

# Effects of Partial Extraction of Light Chain 2 on the $\text{Ca}^{2+}$ Sensitivities of Isometric Tension, Stiffness, and Velocity of Shortening in Skinned Skeletal Muscle Fibers

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**ABSTRACT** Various functional roles for myosin light chain 2 ( $\text{LC}_2$ ) have been suggested on the basis of numerous and predominantly in vitro biochemical studies. Using skinned fibers from rabbit psoas muscle, the present study examines the influence of partial removal of  $\text{LC}_2$  on isometric tension, stiffness, and maximum velocity of shortening at various levels of activation by  $\text{Ca}^{2+}$ . Isometric tension, stiffness, and velocity of shortening were measured at pCa values between 6.6 and 4.5 (*a*) in a control fiber segment, (*b*) in the same fiber segment after partial removal of  $\text{LC}_2$ , and (*c*) after recombination with  $\text{LC}_2$ . The extraction solution contained 20 mM EDTA, 20 or 50 mM KCl, and either imidazole or  $\text{PO}_4^{2-}$  as a pH buffer (pH 7.0). The amount of  $\text{LC}_2$  extracted varied with the temperature, duration of extraction, and whether or not troponin C (0.5 mg/ml) was added to the extraction solution. Extraction of 20–40%  $\text{LC}_2$  resulted in increased active tensions in the range of pCa's between 6.6 and 5.7, but had no effect upon maximum tension. The tension-pCa relationship was left-shifted to lower  $[\text{Ca}^{2+}]$  by as much as 0.2 pCa units after  $\text{LC}_2$  extraction. At low concentrations of  $\text{Ca}^{2+}$ , an increase in stiffness proportional to the increase in tension was observed. Readdition of  $\text{LC}_2$  to these fiber segments resulted in a return of tension and stiffness to near control values. Stiffness during maximal activation was unaffected by partial extraction of  $\text{LC}_2$ .  $\text{LC}_2$  extraction was shown to uniformly decrease (by 25–30%), the velocity of shortening during the high velocity phase but it did not significantly affect the low velocity phase of shortening. This effect was reversed by readdition of purified  $\text{LC}_2$  to the fiber segments. On the basis of these findings we conclude that  $\text{LC}_2$  may modulate the number of cross-bridges formed during  $\text{Ca}^{2+}$  activation and also the rate of cross-bridge detachment during shortening. These results are consistent with the idea that  $\text{LC}_2$  may modulate contraction via an influence upon the conformation of the S1-S2 hinge region of myosin.

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## INTRODUCTION

The myosin molecule consists of two heavy chains, each having a globular domain or S1 "head," a "neck" region termed S2, and a long peptide "tail." The S1 head contains the ATPase and actin binding sites, and has associated with it two classes of low molecular weight proteins termed light chains. One of these classes of light chains can be chemically dissociated with DTNB (5,5'-dithio-2-nitrobenzoic acid) while the other can be dissociated with alkaline solutions (Lowey and Risby, 1971; Weeds and Lowey, 1971). Poly- and monoclonal antibodies have been used to localize the DTNB light chain, or LC<sub>2</sub>, of skeletal muscle myosin at or near the S1-S2 junction (Winkelmann et al., 1983; Tokunaga et al., 1987), while the NH<sub>2</sub> terminus of the alkali light chain is ~10 nm distal to the S1-S2 junctional region (Waller and Lowey, 1985; Winkelmann and Lowey, 1986; Tokunaga et al., 1987). The alkali light chains of mammalian fast-twitch skeletal muscle, LC<sub>1</sub> and LC<sub>3</sub>, differ in molecular weight and are present in molar ratios of 2 mol of light chains/mol of myosin and 2 mol LC<sub>1</sub>/mol LC<sub>3</sub> (Weeds, 1969; Lowey and Risby, 1971). The type of alkali light chain that is present appears to correlate with the velocity of shortening of skinned muscle fibers (Greaser et al., 1988; Sweeney et al., 1988), although this property is determined primarily by the type of myosin heavy chain (Reiser et al., 1985). LC<sub>2</sub> is present in a ratio of 2 mol per mol of myosin (Lowey and Risby, 1971). While it is known that LC<sub>2</sub> is phosphorylated during contraction of striated muscles (Perrie et al., 1973) and that LC<sub>2</sub> possesses a binding site for divalent cations (Werber et al., 1972), there is as yet no clear view as to the overall role of LC<sub>2</sub> in the contraction of striated muscles.

Unlike the striated muscle of molluscs or mammalian smooth muscle, contraction of vertebrate striated muscle is believed to be regulated primarily at the level of the thin filament. An increase in [Ca<sup>2+</sup>] results in the binding of Ca<sup>2+</sup> to troponin C (TnC), a thin filament-associated regulatory protein, which leads to structural changes in the thin filament and allows the cross-bridge interaction cycle to proceed. Phosphorylation of LC<sub>2</sub> is not required for contraction in vertebrate striated muscle but has been shown under a variety of conditions to modulate actin-activated myosin ATPase activity (Persechini and Stull, 1984), tension generation (Sweeney and Stull, 1986; Metzger et al., 1989), and the rate of rise of tension (Metzger et al., 1989). In addition, the ATPase of myosin in the presence of deregulated thin filaments (i.e., lacking troponin) exhibits a sensitivity to Ca<sup>2+</sup> (Lehman, 1978; Pulliam et al., 1983) which decreases with LC<sub>2</sub> extraction (Pemrick, 1977; Oda et al., 1980; Margossian et al., 1983) and may be a manifestation of an effect of Ca<sup>2+</sup> upon the thick filament (see Discussion).

Previous work from this laboratory has shown that partial extraction of LC<sub>2</sub> results in a reversible decrease in unloaded shortening velocity ( $V_{max}$ ) in maximally activated skinned muscle fibers (Moss et al., 1982). These data, along with biochemical studies of the actin-activated ATPase of LC<sub>2</sub>-deficient myosin (Margossian et al., 1983), suggest that LC<sub>2</sub> may have a modulatory role involving the kinetics of interaction of actin and myosin. It has also been shown that LC<sub>2</sub> is required for Ca<sup>2+</sup>-sensitive binding of myosin to the thin filament (Wagner, 1984). Thus, the present study was undertaken to investigate a possible role of LC<sub>2</sub> in the Ca<sup>2+</sup> regulation of contraction of skeletal muscle by determining the effects of partial extraction of LC<sub>2</sub>

on (a) the tension-pCa relation, (b) maximum shortening velocity as a function of pCa, and (c) the stiffness-pCa relationship.

#### METHODS

Bundles of ~50 fibers were dissected from the psoas muscles of adult New Zealand rabbits. The bundles were tied to glass capillary tubes and stored at  $-22^{\circ}\text{C}$  in relaxing solution containing 50% (vol/vol) glycerol for up to 21 d before use (Moss et al., 1983a). An individual fiber was pulled from the end of the bundle, a control segment of the fiber was saved for later protein analysis, and an experimental segment 1.5–2.5 mm in length was mounted to the mechanical apparatus. The dissecting and mounting procedures, as well as the details of the experimental setup, have been described previously (Moss et al., 1983a). Mean sarcomere length in the relaxed fiber segment was initially adjusted to 2.3–2.6  $\mu\text{m}$  by changing overall length ( $L_0$ ). Fiber width, depth, and sarcomere length were measured at rest and during activation directly from photomicrographs at  $160\times$  magnification using methods similar to those described earlier (Moss, 1979). Experimental measurements of tension were done at  $15^{\circ}\text{C}$  in order to minimize the occurrence of sarcomere length nonuniformities. A brief series of experiments was done at  $30^{\circ}\text{C}$ , as described in the Results section.

The fiber segments were activated in solutions containing various concentrations of free calcium between pCa ( $-\log [\text{Ca}^{2+}]$ ) 6.60 and 4.5. The solutions contained 7 mM EGTA, 1 mM free  $\text{Mg}^{2+}$ , 20 mM imidazole (pH 7.00), 4.42 mM total ATP, 14.5 mM creatine phosphate, various free  $\text{Ca}^{2+}$  concentrations and sufficient KCl to adjust ionic strength to 180 mM. The pCa of the relaxing solution was 9.0. The computer program of Fabiato and Fabiato (1979) 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 constant for Ca-EGTA was corrected to  $15^{\circ}\text{C}$  and for an ionic strength of 180 mM (Fabiato and Fabiato, 1979). The solutions for the stiffness measurements at  $10^{\circ}\text{C}$  were made using the  $15^{\circ}\text{C}$  Ca-EGTA constants and used at  $10^{\circ}\text{C}$  without correcting the stability constant for temperature, although pH was adjusted to 7.0 at  $10^{\circ}\text{C}$ .

