Myosin light chain 2 modulates calcium-sensitive cross-bridge transitions in vertebrate skeletal muscle

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ABSTRACT We investigated the mechanism of the Ca^{2+} sensitivity of cross-bridge transitions that limit the rate of force development in vertebrate skeletal muscle. The rate of force development increases with Ca^{2+} concentration in the physiological range. We show here that at low concentrations of Ca^{2+} the rate of force development increases after partial extraction of the 20-kD light chain 2 subunit of myosin, whereas reconstitution with light chain 2 fully restores native sensitivity to Ca^{2+} in skinned single skeletal fibers. Furthermore, elevated free Mg^{2+} concentration reduces Ca^{2+} sensitivity, an effect that is reversed by extraction of the light chain but not by disruption of thin-filament activation by partial removal of troponin C, the Ca^{2+} binding protein of the thin filament. Our findings indicate that the Ca^{2+} sensitivity of the rate of force development in vertebrate skeletal muscle is mediated in part by the light chain 2 subunit of the myosin cross-bridge.

INTRODUCTION

Force development and shortening in muscle result from interaction of the contractile proteins myosin and actin. In all muscle cells, the extent of interaction between actin and myosin is regulated by the concentration of cytosolic Ca^{2+}, although the specific proteins that confer Ca^{2+} sensitivity differ among muscle types. For example, Ca^{2+} regulation of contraction in vertebrate striated muscle is mediated by troponin and tropomyosin associated with the thin filament (1), whereas striated muscles of various invertebrate species are regulated by Ca^{2+} binding directly to myosin (2–5).

The molecular basis of Ca^{2+} activation of contraction in myosin-regulated muscle is not well understood. The Ca^{2+} specific binding site responsible for activation of myosin-regulated muscle is located in the globular head region of myosin. This binding site is lost on removal of the light chain 2 (LC_2) protein subunit of myosin; however, LC_2 itself does not contain the Ca^{2+} specific binding site (4, 5). Recent evidence suggests that the Ca^{2+} specific binding site of myosin involves a three-peptide domain that consists of the regulatory LC (i.e., LC_2), essential LC, and a 10–kD myosin heavy chain fragment (6). It is possible that the Ca^{2+} specific binding site may be defined by a pocket formed by these peptides. In addition, it is known that myosin LC_2 exhibits significant primary sequence homology with Ca^{2+} binding proteins troponin C, parvalbumin, and calmodulin (7–9). Indeed, a high-affinity Ca^{2+}/Mg^{2+} binding site has been localized to the N-terminal region of LC_2 (for review see reference 5). The role of this divalent cation binding site in Ca^{2+} activated contraction of myosin-regulated muscle is unclear. Interestingly, LC_2 is present in the myosin molecule of thin filament-regulated muscle. Myosin LC_2 of vertebrate skeletal muscle also contains the high-affinity Ca^{2+}/Mg^{2+} binding site, although its possible function in the Ca^{2+} sensitivity of contraction has not been established (4). However, in myosin-regulated muscle the inhibitory effect of LC_2 was lost in LC_2 extracted fibers that were reconstituted with a mutant form of vertebrate LC_2 that lacked a functional Ca^{2+}/Mg^{2+} binding site (10). This suggests that the high-affinity binding site of LC_2 is somehow important in regulating the interaction of myosin with actin in these fibers, but whether a similar role exists in vertebrate muscle has not been established.

The idea that vertebrate skeletal muscle contains some form of myosin-based regulation of contraction has been inferred from various studies (11, 12). The applicability of these findings to intact muscle is uncertain because the studies typically used isolated proteins, which eliminated some factors that influence contraction in vivo, such as structural constraints on cross-bridge interaction imposed by the intact filament lattice. Thus, although the results of the earlier studies are convincing for the conditions used, there is as yet no physiological evidence for a myosin component of Ca^{2+} regulation of contraction in vertebrate striated muscle.

Here, we investigated the basis of the Ca^{2+} sensitivity of weak to strong cross-bridge transitions in mammalian skeletal muscle (13–15). Experiments were designed to determine whether myosin LC_2 is involved in conferring Ca^{2+} sensitivity to cross-bridge transitions. Single fibers from rabbit and rat fast-twitch skeletal muscles were treated chemically to permeabilize surface membranes, thereby allowing direct control of intracellular solutions and permitting specific extraction and readdition of contractile and regulatory protein subunits. Results showed that during submaximal Ca^{2+} activation LC_2 is repressive to cross-bridge transitions and that repression is relieved by increasing the concentration of Ca^{2+} or by removal of endogenous LC_2. Furthermore, increased Mg^{2+} concentration decreased Ca^{2+} sensitivity, an effect

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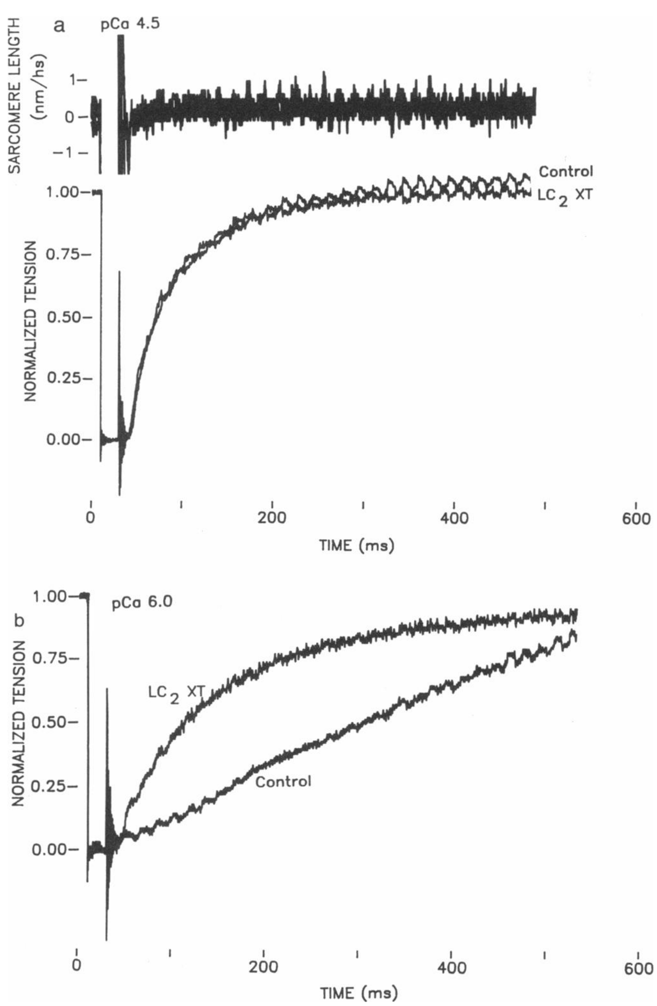

