

Alterations in Ca^{2+} Sensitive Tension Due to Partial Extraction of C-Protein from Rat Skinned Cardiac Myocytes and Rabbit Skeletal Muscle Fibers

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ABSTRACT C-protein, a substantial component of muscle thick filaments, has been postulated to have various functions, based mainly on results from biochemical studies. In the present study, effects on Ca^{2+} -activated tension due to partial removal of C-protein were investigated in skinned single myocytes from rat ventricle and rabbit psoas muscle. Isometric tension was measured at pCa values of 7.0 to 4.5: (a) in untreated myocytes, (b) in the same myocytes after partial extraction of C-protein, and (c) in some myocytes, after readdition of C-protein. The solution for extracting C-protein contained 10 mM EDTA, 31 mM Na_2HPO_2 , 124 mM NaH_2PO_4 , pH 5.9 (Offer et al., 1973; Hartzell and Glass, 1984). In addition, the extracting solution contained 0.2 mg/ml troponin and, for skeletal muscle, 0.2 mg/ml myosin light chain-2 in order to minimize loss of these proteins during the extraction procedure. Between 60 and 70% of endogenous C-protein was extracted from cardiac myocytes by a 1-h soak in extracting solution at 20–23°C; a similar amount was extracted from psoas fibers during a 3-h soak at 25°C. For both cardiac myocytes and skeletal muscle fibers, partial extraction of C-protein resulted in increased active tension at submaximal concentrations of Ca^{2+} , but had little effect upon maximum tension. C-protein extraction also reduced the slope of the tension-pCa relationships, suggesting that the cooperativity of Ca^{2+} activation of tension was decreased. Readdition of C-protein to previously extracted myocytes resulted in recovery of both tension and slope to near their control values. The effects on tension did not appear to be due to disruption of cooperative activation of the thin filament, since C-protein extraction from cardiac myocytes that were 40–60% troponin-C (TnC) deficient produced effects similar to those observed in cells that were TnC replete. Measurements of the tension-pCa relationship in skeletal muscle fibers were also made at a sarcomere length of 3.5 μm which, because of the distribution of C-protein on the thick filament, should eliminate any interaction between C-protein and actin. The effects of C-protein extraction were similar at long and short sarcomere lengths. These data are consistent with a model

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in which C-protein modulates the range of movement of myosin, such that the probability of myosin binding to actin is increased after its extraction.

INTRODUCTION

C-protein is an integral component of the myosin-containing thick filaments of cardiac and skeletal muscles. It is a polypeptide of 130–165 kD molecular mass and occurs in vivo in a 1:8 molar ratio of C-protein to myosin (Offer et al., 1973). With immunocytochemical techniques, C-protein has been localized to discrete regions in each half of the A-band along two 0.3- μm lengths of the thick filament beginning 0.2 μm from its center. Within each 0.3- μm region, C-protein molecules in different thick filaments lie in parallel register in seven or eight transverse, 10-nm wide strips spaced ~ 43 nm apart (Offer, 1972; Rome et al., 1973; Craig and Offer, 1976; Dennis et al., 1984; Bennett et al., 1986). It has been suggested that three C-protein molecules wrap around each thick filament and that an arm of the C-protein would be able to span the thick-thin filament gap (Craig and Offer, 1976; Moos et al., 1978; Hartzell and Sale, 1985; Solaro, 1987). Two binding sites for C-protein have been identified on myosin, one on light meromyosin and the other on heavy meromyosin subfragment-2 (S-2) (Moos et al., 1975; Starr and Offer, 1978).

Previous investigations of the functional importance of C-protein can be categorized into studies that considered C-protein as an organizer or regulator of thick filament structure and those that considered C-protein as a modulator of contractile function (see Solaro, 1987, for a recent review). Purified myosin can self-assemble into thick filaments in the absence of C-protein; however, the addition of C-protein alters the periodicity and length of the final thick filament product (Moos et al., 1975; Koretz et al., 1982). Several investigators have shown that depending on ionic strength and the molar ratio of actin and myosin in solution, the addition of C-protein can modulate actin-activated ATPase activity of skeletal and cardiac myosins (Offer et al., 1973; Moos and Feng, 1980; Yamamoto and Moos, 1983; Hartzell, 1985; Margossian, 1985; Lim and Walsh, 1986).