Before each experiment, the mounted fiber segment was bathed for 15–30 s in a solution of pCa 9.0 containing 0.3–0.4 mg/ml TnC, to insure that the fiber was fully TnC replete (TnC was prepared by the method of Greaser and Gergely, 1971). Measurements of tension at randomly selected submaximal pCa's were then obtained, with first, final, and interspersed contractions at pCa 4.5 to assess any decline in the performance of the fiber. At each pCa steady tension was allowed to develop, at which time the segment was rapidly (within 1 ms) slackened and subsequently relaxed. The difference between the steady developed tension and the tension baseline immediately after the slack step was measured as total tension. Active tension was obtained by subtracting the resting tension obtained at pCa 9.0 from the total tension. Tensions ( $P$ ) at each pCa are expressed as a fraction of the maximum ( $P_0$ ) obtained under the same conditions, i.e., either in the control fiber or after LC<sub>2</sub> extraction.

In-phase cross-bridge stiffness was measured at  $10^{\circ}\text{C}$  by applying a small amplitude ( $<0.1\%$   $L_0$ ) sinusoidal length change at one end of the fiber and measuring the resultant changes in force and sarcomere length. Details of similar experiments, with the exception of the sarcomere length monitoring system, have been presented previously (Allen and Moss, 1987). A sinusoidal frequency of 3.3 kHz was used because previous studies from this laboratory (Allen and Moss, 1987) have shown that in rabbit psoas fibers at  $10^{\circ}\text{C}$ , there is no phase shift between length and force at frequencies above 3 kHz. Sarcomere length was measured using the first-order line of a laser (He-Ne, 3 mW, Spectra Physics, Mountain View, CA) diffraction pattern projected onto a lateral effects photodiode (LSC-5D; United Detector Technologies, Culver City, CA). This system has a resolution of 0.5–1.0 nm/half-sarcomere with sufficient

intensity of the first-order line to allow sarcomere length measurements at all levels of activation (Metzger et al., 1989). The peak-to-peak amplitudes of sarcomere length ( $\Delta SL$ ) and tension ( $\Delta P$ ) were obtained from the averages of 10 consecutive oscillations, and stiffness was calculated as  $\Delta P/\Delta L$ . Relative stiffness was calculated as the percent of maximum stiffness obtained from the same fiber, control or LC<sub>2</sub> extracted, at pCa 4.5. Isometric tensions were determined by rapidly slackening the fiber and measuring between the midpoint of the force sinusoid and the force baseline.

$V_{\max}$  was measured using the slack-test method (Edman, 1979; Moss, et al., 1982). Briefly, slack was introduced at one end of the fiber (length step completed in 0.6–1.0 ms) and the time required to take up this slack was measured. For each fiber segment various amounts of slack were introduced at each pCa, ranging from four measurements during successive activations at pCa 4.5, to 24 measurements during a sustained contraction at pCa 6.3. The amount of length change ( $\Delta L$ ) was plotted vs. the duration ( $\Delta t$ ) of unloaded shortening, and  $V_{\max}$  was calculated as the slope of a straight line fitted to this data. Line fitting was done using the least-squares error method. At low levels of Ca<sup>2+</sup> activation, where slack test plots were biphasic (Moss, 1986), the breakpoint between the two phases was chosen by eye, and  $V_{\max}$  was determined for each phase. If the correlation coefficient ( $r$ ) for any fitted data was <0.95 the data was discarded.  $V_{\max}$  is expressed as muscle lengths ( $ML$ ) per second, calculated as:

$$\frac{\text{absolute velocity (mm s}^{-1}\text{)}}{ML(\text{mm})} \times \frac{SL (\mu\text{m})}{2.40 \mu\text{m}} \quad (1)$$

$V_{\max}$  is normalized to a sarcomere length of 2.40  $\mu\text{m}$  to account for differences between fibers in the numbers of sarcomeres in series and thus to allow comparisons of results among fibers.

In a given fiber, control measurements of the tension-pCa,  $V_{\max}$ -pCa and/or stiffness-pCa relationships were made first and LC<sub>2</sub> was subsequently extracted (Moss et al., 1982, 1983a). The fiber was treated in one of three ways to partially extract LC<sub>2</sub>: (method A) warmed to 30°C and bathed in 20 mM KCl, 20 mM EDTA, 5 mM imidazole (pH 7.0) for 3 h, (method B) warmed to 20°C and bathed in 50 mM KCl, 20 mM EDTA, 5 mM PO<sub>4</sub> (pH 7.0), 0.5 mg/ml TnC for 2 h; or (method C) warmed to 32–34°C and bathed in 20 mM KCl, 20 mM EDTA, 5 mM imidazole (pH 7.0) for 2 h. For each of the extraction solutions pH was adjusted at room temperature. Multiple extraction protocols were tried in an attempt to obtain more consistent results between fibers in terms of the amount of LC<sub>2</sub> extracted (see Results). Rigor tension developed rapidly in all extracting solutions, but this was quickly dissipated to <1%  $P/P_0$  by manually slackening the fiber via a micrometer-driven translator. After extracting for the indicated time, the chamber was cooled to 15 or 10°C, the fiber was returned to relaxing solution and its original length, and a photomicrograph was taken to verify that sarcomere length was similar to the value measured before treatment. Fibers exhibiting tensions in relaxing solution that were increased relative to control were briefly bathed in relaxing solution containing 0.3 mg/ml troponin (prepared according to the method of Greaser and Gergely, 1971). To be certain that the fibers were TnC replete, the fibers were then bathed for 15 s in relaxing solution containing 0.4 mg/ml TnC and activated at pCa 4.5 to assess the extent of TnC recombination. This cycle of TnC soak and maximal activation was repeated until there was no significant increase in active tension with additional TnC soaks. The tension-pCa,  $V_{\max}$ -pCa and/or stiffness-pCa relationships, as appropriate, were then assessed in the LC<sub>2</sub>-deficient fibers. In several instances, LC<sub>2</sub> (prepared according to Wagner, 1982) was recombined into the extracted fiber by bathing the fiber in relaxing solution containing 0.5 mg/ml purified LC<sub>2</sub> for 2–3 h at 15°C (Moss et al., 1982). The Ca<sup>2+</sup> dependence of  $V_{\max}$ , stiffness, and/or tension was then measured. After the mechanical measurements were completed, the experi-

mental fiber segment was placed in sodium deodecyl sulfate (SDS) sample buffer and stored at  $-80^{\circ}\text{C}$  for later analysis of protein composition.

The relative proportions of LC<sub>1</sub>, LC<sub>2</sub>, LC<sub>3</sub>, TnI, and TnC were determined in the control and experimental fibers by SDS-polyacrylamide gel electrophoresis (PAGE) and an ultrasensitive silver stain (Giulian et al., 1983). Densitometric scans of the gels were done, and the areas under the peaks were quantitated by planimetry. The amount of LC<sub>2</sub> present was expressed in relative terms as a fraction of the total LC<sub>1</sub> plus LC<sub>3</sub> present, since these light chains are unaffected by the extraction procedure (Moss et al., 1983a).

Statistically, a two-way analysis of variance was used to test whether LC<sub>2</sub> extraction and/or  $[\text{Ca}^{2+}]$  significantly affected either isometric force, stiffness or velocity. When significant interactions were found, a Student's two-tailed *t* test was used to test for significant differences between two means. A level of  $P < 0.05$  was chosen as indicating significance.

To determine whether there were gross effects of the extraction upon fiber ultrastructure, electron micrographs of longitudinal sections were obtained from control and LC<sub>2</sub>-extracted fibers (Fig. 1). In each case, fibers were tied into the experimental chamber, sarcomere length was adjusted as described, and the fiber was then fixed in relaxing solution containing 4% (vol/vol) glutaraldehyde. Fibers were either untreated or LC<sub>2</sub> extracted for 3 h at  $30^{\circ}\text{C}$  (20 mM KCl, 20 mM EDTA, 5 mM imidazole) before glutaraldehyde fixation. The main conclusion reached on the basis of these micrographs was that myosin did not dissociate from the ends of the thick filament as a result of the LC<sub>2</sub> extraction procedure. In random measurements it was established that the thick filament length in LC<sub>2</sub>-extracted fibers was  $100.9 \pm 0.4\%$  ( $\bar{x} \pm \text{SEM}$ ,  $n = 25$ ) of the control thick filament length (100.0%). No other structural changes were apparent from the electron micrographs of control and LC<sub>2</sub>-extracted fibers.