FIGURE 1 (*A*) Records of sarcomere length (*upper traces*) and tension (*lower traces*) obtained in the determination of k_{tr} during maximal activation (pCa 4.5) of a psoas fiber before and after extraction of 25% LC_2. k_{tr} was 20 s^{-1} both before and after partial extraction of LC_2. Sarcomere length was clamped to 2.44 μm during tension redevelopment. P_0 was 128 kN/m^{-2}. To extract LC_2, the fiber was placed in a solution containing, in mM, 20 KCl, 20 EDTA, 5 imidazole, pH 7.00, for 3 h at 30°C. hs, half sarcomere. All tension traces are normalized to the maximum tension generated in each fiber under the particular experimental conditions (i.e., control, extracted; high Ca^{2+}, low Ca^{2+}, etc.). (*B*) Records of tension obtained during the protocol to determine k_{tr} in a psoas fiber during submaximal Ca^{2+} activation both before and after partial extraction of LC_2. k_{tr} was 3 s^{-1} before extraction of LC_2 and 10 s^{-1} after extraction. Sarcomere length was clamped as in *A* (records not shown). Results were obtained from the same fiber as in *A*.

that was reversed by extraction of LC_2. Our findings suggest a role for LC_2 in modulating Ca^{2+} sensitivity of contraction in mammalian skeletal muscle.

MATERIALS AND METHODS

Skinned fiber preparations and experimental apparatus

Fast-twitch skeletal muscle fibers were obtained from the superficial portion of the vastus lateralis (svl) muscles of adult female Sprague-Dawley rats and from psoas muscles of adult male New Zealand rabbits. Results obtained using psoas and svl fibers were qualitatively similar. Bundles of ~50 fibers were dissected from each muscle while in relaxing solution (below) and were then tied with surgical silk to glass capillary tubes. Bundles were stored for up to 3 wk at −23°C in relaxing solution containing 50% (vol/vol) glycerol. Before each experiment, bundles were placed in relaxing solution containing 0.5% (wt/vol) Brij-58 for 30 min to disrupt the sarcoplasmic reticulum (16). Individual fibers were carefully pulled free from one end of the fiber bundle and mounted between a force transducer (model 407; Cambridge Technology, Inc., Cambridge, Massachusetts; sensitivity, 0.2 mV/μN; 1–99% response time, 100 μs; resonant frequency, ~5 kHz; noise level at the output equivalent to 1 mg peak-to-peak) and DC torque motor (model 300s; Cambridge Technology, Inc.). The fiber was viewed through an inverted microscope (model IM; Carl Zeiss, Inc., Thornwood, New York), and its overall length was adjusted with a mechanical translator to set resting sarcomere length. Complete details of the mounting procedure and experimental set-up have been reported elsewhere (16, 17).

Solutions

Relaxing and activating solutions contained (mM) 7 ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 1 or 10 free Mg^{2+}, 4.4 total ATP, 14.5 creatine phosphate, 20 imidazole, and sufficient KCl to yield a total ionic strength of 180 mM. Solution pH was adjusted to 7 with KOH. Relaxing solution had a pCa (i.e., $-\log [Ca^{2+}]$) of 9.0, whereas the pCa of the solution for maximal activation was 4.5. The computer program of Fabiato and Fabiato (18) was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex, using the stability constants listed by Godt and Lindley (19). The apparent stability constant for Ca^{2+}-EGTA was corrected for ionic strength, pH, and an experimental temperature of 15°C (18).

Rate constant of tension redevelopment and sarcomere length control system

The experimental protocol for measuring the rate constant of tension redevelopment (k_{tr}) was a modification of the multistep protocol developed by Brenner and Eisenberg (20). Measurement of k_{tr} involved a mechanical maneuver to dissociate myosin cross-bridges from actin in a steadily activated fiber so that the subsequent rate of tension redevelopment reflects the forward and reverse rate constants for the rate limiting transition(s) in the cross-bridge cycle leading to formation of strongly bound force-generating states. The fiber was first transferred from relaxing solution to an activating solution, and steady isometric tension was developed. The fiber was then rapidly (within 0.5 ms) shortened by ~200–300 nm per half-sarcomere, resulting in an abrupt reduction of force to zero, and the fiber shortened for 5–40 ms under unloaded conditions (i.e., at maximum velocity). Before the imposed slack was taken up, the fiber was rapidly reextended to its initial length. Coincident with the restretch, force transiently increased and then rapidly declined to zero or very nearly zero. The redevelopment of force after this maneuver reflects the rate of reattachment of cross-bridges and the transition to strongly bound force-producing states. During redevelopment of force, sarcomere length was held constant, because in the absence of sarcomere length control k_{tr} would be underestimated due to end compliance (20). Sarcomere length was clamped to within 0.5 nm per half-sarcomere by servocontrol of the position of the first order line of the laser diffraction pattern (Fig. 1). Records of tension redevelopment were best fit by a single exponential equation. Complete details of the experimental protocol, curve fitting procedure, mechanical set-up, and sarcomere length control system are described elsewhere (17).