In the present study, the effects of partial extraction of C-protein on the tension-pCa relationship were investigated in single skinned cardiac myocytes and skeletal muscle fibers. These studies used near-physiological conditions to examine the function of C-protein in isolated muscle cells. The skinned single cell allows the study of functions of myofilament proteins in the context of an intact filament lattice, but without confounding effects due to intercellular connective tissue and protein heterogeneity between cells of multicellular preparations. Our results indicate that partial extraction of C-protein leads to a reversible increase in isometric tension at submaximal concentrations of Ca^{2+} and a decrease in the cooperative activation of Ca^{2+} -dependent tension, supporting the view that contractile function is affected by C-protein.

METHODS

Tissue Preparation and Attachment Procedures

Adult female Sprague-Dawley rats were anesthetized by either inhalation of Metofane or injection of sodium pentobarbital. The heart was excised, and the ventricles were placed in

relaxing solution (see below) and then minced. Groups of cardiac cells, single myocytes, and cell fragments were obtained by dissociating the minced ventricle in a blender. The homogenization procedure permeabilized the surface membranes of the myocytes, since immersion in a maximally activating solution induced immediate contraction, and no change in the tension-pCa relationship was observed in cells subsequently treated for 30 min with 3% Triton X-100 in relaxing solution. A drop of relaxing solution containing dissociated myocytes was placed on a coverslip. A cell was selected for attachment only if it was rod-shaped and had a striation pattern that was visible along its entire length. Attachment of the cell to the experimental apparatus was similar to the procedure described by Sweitzer and Moss (1990). In brief, cells were attached with micropipettes to a piezoelectric translator at one end and a force transducer at the other (see below). Once a cell was selected, the glass micropipettes ($\sim 20 \mu\text{m}$ tip), coated with uncured silicone adhesive (Dow Corning Corp., Midland, MI), were gently placed against its ends. After allowing the adhesive to cure for 45 min, the micropipettes with cell attached were lifted to the center of the drop of relaxing solution.

Procedures for preparation of rabbit skinned psoas fibers and mounting of an individual fiber to the experimental apparatus were identical to those described previously (Moss et al., 1983a).

Experimental Apparatus

One of the attachment micropipettes (Glass Co. of America, Bargaintown, NJ, 300–400 μm o.d.) was fixed to the force transducer with paraffin. The force transducer (Model 406; Cambridge Technology, Watertown, MA) had a sensitivity of 200 mV/mg, a resonant frequency of 100 Hz, and a compliance of 100 $\mu\text{m}/\text{gm}$. The force transducer was mounted on a micromanipulator (No. MM-31; Narishige Scientific Instrument Laboratory, Tokyo, Japan) for precise three-way positioning. The signal from the force transducer was recorded on a digital oscilloscope (No. 2090, Nicolet Instruments, Inc., Madison, WI) and an X-Y recorder (No. 7015B, Hewlett-Packard, San Diego, CA).

The second attachment micropipette was formed from pyrex capillary tubing (1.0 mm o.d.) and then fixed to the piezoelectric translator. The translator (No. 173; Physik Instrumente, Waldbronn, FRG) completed length changes of 0–50 μm in < 1 ms. A power supply/amplifier (No. BOP 1000M, Kepco, Inc., Flushing, NY) driven by a pulse generator (series 1800; World Precision Instruments, New Haven, CT) was used to control displacement of the translator. The position of the translator was adjusted with a Narishige micromanipulator.

Myocytes were observed and photographed through a Zeiss inverted microscope (IM) fitted with a long working distance $\times 40$ objective (CDPlan 40, numerical aperture 0.50, working distance 2 mm; Olympus Optical Co. Ltd., Tokyo, Japan) and a filar micrometer $\times 10$ eyepiece (Reichert Scientific Instruments, Buffalo, NY), calibrated with a stage micrometer. This eyepiece has a graduated micrometer drum which controls the position of a fiducial line, permitting rapid measurement of sarcomere length. Photographs were taken through a separate lens configuration using the $\times 40$ objective, a $\times 7$ eyepiece, and an Olympus model PM-10 camera system with a 35 mm camera back. Photographs were taken both while the cells were relaxed and during steady contractions. Developed negatives were projected with a photo-enlarger, yielding final magnifications between $\times 400$ and $\times 800$ depending on projection distance, and striation spacing was measured from the entire length of the cell.