## RESULTS

### *SDS-PAGE of Control and Partially LC<sub>2</sub>-extracted Fibers*

As indicated in the Methods three different procedures were used to extract LC<sub>2</sub>. This was done in an attempt to obtain more consistent results between fibers in terms of the amount of LC<sub>2</sub> extracted. The three methods resulted in the extraction of different amounts of LC<sub>2</sub>, with no apparent differences between methods with regard to variability. After mechanical measurements were made the fiber segments were run on SDS polyacrylamide gels and protein composition was determined. Densitometric scans of the low molecular weight proteins (<30 kD) are shown in Fig. 2 for one fiber both before and after the LC<sub>2</sub> extraction procedure. The percent depletion of LC<sub>2</sub> was calculated from the change in the LC<sub>2</sub>/(LC<sub>1</sub> + LC<sub>3</sub>) ratio upon extraction. For the fiber in Fig. 2, 24.3% of the LC<sub>2</sub> was removed, while cumulative data from all fibers indicate that  $27.8 \pm 3.7\%$  LC<sub>2</sub> ( $\bar{x} \pm \text{SEM}$ ,  $n = 9$ ) was removed using extraction method C. From these gels it was also apparent that the fibers were TnC replete during the mechanical measurements since there were no significant differences in the TnC/TnI ratios between the control and LC<sub>2</sub>-extracted segments of the same fibers. As noted previously (Moss et al., 1982) the LC<sub>2</sub>/(LC<sub>1</sub> + LC<sub>3</sub>) and LC<sub>3</sub>/LC<sub>1</sub> ratios of 0.70 and 0.15 were below the expected values of 1.0 and 0.5, respectively, due to differential staining intensity of the light chains by silver when compared with Coomassie brilliant blue. The differential staining properties of silver stain also account for the apparent low quantities of troponin subunits. The intensities of the troponin subunit bands could have been enhanced by additional cycles of silver staining; instead, the staining protocol was optimized for light

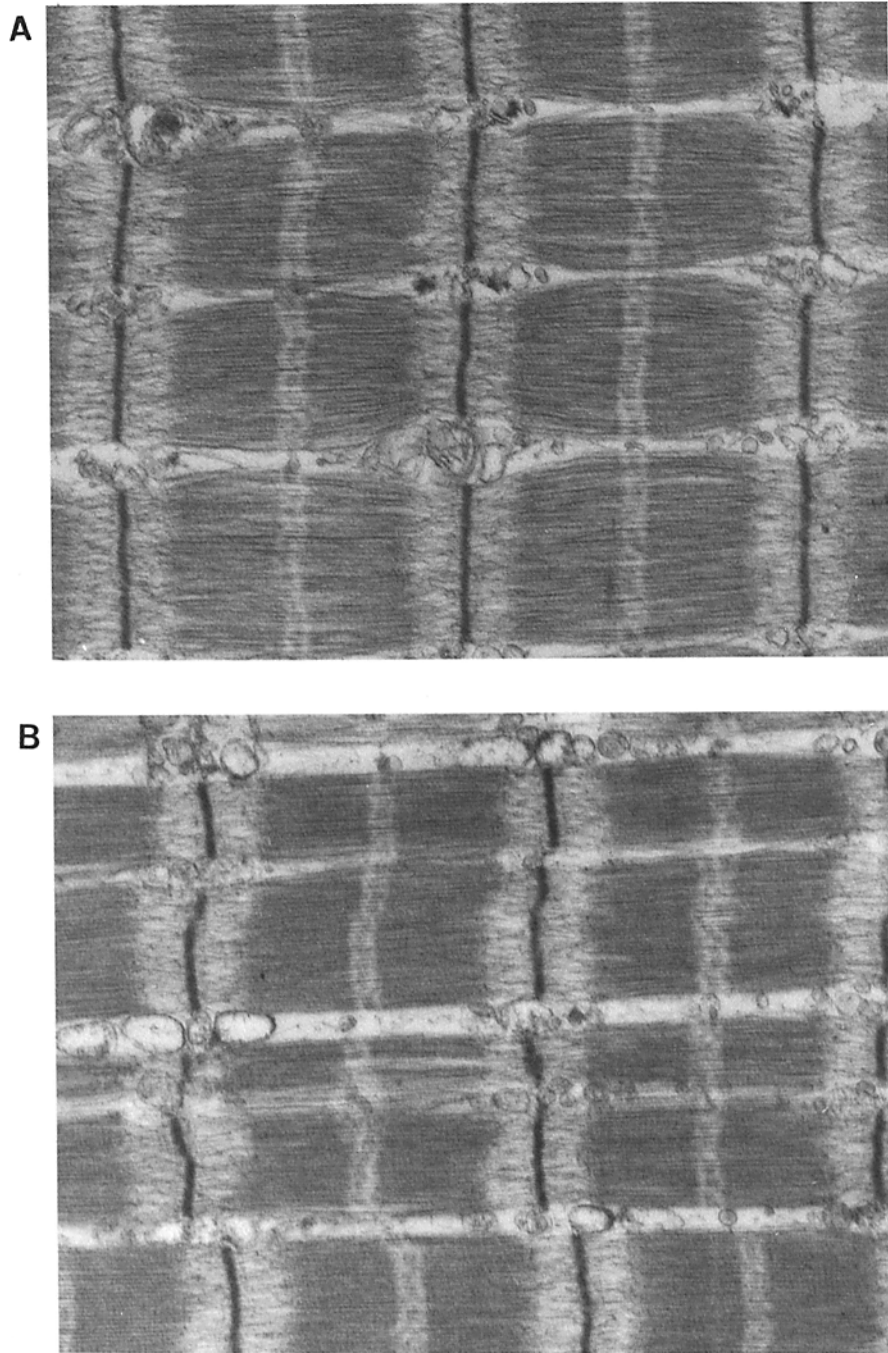


FIGURE 1. Electron micrographs (25,000 $\times$ ) of skinned fibers from rabbit psoas muscle (*A*) before treatment and (*B*) after partial extraction of LC<sub>2</sub> using method A. Sarcomere length before extraction was 2.32  $\mu\text{m}$ .

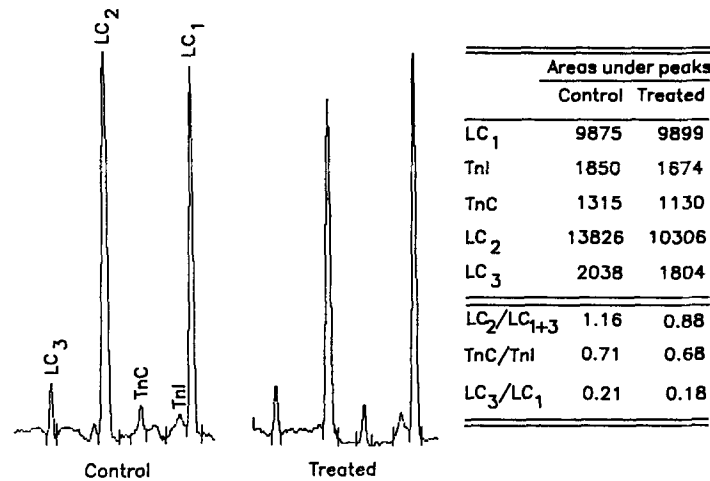


FIGURE 2. Densitometric scans of the low molecular weight region of SDS polyacrylamide gels from segments of a single psoas fiber before (control) and after (treated) LC<sub>2</sub> extraction. LC<sub>2</sub> was partially extracted using method C, and the fiber was subsequently recombined with troponin and TnC. The areas under the peaks (in arbitrary area units) corresponding to the identified proteins are listed in the adjacent table. Approximately 25% of the LC<sub>2</sub> was extracted as determined from the change in the LC<sub>2</sub>/(LC<sub>1</sub> + LC<sub>3</sub>) ratio; the content of TnC normalized to the TnI peak in each scan was equivalent in the control (TnC/TnI = 0.71) and extracted (TnC/TnI = 0.68) segments.

chain staining. Additional gels showed that the LC<sub>2</sub> content of segments extracted using method A was reduced by  $37.0 \pm 4.9\%$  ( $\bar{x} \pm \text{SEM}$ ,  $n = 6$ ) relative to control segments, and segments extracted using method B typically showed a 20% depletion of LC<sub>2</sub> relative to control values.