Tension–pCa relationship

At each pCa, steady isometric tension was allowed to develop, after which the fiber was slackened to obtain the tension baseline. The fiber

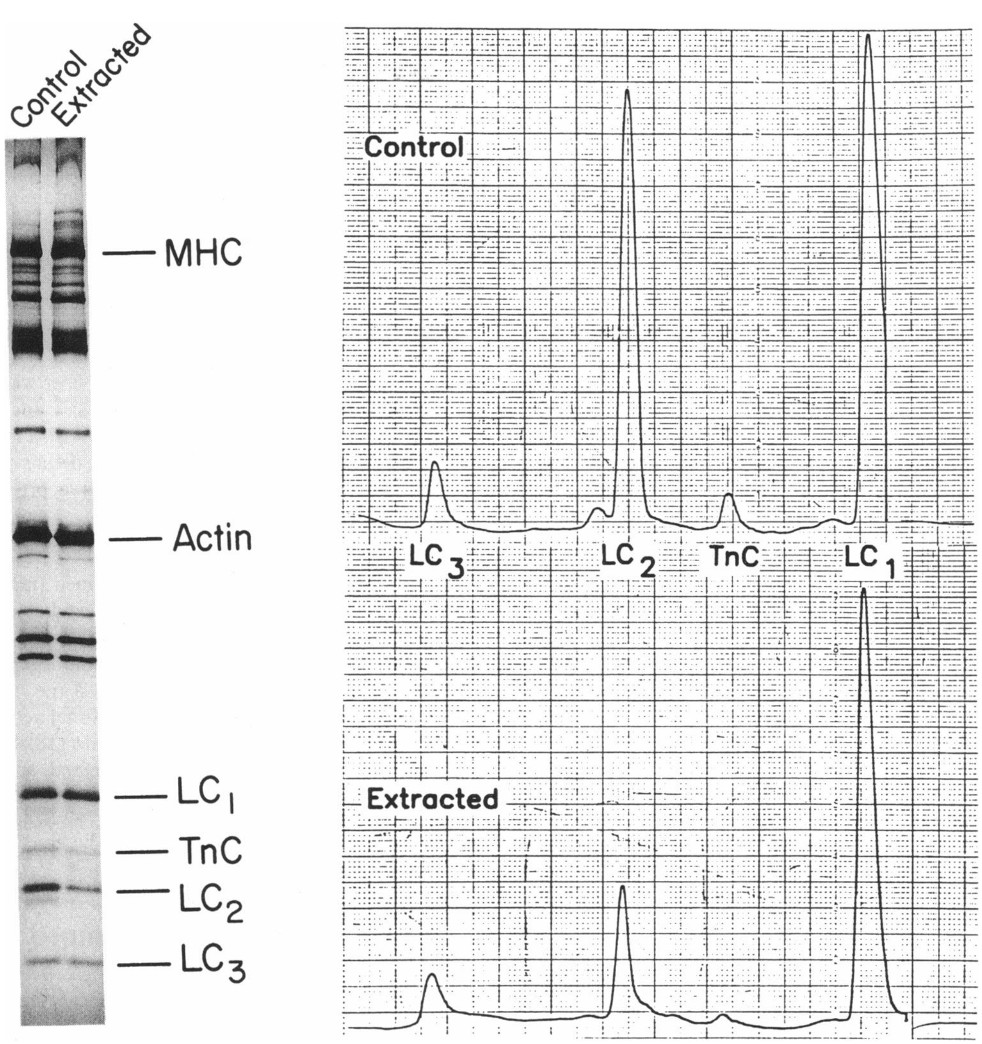

FIGURE 2 SDS-polyacrylamide gels and densitometric scans of gels obtained from segments of a psoas fiber before and after the procedure to extract LC_2. To quantitate the amount of LC_2 extracted, the ratio $LC_2/(LC_1 + LC_3)$ was determined for both control and extracted fibers by measuring the areas under the peaks corresponding to these proteins. The extracted ratio was then divided by the control ratio to determine the reduction in LC_2 content. Calculated in this way, 61% of LC_2 was extracted from this fiber, which was the largest amount removed in this study. Importantly, exogenous troponin C was first added back to the fiber before mechanical measurements were made because the LC_2 extraction procedure also removes troponin C. Comparison of troponin C/(LC_1 + LC_3) ratios from control and extracted segments showed that readdition of troponin C to extracted fiber was stoichiometric. TnC, troponin C; MHC, myosin heavy chain.

was then relaxed. The difference between steady tension and the tension baseline after the slack step was measured as total tension. To obtain active tension, resting tension measured at pCa 9.0 (~1% of total tension) was subtracted from total tension. The fiber was transferred to relaxing solution after each activation at a given pCa. Tension–pCa relations were determined in each fiber by expressing tensions (P) at various submaximal Ca^{2+} concentrations as fractions of the maximum value, P_0 (i.e., isometric tension at pCa 4.5 and 1 mM free Mg^{2+}) obtained in each fiber. Every fourth contraction was at pCa 4.5 to monitor any decline in fiber performance (16).

Protocols for extraction and readdition of LC_2

Methods to extract LC2 from skinned skeletal muscle fibers have been published previously and briefly described here (21). To extract LC_2, the fiber was transferred from relaxing solution to a solution containing (mM): 20 KCl, 20 ethylenediaminetetraacetate (EDTA), 5 imidazole, pH 7.00, for 1–4 h at 20–30°C. After extraction, the fiber was transferred to relaxing solution and temperature was returned to 15°C. Because there was a variable amount of extraction of troponin C during this procedure, exogenous troponin C was added back to the fibers before data collection. In this study, maximum isometric tension averaged $0.98 \pm 0.02\ P_0$ after extraction of LC_2 and add back of troponin C.