After attachment, the myocyte was suspended in a drop of relaxing solution on a glass slide that could be moved laterally to allow changes of solution. The position of the glass slide was controlled by a translating stage mounted on the fixed stage of the inverted microscope. To change solution the entire glass slide was moved to bring a 20- μl drop of fresh solution into position around the attached myocyte.

The experimental apparatus used for the skeletal muscle studies was identical to that described earlier (Moss, 1979).

Solutions

The cardiac myocytes and skeletal muscle fibers were activated in solutions containing various concentrations of free calcium between pCa 7.0 and pCa 4.5 (pCa = $-\log [\text{Ca}^{2+}]$). The solutions contained 7 mM EGTA, 1 mM free Mg^{2+} , 20 mM imidazole (pH 7.00), 4.42 mM ATP, 14.5 mM creatine phosphate, various free Ca^{2+} concentrations, and KCl to adjust ionic strength. Creatine phosphokinase was not added to these solutions since endogenous kinase activity in our preparation is sufficient to maintain ATP levels (Sweitzer and Moss, 1990). The pCa of relaxing solution was 9.0. The apparent stability constant for Ca-EGTA was corrected to 15°C and an ionic strength of 180 mM (Fabiato, 1988). The computer program of Fabiato (1988) was used to calculate the concentrations of each metal, ligand and metal-ligand complex, based on the stability constants listed by Godt and Lindley (1982). The same solutions were used in the experiments on cardiac myocytes at 22°C, except that the pH of the solutions was adjusted to 7.00 at 22°C.

The solution for extracting C-protein from cardiac myocytes consisted of 10 mM EDTA, 31 mM Na_2HPO_4 , 124 mM NaH_2PO_4 , pH 5.90 (Offer et al., 1973; modified by Hartzell and Glass, 1984), and 0.20 mg/ml bovine cardiac troponin. Cardiac troponin was added to this solution to prevent loss of Tn during the period of extraction, since extractions in the absence of troponin frequently resulted in increased resting tension. The extracting solution for psoas fibers was similar except that skeletal Tn was added instead of cardiac, and 0.20 mg/ml of rabbit skeletal light chain-2 (LC_2) was also added. For psoas fibers extracted in the absence of LC_2 , some of the increase in tension at submaximal concentrations of Ca^{2+} was due to partial extraction of LC_2 , since the increase was partially reversed by readdition of LC_2 to the fibers. For cardiac cells, the addition of LC_2 to the extracting solution had no effect on the results.

To recombine C-protein, the myocytes were bathed in relaxing solution containing 0.20–0.30 mg/ml rabbit or rat cardiac C-protein, 0.20 mg/ml bovine cardiac troponin (Tn), 1.0 mg/ml leupeptin, and 10 mM MgCl_2 . Skeletal muscle fibers were bathed in relaxing solution containing 0.30 mg/ml bovine skeletal C-protein.

The solution for extracting troponin C (cTnC) from cardiac myocytes was similar to that of Cox et al. (1981), as modified by Moss et al. (1985) for extraction of TnC from skeletal muscle fibers. The solution consisted of 20 mM Tris (pH 7.80), 5 mM EDTA, 0.5 mg/ml bovine cardiac troponin, and 0.1 mg/ml cardiac LC_2 . Babu et al. (1987) used a similar solution to extract ~75% of endogenous TnC from hamster skinned trabeculae. In the present studies on single skinned myocytes, both Tn and LC_2 were added to the solution to minimize the loss of these subunits during extraction of cTnC. Cardiac myocytes were bathed in TnC-extracting solution for 2–3 h at room temperature. Cells to which cTnC was readded were bathed for 3 s in a solution of pCa 9.0 containing 0.15 mg bovine cTnC per milliliter. The myocytes were then activated at pCa 4.5 to assess the extent of recombination of cTnC. This cycle of cTnC soak and maximal activation was repeated until additional soaks resulted in no further increases in active tension. This procedure assured that sufficient cTnC recombined into the myocyte.

Cardiac C-protein was prepared using the method of Hartzell and Glass (1984). C-protein prepared in this way had no endogenous phosphatase activity and kinase activity could be detected only in the presence of Ca^{2+} and calmodulin (Hartzell and Glass, 1984). Skeletal C-protein was a gift from Dr. Marion Greaser and was prepared using the method of Offer et al. (1973). Cardiac and skeletal C-protein preparations were examined by SDS-PAGE gel electrophoresis to determine extent of contamination and migration pattern (Fig. 1A). Cardiac and skeletal TnC and troponin were prepared using the methods of Greaser and Gergely (1971). LC_2 was prepared using the method of Wagner (1982).