*Alteration in the Tension-pCa Relation after Partial LC<sub>2</sub> Extraction*

The effect of partial extraction of LC<sub>2</sub> on developed isometric tension is shown for one fiber in Fig. 3. The cumulative results from five fibers that were extracted with method A are shown in Fig. 4. Maximum tension after extraction decreased to  $92.0 \pm 1.0\%$  ( $n = 5$ ) of control values and was unaffected by readdition of TnC or LC<sub>2</sub>; in two of these fibers the tensions measured at pCa 4.5 after extraction were identical to control. In later studies using the LC<sub>2</sub> extraction method B, no decrease

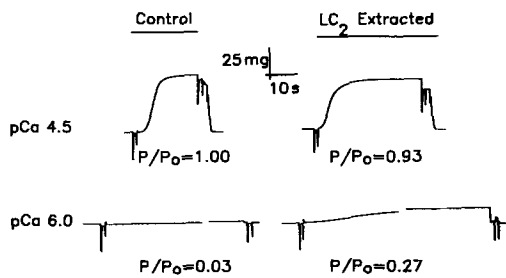
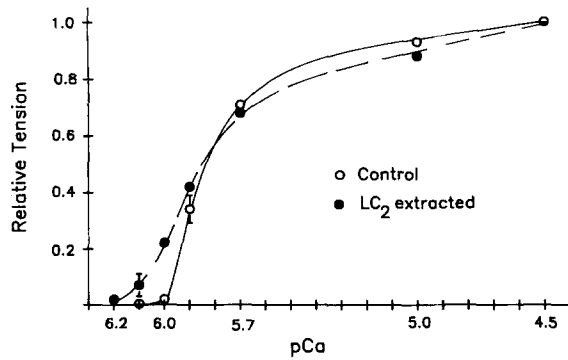


FIGURE 3. Original tension records at pCa 4.5 and 6.0 before treatment (control) and after LC<sub>2</sub> extraction in a single psoas fiber. Using method A, 35.2% of the endogenous LC<sub>2</sub> was extracted from the fiber. The break in the tension records at pCa 6.0 represents 100 s. Fiber No. 32488; overall fiber length, 1.76 mm; sarcomere length, 2.40  $\mu\text{m}$ .



	pCa <sub>50</sub>	n <sub>1</sub>	n <sub>2</sub>
Control	5.81	0.98	10.00
LC <sub>2</sub> extracted	5.89	1.30	5.36

FIGURE 4. Cumulative data from five fibers showing the variation of tension with pCa both before (i.e., control) (○) and after partial extraction of LC<sub>2</sub> (●). An average of 35% of the LC<sub>2</sub> was extracted using method A. Relative tension was obtained by scaling absolute tension to the control value obtained in the same fiber at pCa 4.5. Each point represents the mean ± SEM ( $n = 5$ ); SEM bars smaller than symbol size are not shown. The pCa at half-maximal tension (pCa<sub>50</sub>) and the Hill coefficients ( $n_1$  and  $n_2$ ) are as indicated below (for a review of Hill coefficients see Cornish-Bowden and Koshland, 1975).

in maximally activated tension was observed after LC<sub>2</sub> extraction (Table I). Thus, the fall in mean maximum tension probably represents a nonspecific decline in the performance of some of the fibers rather than being a specific result of LC<sub>2</sub> extraction. Of greater interest were the consistently observed increases in tension developed in the range of pCa's between 5.9 and 6.2, increases that were unaffected by bathing the fibers in troponin or TnC. It should be noted that this effect of LC<sub>2</sub> extraction is opposite from the previously observed decrease in tension at partial activation after

TABLE I  
Absolute Values of P<sub>o</sub>/Cross-sectional Area, Stiffness, and Elastic Modulus  
for Individual Fibers at pCa 4.5

Fiber No.	P <sub>o</sub> /CSA		Stiffness		Elastic Modulus	
	Control	LC <sub>2</sub> Extr.	Control	LC <sub>2</sub> Extr.	Control	LC <sub>2</sub> Extr.
	<i>N/m<sup>2</sup></i>		<i>N/m</i>		<i>N/m<sup>2</sup></i>	
8188	0.75 × 10 <sup>5</sup>	0.78 × 10 <sup>5</sup>	28.61	32.52	1.45 × 10 <sup>7</sup>	1.65 × 10 <sup>7</sup>
8288	1.22 × 10 <sup>5</sup>	1.02 × 10 <sup>5</sup>	22.65	26.47	1.78 × 10 <sup>7</sup>	1.90 × 10 <sup>7</sup>
8488	1.09 × 10 <sup>5</sup>	0.96 × 10 <sup>5</sup>	16.65	18.48	1.08 × 10 <sup>7</sup>	1.20 × 10 <sup>7</sup>
8588	1.04 × 10 <sup>5</sup>	1.01 × 10 <sup>5</sup>	23.62	24.42	2.29 × 10 <sup>7</sup>	2.37 × 10 <sup>7</sup>
83088	1.21 × 10 <sup>5</sup>	1.20 × 10 <sup>5</sup>	34.99	33.11	2.80 × 10 <sup>7</sup>	2.65 × 10 <sup>7</sup>
83188	1.03 × 10 <sup>5</sup>	1.04 × 10 <sup>5</sup>	31.85	30.84	2.50 × 10 <sup>7</sup>	2.42 × 10 <sup>7</sup>
9188	1.39 × 10 <sup>5</sup>	1.36 × 10 <sup>5</sup>	57.80	56.42	3.97 × 10 <sup>7</sup>	3.88 × 10 <sup>7</sup>
9288	1.42 × 10 <sup>5</sup>	1.64 × 10 <sup>5</sup>	11.18	10.53	1.55 × 10 <sup>7</sup>	1.46 × 10 <sup>7</sup>
Means	1.14 × 10 <sup>5</sup>	1.13 × 10 <sup>5</sup>	28.42	29.10	2.18 × 10 <sup>7</sup>	2.19 × 10 <sup>7</sup>
± SEM	0.08 × 10 <sup>5</sup>	0.09 × 10 <sup>5</sup>	5.02	4.75	0.33 × 10 <sup>7</sup>	0.30 × 10 <sup>7</sup>

Partial extraction of LC<sub>2</sub> was done using method B. Cross-sectional area (CSA) was calculated from width and depth measurements, assuming an elliptical area. Stiffness was calculated as:  $\Delta F/[(\Delta SL) \cdot (\text{Number of sarcomeres})]$ . Elastic modulus was calculated as:  $\Delta \text{force (N)}/\text{CSA (m}^2) \div \Delta \text{length (m)}/\text{total fiber length (m)}$ .



the specific extraction of TnC (Moss et al., 1985). The increases in submaximal Ca<sup>2+</sup>-activated tension observed with partial extraction of LC<sub>2</sub> were shown to be reversible in a number of fibers by recombination of purified LC<sub>2</sub> into the fiber segment. Fig. 5 illustrates one fiber in which LC<sub>2</sub> recombination led to reversal of the effects of LC<sub>2</sub> extraction.

As described in the Methods, experiments were normally conducted at 10 or 15°C to maintain uniformity of sarcomere length. (At 15°C, mean sarcomere length was  $2.56 \pm 0.06 \mu\text{m} \pm \text{SD}$ ). However, one series of experiments was done at 30°C to determine whether the effects observed at 10 and 15°C due to LC<sub>2</sub> extraction would also occur at more nearly physiological temperatures. Each of these fibers was activated a total of only five times at 30°C, since sarcomere length uniformity rapidly deteriorated during successive activations. The untreated fiber was first activated maximally at pCa 4.5, then partially activated at pCa 6.5, and again maximally activated. The use of pCa 6.5 was necessary to obtain the desired low level of activation at 30°C in the control fiber and is consistent with the leftward shift of the tension-pCa relation observed when temperature is increased (Sweitzer, N., and R. Moss,

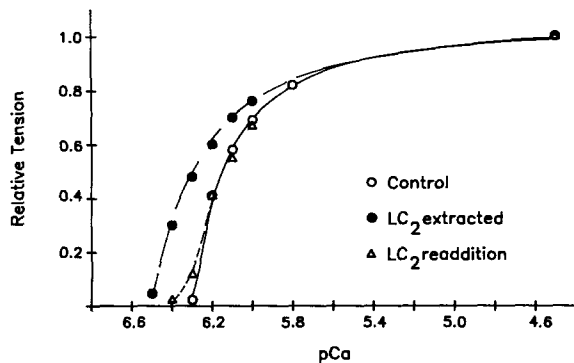


FIGURE 5. Tension-pCa relationships obtained from a single psoas fiber before (○) and after LC<sub>2</sub> extraction (●) by method B and, finally, after readdition of LC<sub>2</sub> (△). Fiber No. 71488J; overall fiber length 2.14 mm; sarcomere length 2.63  $\mu\text{m}$ .

unpublished observation). The fiber was then cooled to 20°C, and LC<sub>2</sub> was extracted for 2 h according to method B. After extraction, the fiber was briefly bathed in troponin- and TnC-containing relaxing solutions. After warming to 30°C, the fiber was again activated, first at pCa 6.5 and then at pCa 4.5. Photomicrographs of maximally activated fibers indicated that average sarcomere length at 30°C was  $2.58 \pm 0.19 \mu\text{m}$  ( $\bar{x} \pm \text{SD}$ ), with considerable variation in the uniformity of the sarcomere length pattern. Before LC<sub>2</sub> extraction, the fibers generated a tension of  $16.0 \pm 0.0\% P_0$  ( $\bar{x} \pm \text{SEM}$ ,  $n = 4$ ) at pCa 6.5, while after extraction tension at the same pCa increased to  $32.0 \pm 1.0\%$  ( $\bar{x} \pm \text{SEM}$ ,  $n = 4$ ). Maximal tension after extraction decreased to  $92.0 \pm 2.0\%$  of control ( $\bar{x} \pm \text{SEM}$ ,  $n = 4$ ).