Readdition of exogenous LC_2 involved transferring the fiber to a relaxing solution containing 0.5 mg/ml purified LC_2 for 2–3 h at 15°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Before attachment of the fiber to the experimental apparatus, a 0.5–1-mm segment of the fiber was removed for analysis of the control protein composition by SDS-PAGE. The remaining portion of the fiber, ~2–3 mm in length, was mounted into connectors (16). Contractile properties were determined in the control fiber and after extraction of

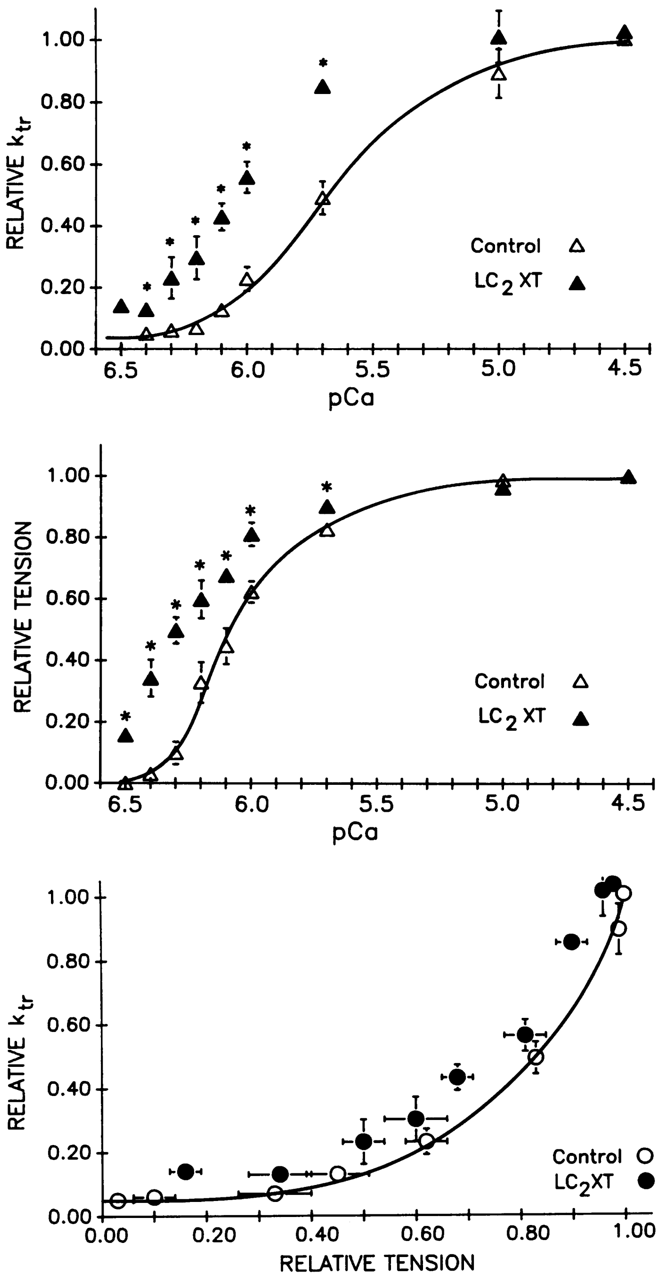

FIGURE 3 Summaries of the effects of partial extraction of LC_2 on the k_{tr}–pCa (*upper*), isometric tension–pCa (*middle*), and k_{tr}–isometric tension (*lower*) relationships in rabbit psoas fibers. Maximum isometric tension averaged $0.98 \pm 0.02\ P_0$ after extraction of LC_2 and add back of troponin C (see Fig. 2). Values are mean ± SE with 6–10 observations per point. In some instances, error bars were smaller than the symbol size. Asterisks indicate experimental values that were significantly greater than control ($P < 0.05$). Qualitatively similar results were obtained from svl fibers.

LC_2. After extraction, a 0.5–1-mm segment of the fiber was removed for protein analysis. Fibers were subsequently reconstituted by bathing in relaxing solution containing purified LC_2. Mechanical measurements were obtained after readdition of LC_2. Each fiber segment was placed in a 0.5-ml microfuge tube containing SDS sample buffer (10 μl/mm length of fiber segment) and stored at −80°C for subsequent analysis of contractile and regulatory protein content by SDS-PAGE and scanning densitometry, as described previously (21, 22). Thus, gels obtained from segments of the same fiber were analyzed to quantitate protein composition at the different stages of an experiment (Fig. 2). Gels of LC_2 reconstituted fibers have been published (22).

RESULTS AND DISCUSSION

To probe cross-bridge transitions that limit the rate of formation of the strongly bound force-bearing state, we used a mechanical maneuver to determine the rate constant of tension redevelopment (k_{tr}) after rapid release and reextension of fiber length during steady Ca^{2+} activation (13–15, 20) (Fig. 1). k_{tr} is sensitive to Ca^{2+} in the physiological range (i.e., 10^{-6}–10^{-4} M) and is thought to be determined by the step or steps in the actin-myosin ATP hydrolysis reaction that limit the rate of formation of the strongly bound force-generating cross-bridge state (13–15). In earlier studies, the Ca^{2+} sensitivity of k_{tr} was unchanged when thin filament activation was disrupted by partial extraction of troponin C, whereas the Ca^{2+} sensitivity of steady-state isometric tension was markedly reduced (23). Partial extraction of troponin C results in a rightward shift (i.e., to higher [Ca^{2+}]) and reduction in the steepness of the tension–pCa relationship due to a disruption of near-neighbor cooperativity between adjacent functional groups (defined structurally as 1 tropomyosin, 1 troponin complex, and 7 actin monomers) on the thin filament (24, 25). This dissociation of Ca^{2+} sensitivities of k_{tr} and isometric tension provides evidence that k_{tr} is significantly affected by an apparent rate constant that characterizes the kinetics of transitions

