effects of a non-divalent cation binding mutant of myosin regulatory light chain on tension generation in skinned skeletal muscle fibers. Biophys. J. 68: 1443–1452.

Fabiato, A. 1988. Computer programs for calculating total from specified free and free from specified total ionic concentrations in aqueous solutions containing multiple metals or ligands. Methods Enzymol. 157: 378–417.

Godt, R. E., and B. D. Lindley. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. J. Gen. Physiol. 80:279–297.

Goodwin, E. B., L. A. Leinwand, and A. G. Szent-Gyorgi. 1990. Regulation of scallop myosin by mutant regulatory light chains. J. Mol. Biol. 216:85–93.

Greaser, M. L., and J. Gergely. 1971. Reconstitution of troponin activity from three protein components. J. Biol. Chem. 246:4226–4233.

Hofmann, P. A., J. M. Metzger, M. L. Greaser, and R. L. Moss. 1990. Effects of partial extraction of light chain 2 on the Ca^{2+} sensitivities of isometric tension, stiffness, and velocity of shortening in skinned skeletal muscle fibers. J. Gen. Physiol. 95:477–498.

Holroyde, M. J., J. D. Potter, and R. J. Solaro. 1979. The calcium binding properties of phosphorylated and unphosphorylated cardiac and skeletal myosins. J. Biol. Chem. 254:6478–6482.

Kamm, K. E., and J. T. Stull. 1985. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Annu. Rev. Pharmacol. Toxicol. 25:593–620.

Kaplan, J. H., and G. C. R. Ellis-Davies. 1988. Photolabile chelators for the rapid photorelease of divalent cations. Proc. Natl. Acad. Sci. USA. 85:6571–6575

Kwon, H., E. B. Goodwin, L. Nyitray, E. Berliner, E. O'Neall-Hennessey, F. D. Melandri, and A. G. Szent-Gyorgi. 1990. Isolation of the regulatory domain of scallop myosin: role of the essential light chain in calcium binding. Proc. Natl. Acad. Sci. USA. 87:4771–4770.

Lenart, T. D., T. StC. Allen, R. J. Barsotti, G. C. R. Ellis-Davies, J. H. Kaplan, C. Fanzini-Armstrong, and Y. E. Goldman. 1993. Mechanics and structure of cross-bridges during contractions initiated by photolysis of caged Ca^{2+}. In Mechanism of Myofilament Sliding in Muscle Contraction. H. Sugi and G. H. Pollack, editors. Plenum Press, New York.

Lowey, S., and D. Risby. 1971. Light chains from fast and slow myosins. Nature. 234:81–85.

Lowey, S., G. S. Waller, and K. M. Trybus. 1993. Skeletal muscle light chains are essential for physiological speeds of shortening. Nature. 365:454–456.

Metzger, J. M., and R. L. Moss. 1991. Kinetics of a Ca^{2+} sensitive cross-bridge state transition in skeletal muscle fibers: effects due to variations in thin filament activation by extraction of troponin C. J. Gen. Physiol. 98:233–248.

Metzger, J. M., and R. L. Moss. 1992. Myosin light chain 2 modulates calcium-sensitive cross-bridge transitions in vertebrate skeletal muscle. Biophys. J. 63:460–468.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1982. Physiological effects accompanying the removal of LC$_2$ from skinned skeletal muscle fibers. J. Biol. Chem. 257:8588–8591.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1983. Effects of EDTA treatment upon the protein subunit composition and mechanical properties of mammalian single skeletal muscle fibers. J. Cell Biol. 96: 970–978.

Rayment, I., W. R. Rypniewski, K. Schmidt-Bäse, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg, and H. M. Holden. 1993. Three-dimensional structure of myosin subfragment-1: a molecular motor. Science. 261:50–58.

Simmons, R. M., and A. G. Szent-Gyorgi. 1978. Reversible loss of calcium control of tension in scallop striated muscle associated with the removal of regulatory light chain. Nature. 273:62–64.

Swartz, D. R., and R. L. Moss. 1992. Influence of a strong-binding myosin analogue on calcium-sensitive properties of skinned skeletal muscle fibers. J. Biol. Chem. 267:20497–20506.

VanBuren, P., G. S. Waller, D. E. Harris, K. M. Trybus, D. M. Warshaw, and S. Lowey. 1994. The essential light chain is required for full force production by skeletal muscle myosin. Proc. Natl. Acad. Sci. USA. 91:12403–12407

Wagner, P. D. 1982. Preparation and fractionation of myosin light chains and exchange of the essential light chain. Methods Enzymol. 85:72–81.

Weeds, A. G., and S. Lowey. 1971. Substructure of the myosin molecule. II. The light chains of myosin. J. Mol. Biol. 61:701–725.

Wolff, M. W., K. S. McDonald, R. L. Moss. 1995. Rate of tension development in cardiac muscle varies with the level of activator calcium. Circ. Res. 76:154–160.