There was some variation in the absolute positions of control tension-pCa relationships obtained in different fibers at 15°C. For example, in Fig. 5 a pCa<sub>50</sub> value of 6.17 was obtained from the control measurements while for Fig. 4 the control pCa<sub>50</sub> was 5.81. These differences in pCa<sub>50</sub> values have occasionally been observed in this laboratory and are associated with different rabbits. Addition of troponin or TnC to otherwise untreated control fibers did not alter the position of these rela-

tionships, indicating that the fibers are replete in these subunits. While  $pCa_{50}$ 's may vary significantly between animals, there are no significant differences in the control tension-pCa relations obtained in different fibers from the same animal, as is the case in comparing the control data of Fig. 5 ( $pCa_{50} = 6.17$ ) and Fig. 7, below ( $pCa_{50} = 6.16$ ). In the present study, for a given figure, the control,  $LC_2$ -extracted and  $LC_2$ -readded data is paired such that the data for each condition is obtained from the same single muscle fiber. It is possible that between-animal variations in  $pCa_{50}$  are due to differences in contractile or regulatory protein isoforms, but we have not systematically investigated this point.

#### *Effect of Partial Extraction of $LC_2$ on Stiffness*

Fig. 6 shows tracings of original records of force and sarcomere length obtained while imposing a 3.3 kHz sinusoid ( $\Delta L < 0.1\%$  of total muscle length or  $\sim 2$  nm/half sarcomere) during activations at pCa 4.5 and 6.2. The averaged tension and stiffness results from control and  $LC_2$ -extracted (method B) fibers are shown in Fig. 7. As described above,  $LC_2$  extraction led to an increase in tension at low  $[Ca^{2+}]$ , which was reversed by readdition of purified  $LC_2$ . An increase in relative stiffness was also

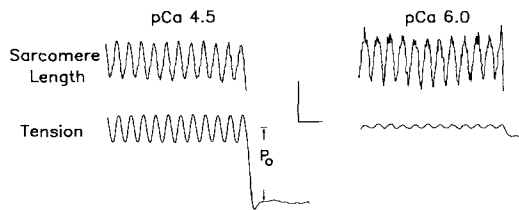


FIGURE 6. Original records of tension and sarcomere length obtained during stiffness measurements at pCa 4.5 and 6.0. The vertical calibration bar represents a force of  $0.5 \times 10^5$  N/m<sup>2</sup> and a length change of 2 nm/half sarcomere, while the horizontal bar represents 0.6 ms. Fiber No. 92288; overall fiber length 3.18 mm; sarcomere length 2.58  $\mu$ m; experimental temperature 10°C.

observed (Fig. 7 B) at pCa values of 6.0 and greater, and this effect was reversed by  $LC_2$  readdition. As shown in Table I,  $P_o$ /cross-sectional area, stiffness, and elastic modulus at pCa 4.5 were unaffected by extraction of 20% of  $LC_2$ . The mean elastic modulus of these fibers is in good agreement with the values reported by Tawada and Kimura (1984):  $2.5 \times 10^7$  N/m<sup>2</sup> (rabbit psoas in rigor), and Allen and Moss (1987):  $2.1 \times 10^7$  N/m<sup>2</sup> (rabbit psoas, pCa 4.5). The increases in tension and stiffness observed at low  $Ca^{2+}$  after  $LC_2$  extraction were proportionate, as can be seen in plots of relative stiffness vs. relative force measured in both control and  $LC_2$ -extracted fibers (Fig. 8). The lines drawn in Fig. 8 are the linear best fits to the data, with a slope of 1.03 for both the control ( $r = 0.98$ ) and  $LC_2$ -extracted ( $r = 0.97$ ) fibers.

#### *Differential Effects of $LC_2$ Extraction on the $Ca^{2+}$ Sensitivities of the High and Low Velocity Phases of Shortening*

The control measurements of maximum shortening velocity ( $V_{max}$ ) as a function of pCa, illustrated for one fiber in Fig. 9, confirm earlier work from this laboratory (Moss, 1986; Metzger and Moss, 1988) in which  $V_{max}$  was observed to decrease when

[Ca<sup>2+</sup>] was decreased. Plots of  $\Delta L$  vs.  $\Delta t$  were biphasic at low concentrations of Ca<sup>2+</sup> (i.e., pCa  $\geq$  5.9). The initial high velocity phase of shortening was relatively insensitive to [Ca<sup>2+</sup>], in that  $V_{\max}$  decreased only at levels of Ca<sup>2+</sup> corresponding to steady tensions less than  $\sim 0.3 P_0$ .  $V_{\max}$  in the subsequent low velocity phase decreased

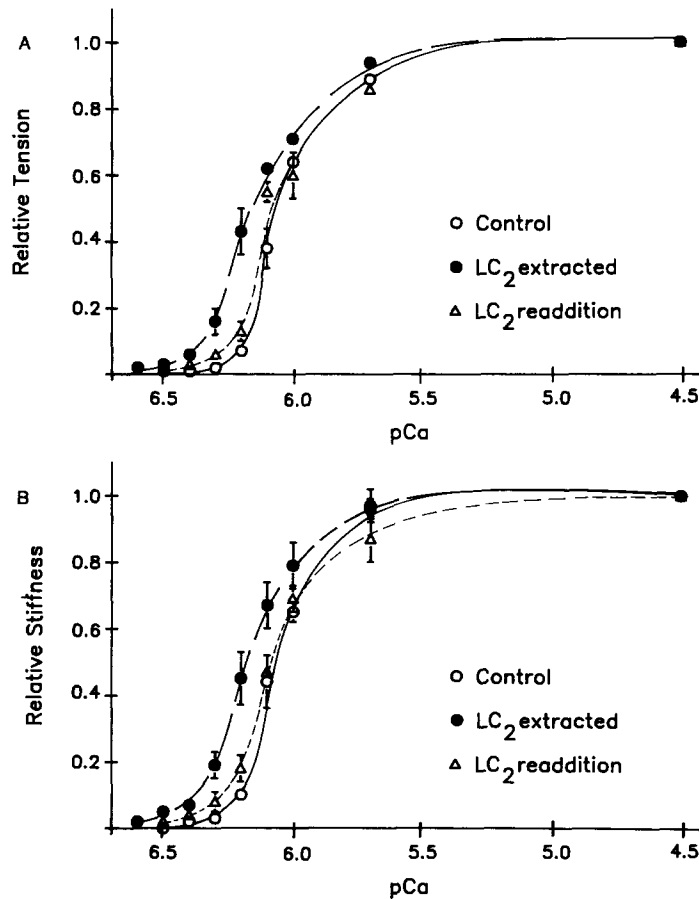


FIGURE 7. Cumulative (A) tension-pCa and (B) stiffness-pCa data from control (○), LC<sub>2</sub> extracted (●), and LC<sub>2</sub> recombined (△) fibers. Approximately 20% of the LC<sub>2</sub> was extracted using method B. Control data is from nine fibers, five of which yielded data after LC<sub>2</sub> extraction, and of these, three fibers recovered to control values upon readdition of LC<sub>2</sub>. All data are scaled to the value obtained at pCa 4.5 under the same experimental condition (i.e., control, extracted, or recombined). Each point is the mean  $\pm$  SEM (SEM bars smaller than the symbol size are not shown).

markedly as pCa was increased. The transition from high to low velocity shortening occurred after shortening equivalent to 40–80 nm/half sarcomere.

The effects of partial extraction of LC<sub>2</sub> (method C) on  $V_{\max}$  at various pCa's are shown for one fiber in Fig. 10. For this particular fiber,  $\sim 31\%$  of the endogenous LC<sub>2</sub> was extracted. At pCa 4.5 (Fig. 10 A), such extraction resulted in a decrease in

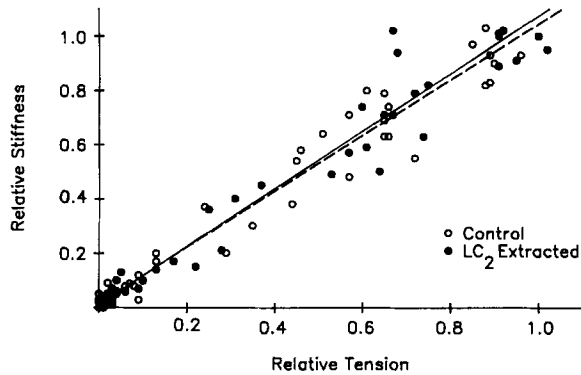


FIGURE 8. Plot of relative stiffness vs. relative tension measured in control fibers and after partial extraction of  $LC_2$ . Each point is an individual measurement, with lines drawn to the first-order best fit for each of the two sets of data. This same stiffness and tension data are plotted cumulatively vs. pCa in Fig. 7.