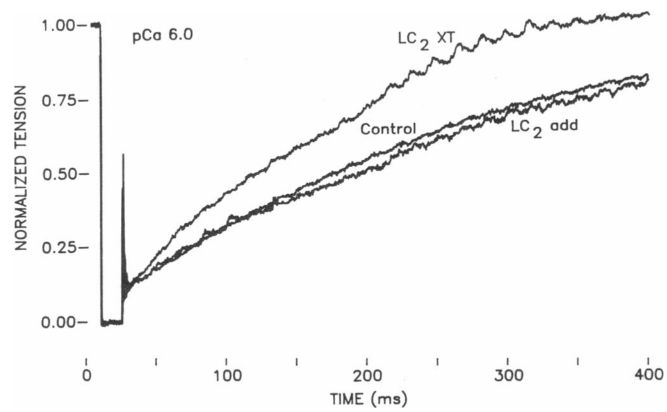

FIGURE 4 Records of tension obtained during determination of k_{tr} at a submaximal concentration of Ca^{2+} in a psoas fiber before and after extraction of LC_2 and after the readdition of exogenous LC_2. k_{tr} was 4.8 s^{-1} in control, 6.5 s^{-1} after partial extraction of LC_2, and 4.4 s^{-1} after the readdition of purified LC_2 into the fiber. In each trial, sarcomere length was clamped to 2.58 μm during tension redevelopment (records not shown). P_0 was 131 kN/m^{-2}. After extraction of LC_2 and readdition of troponin C, isometric tension at pCa 4.5 was 1.01 P_0. The fiber was placed in LC_2 extracting solution (Fig. 1) for 2.5 h at 20°C. To reconstitute the extracted fiber with LC_2, the fiber was bathed for 3.5 h at 15°C in relaxing solution containing 0.58 mg/ml purified LC_2. SDS-PAGE gels of segments obtained from a single fiber before and after reconstitution with LC_2 have been published (22).