$V_{max}$  from 3.85  $ML/s$  (control) to 2.38  $ML/s$ , an effect which is in quantitative agreement with the results of Moss et al. (1982). At submaximal pCa's (Fig. 10, B and C),  $V_{max}$  in the initial, high velocity phase decreased after partial extraction of  $LC_2$ , even while isometric tension generating capability increased.  $V_{max}$  in the low velocity phase was unaffected by the extraction. After extraction, the amount of shortening in the high velocity phase decreased so that the breakpoint between the high and low velocity phases occurred earlier (Fig. 10).

The results of the velocity measurements from all fibers are summarized in Fig. 11. At each level of thin filament activation, obtained by varying the pCa of the activating solution, partial extraction of  $LC_2$  resulted in a uniform 25–30% decrease in the velocity of shortening during the high velocity phase (Fig. 11 A). Again,  $V_{max}$  in the low velocity phase was unaffected by  $LC_2$  extraction (Fig. 11 B).

The observed decrease in the amount of shortening to the breakpoint between high and low velocity phases due to  $LC_2$  extraction (Fig. 10) was examined further in

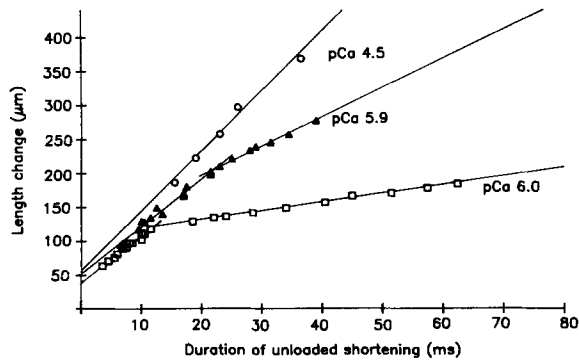


FIGURE 9. Plots of slack test data obtained from a single control psoas fiber segment at pCa's 4.5, 5.9, and 6.0. Fiber No. 12088; overall fiber length of 2.08 mm; initial sarcomere length of 2.38  $\mu m$ .

	pCa		
	4.5	5.9	6.0
$V_{max}$ , high V phase ( $ML/s$ )	4.18	3.43	3.32
$V_{max}$ , low V phase ( $ML/s$ )	—	2.09	0.61
Shortening to breakpoint (nm/half sarcomere)	—	38.7	33.7

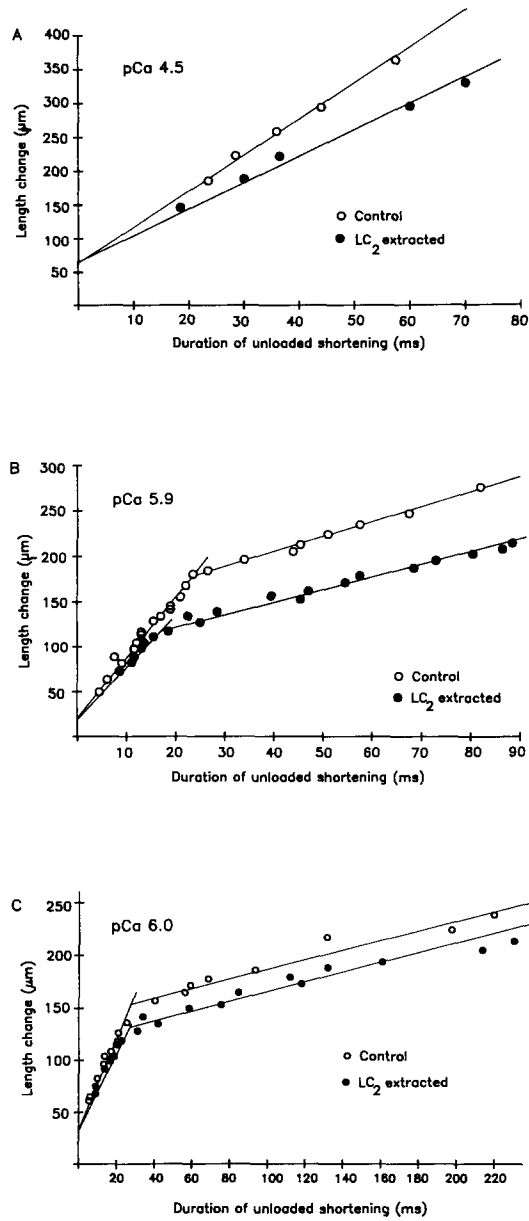


FIGURE 10. Plots of slack-test data obtained from a psoas fiber segment at (A) pCa 4.5, (B) pCa 5.9, and (C) pCa 6.0 before and after extraction (method C) of 31% of the total LC<sub>2</sub>. Fiber No. 12188; overall fiber length of 1.45 mm; initial sarcomere length of 2.34 µm.

	Control			LC <sub>2</sub> extracted		
	4.5	5.9	6.0	4.5	5.9	6.0
pCa	4.5	5.9	6.0	4.5	5.9	6.0
High V phase (ML/s)	3.85	3.95	2.54	2.38	3.09	2.25
Low V phase (ML/s)	—	1.06	0.29	—	0.80	0.26
Breakpoint (nm/half sarcomere)	—	83.6	74.0	—	53.6	64.2

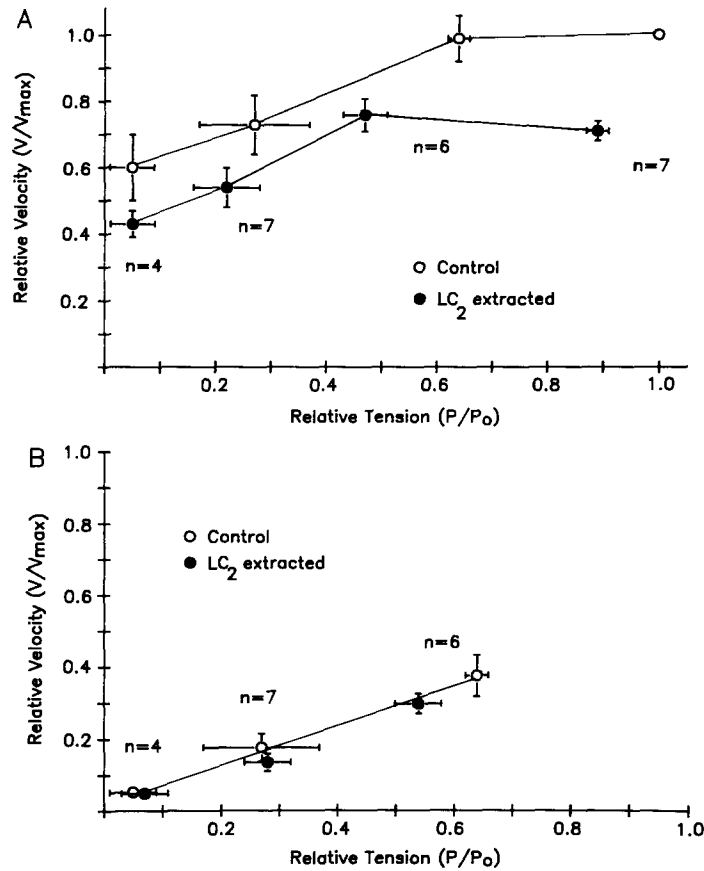


FIGURE 11. Relative  $V_{max}$  normalized to  $V_{max}$  at pCa 4.5, as a function of the level of thin filament activation (i.e., isometric tension scaled to  $P_0$ ) in paired control and  $LC_2$ -extracted fiber segments for the high (A) and low (B) velocity phases of shortening. Each point is the mean  $\pm$  SEM of four to seven determinations.