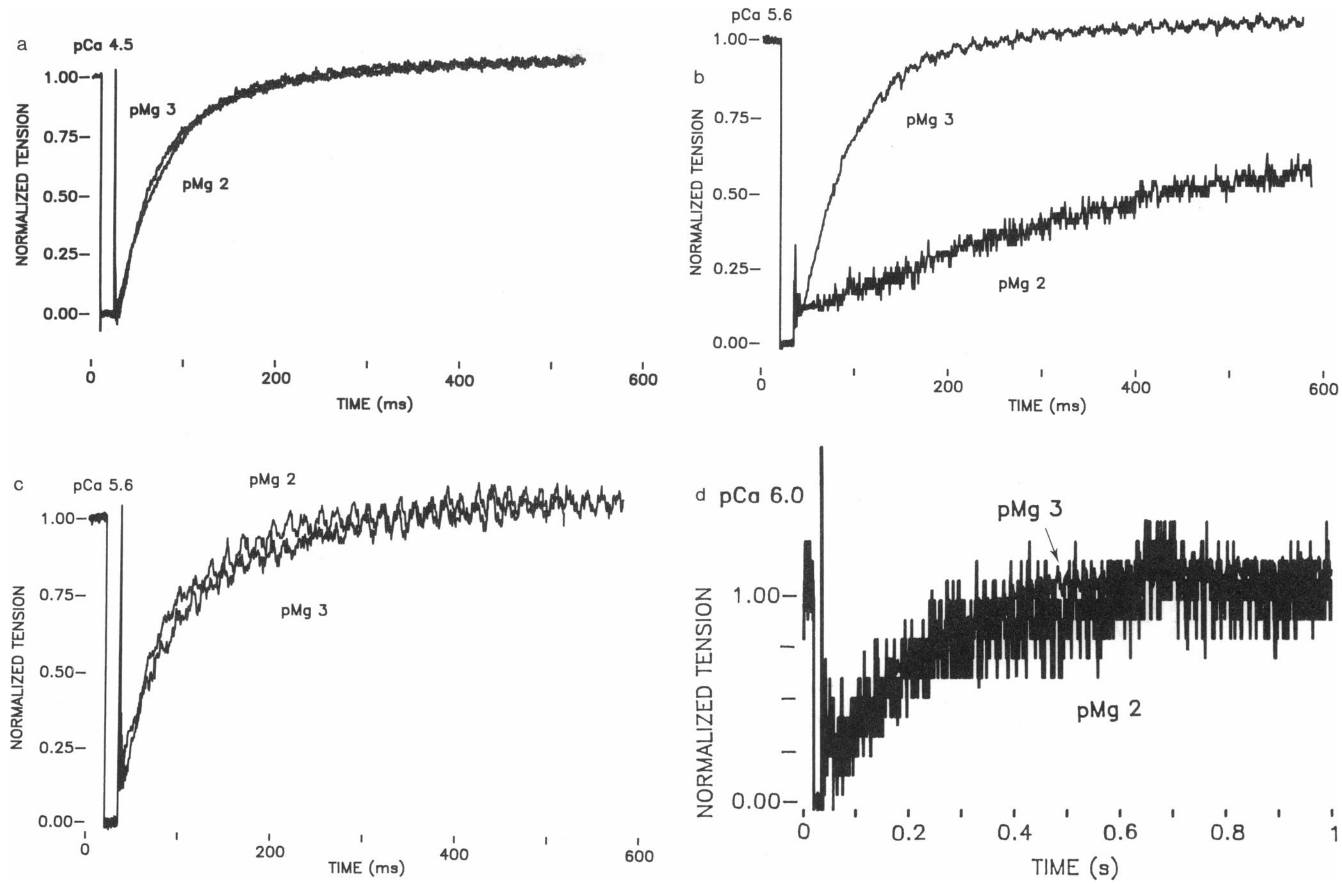

FIGURE 5 Effects on the Ca^{2+} sensitivity of k_{tr} due to elevated [Mg^{2+}] in control and partially LC_2 extracted fibers. Results in *B–D* were obtained from the same fiber. *A* and *B* are controls, *C* and *D* are after partial extraction of LC_2. (*A*) Records of tension obtained during determination of k_{tr} at maximal concentration of Ca^{2+} (pCa 4.5) at pMg 3 and 2 in a svl fiber. k_{tr} was 20 s^{-1} at pMg 3 and 19.5 s^{-1} at pMg 2. Qualitatively similar results were obtained from psoas fibers. P_0 = 115 kN/m^2. (*B*) Records of tension obtained during determination of k_{tr} at a submaximal concentration of Ca^{2+} (pCa 5.6) at pMg 3 and 2 in a svl fiber. k_{tr} was 17 s^{-1} at pMg 3 and 2.8 s^{-1} at pMg 2. Relative tensions were 0.84 P_0 at pMg 3 and 0.33 P_0 at pMg 2. k_{tr} at pCa 4.5 was 23 s^{-1}. Average pCa_{50}'s from k_{tr}–pCa and tension–pCa data were 5.80 and 6.03 at pMg 3 and 5.46 and 5.55 at pMg 2, respectively. Qualitatively similar results were obtained from psoas fibers. (*C*) Records of tension obtained during determination of k_{tr} at pCa 5.6 and pMg 3 and 2 after the partial extraction of LC_2 from the same fiber as in *B*. k_{tr} was 17 s^{-1} at pMg 2 and pMg 3. (*D*) Records of tension obtained during determination of k_{tr} at pCa 6.0 and pMg 3 and 2 after partial extraction of LC_2 from the same fiber as in *B* and *C*. k_{tr} was 5 s^{-1} at pMg 3 and 4.5 s^{-1} at pMg 2 (noisy trace). For all records, sarcomere length (records not shown) was clamped during tension redevelopment. LC_2 extraction solution and protocol were as described in Fig. 1.

among cross-bridge states. Furthermore, k_{tr} increases in the presence of added phosphate, suggesting that the specific cross-bridge transition(s) that underlies k_{tr}, at least in part, includes the phosphate release step of the actomyosin ATP hydrolysis reaction and/or a step in rapid equilibrium with phosphate release (26, 27). Based on analysis of tension transients resulting from photogeneration of phosphate from caged phosphate, it has been proposed that phosphate release from actomyosin is a two-step process involving an isomerization step followed by the phosphate release step (28, 29).

Because LC_2 is essential in conferring Ca^{2+} sensitivity to thick filament regulated muscles (2–5), the Ca^{2+} sensitivity of k_{tr} was examined after partial extraction of myosin LC_2 from skeletal muscle fibers (Fig. 1). Partial extraction of LC_2 had no effect on k_{tr} at maximal [Ca^{2+}], but k_{tr} increased markedly at submaximal [Ca^{2+}] (Fig. 1). In the example shown, there was an approximate threefold increase in k_{tr} at pCa 6.0 (where pCa is $-\log[Ca^{2+}]$) after extraction of 25% of LC_2, determined from SDS-polyacrylamide gels of segments of the same fiber obtained before and after extraction. Densitometric scans of SDS gels of these fiber segments showed that extraction also removed troponin C, but other contractile and regulatory proteins were unchanged by the protocol. It is important to note that before experimental measurements fibers were reconstituted with troponin C (see Fig. 2).

Fig. 3 summarizes the effects of partial extraction of LC_2 on the relationships between k_{tr} and pCa, steady-state tension and pCa, and k_{tr} and steady-state tension. At pCa 4.5, k_{tr} averaged 16.7 ± 0.7 s^{-1} (n = 10) in control fibers and 17.1 ± 0.8 s^{-1} (n = 10) after extraction of an average 28 ± 7% of endogenous LC_2. We have inter-

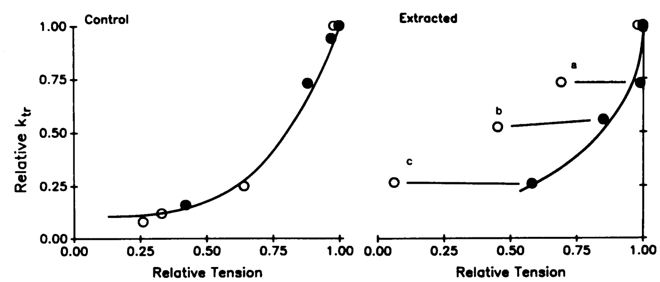

FIGURE 6 Summary of effects on the k_{tr}–tension relationship (data obtained from Fig. 5) due to altered [Mg^{2+}] before (*Control*) and after partial extraction of LC_2 (*Extracted*). ●, pMg 3; ○, pMg 2. Tension and k_{tr} values are scaled to values obtained at pCa 4.5. For the control and extracted data, the pMg 2 values (○ at point 1.0, 1.0 in both plots) were partially covered by the pMg 3 data point (●). For the extracted data, the line marked *a* connects data points obtained at pCa 5.6; at *b* the line connects data points obtained at pCa 5.7; at *c* the line connects data points obtained at pCa 6.0. Qualitatively similar findings obtained in both psoas (n = 2) and svl fibers (n = 5).

preted our results (below) based on the assumption that the extraction of LC_2 is random along the thick filament. This assumption is based on the observation that the extraction of troponin C is random along the length of the thin filament (30). Isometric tension and k_{tr} values at pCa 4.5 were not significantly different than control values (Fig. 3), and this is taken as evidence that the extraction procedure did not adversely affect contractile function. At pCa above 5.0, k_{tr} increased significantly after extraction of LC_2. The pCa required for half maximal k_{tr}, that is pCa_{50}, increased from an average of 5.71 in control fibers to 6.07 after partial extraction of LC_2, indicating that the sensitivity of k_{tr} to Ca^{2+} increased due to extraction of the light chain. For example, at pCa 6.0, k_{tr} was $3.8 \pm 0.7\ s^{-1}$ (n = 9) in controls and $9.3 \pm 1.0\ s^{-1}$ (n = 9) after partial extraction of LC_2.

An additional effect of LC_2 extraction was an increase in the minimum value of k_{tr} obtained at low [Ca^{2+}]. This suggests that partial removal of LC_2 altered the fundamental (i.e., apparent Ca^{2+} independent) value of an apparent rate constant, as well as affecting the sensitivity of the rate constant to Ca^{2+}. In agreement with previous work (21), partial extraction of LC_2 increased the Ca^{2+} sensitivity of steady-state tension, with pCa_{50} increasing from 6.10 in control fibers to 6.25 after extraction.

Taking these results together, partial extraction of LC_2 resulted in a left-shift of the relationship between k_{tr} and steady-state tension (Fig. 3). Thus, the effects on k_{tr} subsequent to extraction of LC_2 are not simply due to enhanced activation of the thin filament as a result of increased cross-bridge binding (21) but rather indicate that LC_2 has a direct effect to modulate k_{tr}. For example, our earlier study showed that tension and instantaneous stiffness were increased at submaximal concentrations of Ca^{2+} after partial extraction of LC_2 (21). This suggested that one effect of removal of LC_2 was to increase the activation of the thin filament at submaximal concentrations of Ca^{2+}. If the effects of LC_2 extraction on k_{tr} were solely related to the increase in thin filament activation, then the k_{tr}–relative tension relationship should be unaffected by extraction. However, we found the k_{tr}–tension relationship to be altered by extraction, which we take to indicate that removal of LC_2 additionally affects cross-bridge kinetics.

That the effects on k_{tr} are specific to the extraction of LC_2 is clearly demonstrated in experiments in which LC_2 readdition to extracted fibers resulted in complete restoration of native Ca^{2+} sensitivity of k_{tr} (Fig. 4). Similar findings were obtained in five separate experiments and provide strong evidence that the observed effects on k_{tr} can be directly attributed to LC_2 rather than a nonspecific effect of the extraction procedure. It should be noted here that these effects of LC_2 are not dependent on phosphorylation of the light chain, because as we showed earlier the phosphate content in these fibers is low (<0.1 mol phosphate/mol LC_2). Effects on k_{tr} and tension due to alterations in phosphorylation of LC_2 have been reported previously (17).

If altered Ca^{2+} sensitivity of k_{tr} is directly related to the extraction of LC_2, then there should be a graded effect of extraction on k_{tr} such that at low amounts of extraction the effect should be small, whereas with greater amounts of extraction the alteration in the Ca^{2+} sensitivity of k_{tr} should correspondingly be increased. To address this point, we examined the relationship between the Ca^{2+} sensitivity of k_{tr}, determined as the ratio of k_{tr} values (extracted/control; $k_{tr,ext/con}$) at a particular pCa, and the amount of LC_2 extracted determined from the same fiber by SDS-PAGE. The data were fit by a straight line using least-squares fit linear regression to obtain the slope of the relationship between $k_{tr,ext/con}$ and percent of LC_2 extracted. In this analysis, the slope was 0.07 and the correlation coefficient was 0.83. Thus, for each 1% of LC_2 extracted, the $k_{tr,ext/con}$ value increased by 7%. This indicates that the Ca^{2+} sensitivity of k_{tr} relates to LC_2 extraction in a graded manner; however, caution must be taken in interpreting the magnitude of this effect because the value of $k_{tr,ext/con}$ is highly dependent on the pCa value used in the comparison. For example, at low pCa values that yield near maximal values of k_{tr}, there would be relatively little or no effect of LC_2 extraction on

TABLE 1 Effects on k_{tr} due to partial extraction of troponin C

	pMg 3		pMg 2	
	Control	Extracted	Control	Extracted
k_{tr} (s^{-1})	11	11	3.5	3.5
P/P_0	0.89	0.30	0.46	0.11

All measurements were made at pCa 5.6. After partial extraction of troponin C, isometric tension at pCa 4.5 was 0.54 P_0. The solution and procedure for extracting troponin C have been published (23, 36). Results were obtained from an sv1 fiber, with sarcomere length servocontrolled to 2.63 μm during measurement of k_{tr}.

the $k_{tr,ext/con}$ value (see Fig. 3). At higher pCa values, the $k_{tr,ext/con}$ value increases so that comparison among fibers with differing extents of LC_2 extraction is complex and involves two factors: the extent of LC_2 extracted and the pCa value used in determining the $k_{tr,ext/con}$ value. This complicating feature was not present in our earlier work that related extent of troponin C extraction to altered contractile function because we were able to use tension at maximal Ca^{2+} concentration as a general marker for extent of protein extraction (25).

LC_2 of vertebrate striated muscle belongs to a large family of Ca^{2+} binding proteins and has considerable sequence homology with the regulatory proteins calmodulin, troponin C, and scallop LC_2 (7–9). Vertebrate skeletal LC_2 has a high affinity divalent cation binding site that preferentially binds Ca^{2+} over Mg^{2+} (K_{Ca} 3×10^7 M^{-1}, K_{Mg} $2.5\text{–}3 \times 10^5$ M^{-1}) (31). However, under resting conditions this site would be occupied predominantly by Mg^{2+} because the concentration of Mg^{2+} is about four orders of magnitude greater than Ca^{2+} (31, 32). Also, due to the slow dissociation rate of Mg^{2+}, this site is not usually thought to be involved in regulation of contraction, because Ca^{2+} would not significantly displace Mg^{2+} during the time course of a twitch (32). However, under conditions that raise the mean Ca^{2+} concentration for prolonged times, such as during successive twitches or tetany, Ca^{2+} binding to LC_2 may be increased to a value of ~0.70 mol Ca^{2+}/mol myosin in the presence of physiological levels of Mg^{2+} and pH 7.00 (31). Presumably, during the protocol to determine k_{tr} in skinned fibers, Ca^{2+} is in equilibrium with the cation binding site of LC_2 because several seconds are required to achieve steady activation of the fibers. If the Ca^{2+} sensitivity of k_{tr} involves this site, increased [Mg^{2+}] should reduce bound Ca^{2+} and decrease k_{tr}. Fig. 5 shows results of the effects on k_{tr} due to increasing free Mg^{2+} from 1 mM, which is near physiological, to 10 mM. Increases in free [Mg^{2+}] had no effect on k_{tr} at pCa 4.5; however, the Ca^{2+} sensitivity of k_{tr} was markedly reduced in that pCa_{50} decreased by 0.34 pCa units at pMg 2 compared with pMg 3. We next examined the possible role of LC_2 in mediating the effect of Mg^{2+} on k_{tr}. In fibers treated to partially extract LC_2, effects on k_{tr} due to increased [Mg^{2+}] were reversed (Figs. 5 and 6). This suggests that LC_2 is critical in conferring Mg^{2+} sensitivity to the modulation of k_{tr} by Ca^{2+}. This effect could involve the Ca^{2+}-Mg^{2+} site of LC_2, although other mechanisms involving LC_2 (see below) are not excluded.