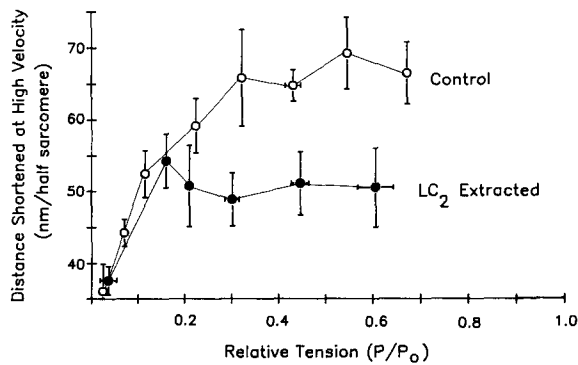


FIGURE 12. Plots of the distance shortened during the high velocity phase as a function of relative tension ( $P/P_0$ ) for control and partially  $LC_2$ -extracted fibers. Each point is the mean  $\pm$  SEM of three to nine measurements.

each of the fibers studied (Fig. 12). Partial extraction of LC<sub>2</sub> consistently reduced the distance shortened in the high velocity phase, with an approximate 20% reduction in the mean shortening distance in this phase. In all fibers tested, extraction of LC<sub>2</sub> reduced the amount of shortening to the breakpoint; however, only at higher

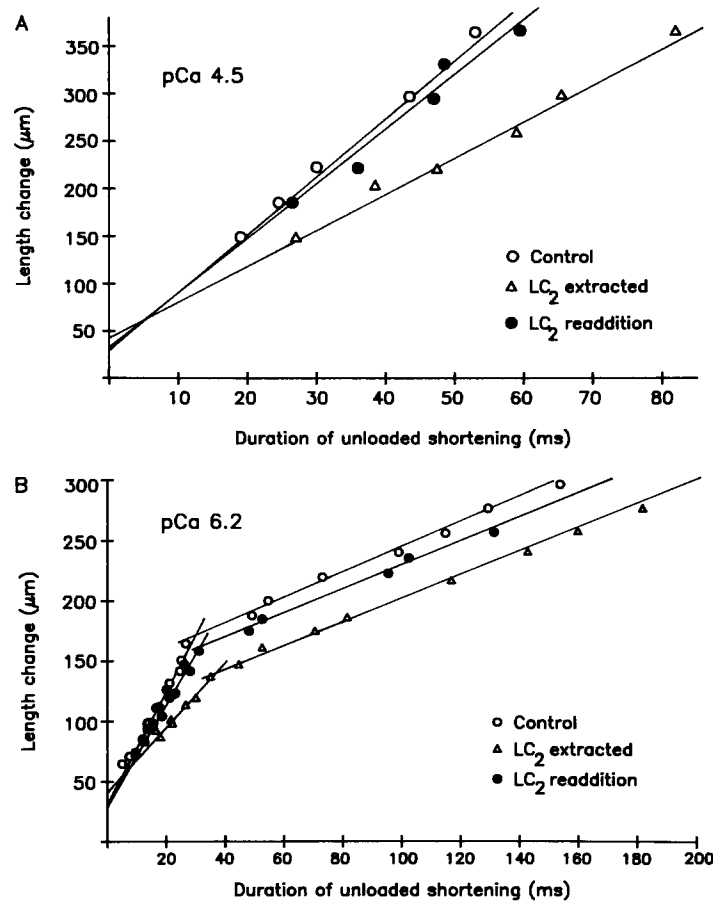


FIGURE 13. Plots of slack-test data obtained from a psoas fiber segment before and after LC<sub>2</sub> extraction (method C) and, finally, after readdition of LC<sub>2</sub>: (A) at pCa 4.5 and (B) at pCa 6.2. The LC<sub>2</sub>/(LC<sub>1</sub> + LC<sub>3</sub>) ratio in the control segment was 0.83 while in the LC<sub>2</sub>-recombined segment this ratio was 0.80.

	Control (ML/s)		<sup>V<sub>max</sub></sup> LC <sub>2</sub> extracted (ML/s)		LC <sub>2</sub> readded (ML/s)	
	High V phase	Low V phase	High V phase	Low V phase	High V phase	Low V phase
pCa 4.5	4.61	—	2.83	—	4.19	—
pCa 6.2	3.06	0.76	1.82	0.73	2.93	0.69

Fiber No. 11288: overall fiber length of 1.35 mm; initial sarcomere length of 2.35 μm.

relative tensions, i.e., 0.30–0.70  $P_o$ , was the difference in the shortening to the breakpoints found to be statistically significant.

Several experiments were done to determine whether the effects of the LC<sub>2</sub> extraction procedure could be specifically reversed by recombination of LC<sub>2</sub> into the fiber segments. The results presented in Fig. 13 are from one such fiber in which sequential measurements of  $V_{max}$  were obtained (a) in the control fiber, (b) in the same fiber after partial extraction of LC<sub>2</sub> and subsequent recombination of TnC and whole troponin, and (c) after a 2-h soak in relaxing solution with purified LC<sub>2</sub> at 15°C. Clearly, the reductions in  $V_{max}$  after LC<sub>2</sub> extraction were reversed upon recombination of LC<sub>2</sub> into the fiber segment.

## DISCUSSION

### *Mechanical Effects of Partial Extraction of LC<sub>2</sub>*

*Tension.* Tension-pCa relationships obtained in control and in LC<sub>2</sub>-extracted fibers (Fig. 4) showed that the Ca<sup>2+</sup> sensitivity of isometric tension increased after partial extraction of LC<sub>2</sub>. A similar leftward shift was observed in earlier studies after partial extraction of whole troponin (Moss et al., 1986), with osmotic compression of the myofilament lattice (Godt and Maughan, 1981; Allen and Moss, 1987; Metzger and Moss, 1988), and by increasing sarcomere length (Endo, 1973; Moss et al., 1983b; Allen and Moss, 1987). Each of these observations, including the present results, can be explained on the basis of an increase in the cooperative activation of the thin filament due to increased cross-bridge formation, which in turn enhances Ca<sup>2+</sup> binding to TnC (Bremel and Weber, 1972; Guth and Potter, 1987) and subsequently increases the number of cross-bridge attachments in adjacent regions of the thin filament. The 1:1 proportionate increases in relative tension and relative stiffness in the LC<sub>2</sub>-extracted fibers (Fig. 8), and the finding that maximum tension and stiffness were unaffected by extraction, strongly support the idea that at low [Ca<sup>2+</sup>] the number of attached cross-bridges increases after extraction of LC<sub>2</sub>. Thus, there appears to be no change in the tension-generating capabilities of the cross-bridges that are LC<sub>2</sub> deficient. As discussed previously in a different context (Moss et al., 1986), the apparent decrease in cooperativity suggested by the decrease in the Hill coefficients after LC<sub>2</sub> extraction does not necessarily reflect a decrease in the cooperative activation of the thin filament by myosin heads. Rather, partial extraction of LC<sub>2</sub> may reduce the Ca<sup>2+</sup> dependence of cooperative activation, resulting in a lower Ca<sup>2+</sup> threshold for activation of tension development.

The finding that LC<sub>2</sub> extraction increases the number of attached cross-bridges at low [Ca<sup>2+</sup>] is consistent with the results of other studies indicating that the Ca<sup>2+</sup> sensitivity of cross-bridge binding to regulated actin requires LC<sub>2</sub> (Wagner and Giniger, 1981; Wagner, 1984). Solution biochemical studies at low [Ca<sup>2+</sup>], low ionic strength ( $\mu = 0.02$  M), and in the presence of MgATP show that the extraction of LC<sub>2</sub> results in a 10-fold greater affinity of binding of myosin to regulated actin (Wagner, 1984). This increase in binding affinity after extraction of LC<sub>2</sub> in proteins reconstituted in solution is a plausible biochemical correlate of the increase in cross-bridge formation observed in our skinned fiber preparations at low [Ca<sup>2+</sup>].



To maintain sarcomere length uniformity, tension-pCa relationships were obtained at 15°C, which is substantially below the in vivo temperature. Stiffness was measured at 10°C to maintain force in phase with length at the sinusoidal frequency (3.3 kHz) used for these measurements. Previous studies have shown that reduced temperature decreases the force developed per attached cross-bridge (Ford et al., 1977; Bressler, 1981; Goldman et al., 1987; Kossler et al., 1987). Also, studies of melting profiles for the S1-S2 hinge region of myosin suggest that during activation,  $\alpha$ -helical melting for the most part occurs between 15 and 25°C (Fig. 7 of Ueno and Harrington, 1986). The results of these earlier studies raised the possibility that the effects of LC<sub>2</sub> extraction observed in this study might occur only at low temperature and thus may not be seen at physiological temperatures. For example, the physical properties of myosin may be affected differently by extraction of LC<sub>2</sub> at 15°C vs. temperatures higher than 25°C due to the greater  $\alpha$ -helical content of myosin at 15°C. With this in mind, the effect of LC<sub>2</sub> extraction on tension at 30°C was studied at one submaximal pCa in several fibers. Partial extraction of LC<sub>2</sub> caused a twofold increase in isometric tension at submaximal activation (pCa 6.5). Comparison with results obtained at 15°C showed that partial extraction of LC<sub>2</sub> caused an estimated 2.6-fold increase in tension from 16%  $P_0$  in the control fibers (16%  $P_0$  corresponds approximately to pCa 6.27 in Fig. 5). Thus, the effects of partial extraction of LC<sub>2</sub> on submaximal tension are qualitatively similar at 15 and 30°C, which suggests that the results obtained at the lower temperature are physiologically relevant. Attempts to assess the entire tension-pCa relationship and to measure velocity at 30°C were not successful due to a consistent loss of sarcomere length uniformity during repeated activations at this temperature.

*Shortening velocity.* The slack test provides a direct measure of  $V_{\max}$ , which in the Huxley (1957) model of contraction is limited by the detachment rate constant ( $g_2$ ) for cross-bridges bearing a compressive load. At pCa 4.5, the plots of  $\Delta L$  vs.  $\Delta t$  from control skinned fibers (Fig. 9) were well fit by a single straight line, indicating that the internal load imposed by cross-bridges under compression remains constant as shortening proceeds. At submaximal pCa's these plots were biphasic but maintained linearity in each phase. As discussed previously (Moss, 1986; Metzger and Moss, 1988), this suggests that during shortening at partial activation, internal load increases rather suddenly. It has been proposed that the amount of shortening to the breakpoint, i.e., 40–80 nm/half sarcomere, represents the compression of the myosin S2 of long-lived cross-bridges formed in zones of transition between active and inactive regions of the thin filament, with a subsequent buckling and compressive strain of S-2 that opposes contraction (Moss, 1986; Metzger and Moss, 1988).  $V_{\max}$  during the low velocity phase of shortening has been shown to be Ca<sup>2+</sup> sensitive (Fig. 9 and Moss, 1986), an effect which according to this hypothesis would be a result of a greater proportion of attached bridges being formed of the long-lived type as the level of activation is reduced.

Upon partial extraction of LC<sub>2</sub>,  $V_{\max}$  in the high velocity phase of shortening was found to decrease by a similar proportion at all pCa's tested (Fig. 11 B). One explanation for this finding is that the rate of dissociation of cross-bridges (i.e., rate constant  $g_2$ ) decreases with extraction of LC<sub>2</sub>, which was suggested previously to account for the decrease in  $V_{\max}$  during maximal activation (Moss et al., 1983a).

Since the low velocity phase of shortening was unaffected by partial extraction of LC<sub>2</sub> (Fig. 11 B), it seems unlikely that the effects of extraction involve alterations in the proposed population of long-lived cross-bridges. The absence of an effect of extraction on the low velocity phase may indicate only that the internal load during this phase is sufficiently great to mask effects due to LC<sub>2</sub> extraction alone.

In terms of a mechanism for the decrease in  $V_{\max}$ , it should be noted that the effect of LC<sub>2</sub> extraction is qualitatively different from alterations in  $V_{\max}$  due to TnC extraction. With TnC extraction, decreases in  $V_{\max}$  in both the high and low velocity phases of shortening were related to concomitant decreases in the level of thin filament activation (Moss, 1986). A change in thin filament activation does not appear to be the mechanism for decreased  $V_{\max}$  after LC<sub>2</sub> extraction, since at similar levels of thin filament activation,  $V_{\max}$  in only the high velocity phase was reduced by partial extraction of LC<sub>2</sub> (Fig. 11 A). Also, with TnC extraction, the slack test plot at pCa 4.5 becomes biphasic (Moss, 1986), an effect that has been attributed to a decrease in the level of thin filament activation. In the present study, the slack test plot at pCa 4.5 remained monophasic after LC<sub>2</sub> extraction.

In considering other mechanisms that might explain the effects of LC<sub>2</sub> extraction on  $V_{\max}$  it is important to take into account our earlier conclusion that LC<sub>2</sub> extraction does not affect the force or stiffness per attached cross-bridge. Thus, the decrease in  $V_{\max}$  and the apparent increase in internal load with LC<sub>2</sub> extraction are not due to an increased stiffness of cross-bridges in compression. In view of previously observed decreases in the actin-activated ATPase after extraction of LC<sub>2</sub> from myosin (Margossian et al., 1983), it is likely that the reductions in mechanical  $V_{\max}$  reflect a decrease in the rate of detachment of cross-bridges during shortening.

The amount of shortening to the breakpoint between high and low velocity phases decreased after LC<sub>2</sub> extraction (Fig. 12). If the breakpoint represents buckling and subsequent strain of S2 (Moss, 1986), our result would suggest that the tendency of S-2 to buckle is influenced by the presence of LC<sub>2</sub>. Since LC<sub>2</sub> is located at or near the junction between S1 and S2 (Winkelman et al., 1983; Winkelman and Lowey, 1986; Tokunaga et al., 1987), LC<sub>2</sub> might be expected to influence the flexibility of this hinge region and the mechanical stability of the cross-bridge in compression. Of additional interest in this regard, studies using enzymatic digestion techniques indicate that intact LC<sub>2</sub> is required for the change in conformation of the S1-S2 region observed upon binding of actin (Miller and Reisler, 1985).

Several investigators have suggested the possibility that divalent cation binding to LC<sub>2</sub> may influence the conformation of the cross-bridge. Morimoto and Harrington (1974) measured sedimentation coefficients of native thick filaments at various pCa's and demonstrated that at physiological levels of Ca<sup>2+</sup>, myosin undergoes conformational changes thought to be due to the binding of Ca<sup>2+</sup> to LC<sub>2</sub>. In addition, Ca<sup>2+</sup>- and Mg<sup>2+</sup>-induced structural changes have been observed in isolated LC<sub>2</sub> (Mrakovic et al., 1979; Alexis and Gretzer, 1978). In synthetic filaments of myosin, changes in the concentration of Mg<sup>2+</sup>, but not of Ca<sup>2+</sup>, in the physiological range appear to cause the cross-bridges to undergo large changes in position with respect to the thick filament backbone (Persechini and Rowe, 1984). As a working model we propose that LC<sub>2</sub> may modulate contraction by the reversible binding of Ca<sup>2+</sup>, although further experiments will be required to explore this possibility. LC<sub>2</sub> has

been shown to bind  $\sim 0.35$  mol of  $\text{Ca}^{2+}$ /mol  $\text{LC}_2$  in the presence of 2.5 mM  $\text{Mg}^{2+}$  (Holroyde et al., 1979); however, in the presence of a physiological  $[\text{Mg}^{2+}]$ , the rate of  $\text{Ca}^{2+}$  binding is too slow for this mechanism to modulate contraction on the time scale of a single twitch (Robertson et al., 1981). If  $\text{Ca}^{2+}$  does modulate contraction by binding to  $\text{LC}_2$ , the time scale of its effect would be on the order of seconds and the magnitude of its effect would be expected to vary with the duration and frequency of prior contractions.

#### *Consideration of Possible Experimental Artifacts*

Previous work has shown that extraction of  $\text{LC}_2$  causes myosin S-1 to aggregate in solution due possibly to the presence of sticky patches (Pastra-Landis and Lowey, 1986). If this was occurring in our skinned fiber preparations, the effects upon mechanical performance were small, since at pCa 4.5, tension in many fibers was unaltered after  $\text{LC}_2$  extraction and tension at submaximal Ca concentrations increased after extraction. Alternatively, and to us more likely, the cross-bridges in our skinned fiber preparations did not aggregate to a significant extent since tension at all pCa's would probably decrease due to a reduced number of cross-bridge interactions.

Another possible concern is that the observed alterations in mechanical performance may not be specifically due to the extraction of  $\text{LC}_2$  but to the extraction of TnC and/or troponin or to a nonspecific effect of the extraction procedure. SDS-PAGE of the fibers determined that 20–40% of the  $\text{LC}_2$  was extracted by our protocols while the concomitant losses of TnC and troponin were reversed by TnC and troponin recombination. Further, the recoveries to control values of tension, stiffness, and  $V_{\max}$  (Figs. 5, 7, and 11) after readdition of  $\text{LC}_2$  to previously extracted fibers indicate that the observed mechanical effects were not simply a nonspecific result of the extraction procedures. This suggests that  $\text{LC}_2$  was appropriately recombined into the filament lattice and provides support for our conclusion that the observed effects were specific to the extraction of  $\text{LC}_2$ .

#### *Summary*

Our results show that  $\text{LC}_2$  reversibly modulates the  $\text{Ca}^{2+}$  sensitivities of tension generation, fiber stiffness, and maximum velocity of shortening. Extraction of 20–40%  $\text{LC}_2$  led to increased active tension in the pCa range of 6.6–5.7, but had little or no effect on maximum tension. At these same submaximal  $\text{Ca}^{2+}$  concentrations an increase in stiffness proportional to the increase in tension was observed.  $\text{LC}_2$  extraction also resulted in a uniform 25–30% decrease in the high velocity phase of shortening. These results are consistent with a molecular model in which  $\text{LC}_2$  has its effects by modifying the enzymatic properties of myosin, possibly through an effect on the S1-S2 hinge region, leading to alterations in the interaction of myosin with actin.

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