The possibility that the effects on Ca^{2+} sensitivity of k_{tr} due to increased Mg^{2+} are mediated by thin filament regulatory proteins also has been considered. It is known that Mg^{2+} depresses the Ca^{2+} sensitivity of steady-state tension (33), thought to be due to an effect of Mg^{2+} to reduce Ca^{2+} binding to the low-affinity sites of troponin C (34). Results in Fig. 6 show that in control fibers the k_{tr}–relative tension relationships are similar at pMg 3 and 2. This might be expected if the Ca^{2+} sensitivity of k_{tr} is due to variations in thin filament activation when the concentrations of Ca^{2+} and Mg^{2+} are altered. However, after partial extraction of LC_2, high Mg^{2+} depressed tension to a disproportionately greater extent than k_{tr} (Fig. 6). Similar results were observed in a total of seven experiments. These findings argue against the idea that thin filament activation is the sole determinant of k_{tr}, but instead suggest that the effect of increased Mg^{2+} to reduce the Ca^{2+} sensitivity of k_{tr} is mediated by LC_2. It is possible, however, that effects of Ca^{2+} and Mg^{2+} on k_{tr} also involve the thin filament regulatory system, but in this case the expression of the thin filament component appears to depend on the presence of LC_2 on the myosin molecule.

The marked reduction in the effect of high Mg^{2+} concentration to alter the Ca^{2+} sensitivity of k_{tr} in LC_2-extracted fibers is at first glance surprising, because in our experiments only ~30% of total LC_2 was extracted. However, previous work suggests that for any given myosin molecule one LC_2 can be removed relatively easily with EDTA, but removal of the second LC_2 is more difficult and requires harsher conditions (35). Thus, in our experiments, an average 30% reduction in LC_2 content could indicate that as much as 60% of myosin molecules have lost an LC_2 subunit.

As a control, we investigated the possibility that troponin C mediates the effects of Mg^{2+} on k_{tr}. Partial removal of troponin C resulted in reduced Ca^{2+} sensitivity of tension due to the disruption of near-neighbor molecular cooperativity within the thin filament. In partially troponin C-extracted fibers, the effect of Mg^{2+} to depress the Ca^{2+} sensitivity of steady-state isometric tension remained, i.e., effects on tension due to troponin C extraction and to high [Mg^{2+}] were additive (Table 1). In contrast, at both 1 and 10 mM Mg^{2+}, the Ca^{2+} sensitivity of k_{tr} was unchanged due to the partial extraction of troponin C. These findings are consistent with the idea that LC_2 mediates the effect of altered [Mg^{2+}] on the k_{tr}–pCa relationship.

The detailed mechanism of LC_2 modulation of the Ca^{2+} sensitivity of k_{tr} is not clear. There is evidence to indicate that binding of divalent metals to the high-affinity Ca^{2+}/Mg^{2+} site of LC_2 induces a conformational change in myosin. In the presence of Ca^{2+}, there is a decrease in the Stokes radius of LC_2 in solution (37), providing evidence of a marked alteration in the tertiary structure of this protein due to Ca^{2+} binding. Furthermore, a conformational change in myosin due to Ca^{2+} binding is suggested in experiments where Ca^{2+}, but not Mg^{2+}, altered the sedimentation coefficient and viscosity of isolated native and synthetic myosin filaments in solution (11). It is possible that a conformational change on Ca^{2+} binding to LC_2 underlies modulation of a step or steps that limit the rate of formation of the strongly bound force-bearing cross-bridge state. Myosin LC_2 is noncovalently bound to the globular head of myosin near the head-tail junction or "hinge" region (38). A potential, although speculative, consequence of Ca^{2+} binding to LC_2 could be altered flexibility of the hinge region, an effect that may underlie modified cross-bridge kinetics. Another possibility is that the thin filament regulatory system mediates the effect of Ca^{2+} on k_{tr}, but the inhibition of cross-bridge kinetics by the thin filament requires that LC_2 be present on myosin so that the cross-bridge somehow senses or modifies the state of thin filament activation. Thus, after removal of LC_2, the inhibition of cross-bridge kinetics due to the thin filament is removed and the rate of the force generating transition is disinhibited. Consistent with this idea, Wagner (39) showed in solution biochemical studies that LC_2 was required for Ca^{2+} sensitive binding of myosin to regulated thin filaments. However, such a mechanism does not easily explain our finding that in troponin C-extracted, but LC_2-replete fibers, k_{tr} was unchanged even though thin filament activation, which was evident as a marked reduction in the Ca^{2+} sensitivity of tension, was substantially decreased.

In conclusion, based on our findings we propose that LC_2 represses Ca^{2+}-sensitive weak to strong transitions of cross-bridges and that this repression can be relieved by added Ca^{2+}, reduced Mg^{2+}, or removal of LC_2. In this respect, vertebrate striated muscle may be somewhat similar to thick-filament regulated systems where removal of LC_2 or addition of Ca^{2+} activates myosin-actin interactions (2–5). Our working hypothesis is that in vertebrate skeletal muscle there are at least two effects of Ca^{2+}: regulation of cross-bridge formation mediated by Ca^{2+} binding to thin filament regulatory proteins and modulation of the rate of formation of force-bearing cross-bridges somehow involving LC_2. Ca^{2+} modulation of the kinetics of force development that are mediated by LC_2 could provide a basis for frequency-dependent potentiation of twitch tension in living muscle.

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