Experimental Protocol

After attachment to the apparatus, the cardiac myocytes were usually immersed for 30 s in relaxing solution containing 0.2% ultra-pure Triton X-100 (Surfact-Amps X-100; Pierce

Chemical Co., Rockford, IL) to enhance the contrast of the striation pattern. Sarcomere length was adjusted to 2.1–2.3 μm and cell length and width were measured (Table I). Typically, a tension-pCa relationship was obtained by initially measuring force during maximal activation (pCa 4.5), followed by contractions at randomly chosen submaximal pCa's, and again at pCa 4.5 to assess any decline in the performance of the cell. On average, maximum tension declined by $1.9 \pm 1.5\%$ ($n = 17$) during the measurement of an entire tension-pCa relationship. After obtaining the control tension-pCa relationship, the myocyte was bathed in C-protein extracting solution for 60 min at room temperature (20–23°C). Since the extracting solution contained no added ATP, rigor tension developed during extraction, but this tension was immediately dissipated by slightly decreasing the length of the myocyte. The cell was re-extended to its original length after extraction. The myocyte was then briefly bathed in a relaxing solution containing 0.25 mg/ml cTnC to replenish any cTnC that may have been nonspecifically lost, and the experimental tension-pCa relationship was measured. For cells to which C-protein was readded, the myocyte was bathed in C-protein readdition solution for 60–120 min at 22°C. After this procedure, the cells were briefly soaked in relaxing solution containing cardiac TnC before obtaining the final tension-pCa relationship.

The experimental protocols for skeletal muscle fibers were similar to those for cardiac myocytes, with a few exceptions. Sarcomere length was set to 2.47–2.61 μm , and there was no treatment with Triton X-100 since the striation pattern was clear. For skeletal muscle, due to its greater stability during activation, we interspersed activations at pCa 4.5 to monitor fiber

TABLE I
Characterization of Cardiac Myocyte Preparation

Characteristic	Mean \pm SD (n)	Range of values
Maximum tension (mg)	1.06 \pm 0.31 (50)	0.66–1.60
Sarcomere length (μm)	2.20 \pm 0.09 (50)	2.01–2.33
Overall length (μm)	89 \pm 22 (50)	52–147
Width (μm)	25 \pm 5 (30*)	14–36

*Widths were not measured in the early phases of this study.

performance during the measurement of tension-pCa relations. Skeletal muscle fibers were bathed in extracting solution for 3 h at 25°C, and after extraction, as well as after readdition of C-protein, the fiber was briefly bathed in relaxing solution with both skeletal TnC and whole troponin to insure that the fibers had full complements of these proteins.

For both cardiac and skeletal muscle preparations, tensions measured at submaximal Ca^{2+} concentrations were expressed as a fraction of the maximum tension (P_o) developed by the same fiber at pCa 4.5 under the same conditions (e.g., control or C-protein extracted). Thus, the tension-pCa relationships presented in this report are actually normalized *relative* tension-pCa relations. Presentation of data in this form allows direct comparisons of the forms and midpoints of the tension-pCa relations under a variety of experimental conditions.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To quantitate the extraction of C-protein and to monitor the content of LC₂ and TnC, cardiac myocytes and segments of the skeletal muscle fibers were analyzed by slab SDS-PAGE and subsequent silver staining (Giulian et al., 1983; Reiser et al., 1988). Before attachment, a 1–2-mm length of each skeletal muscle fiber was cut free and placed in SDS sample buffer (10 $\mu\text{l}/\text{mm}$) for later analysis. The remainder of the same fiber was mounted to the apparatus for experimentation and at the conclusion of the experiment was placed in sample buffer.

When gels of skeletal muscle fibers were run, 6 μ l of sample buffer was loaded into each lane. Gels of individual cardiac myocytes were either control cells that were attached to the apparatus but not experimentally modified or cells that were attached, activated, extracted, and in some cases recombined with C-protein. Because we were not always able to detect intercalated disks, it is possible that some preparations were composed of two or three cells rather than one. To transfer the myocytes to sample buffer, the cell was left attached to one micropipette that was transferred to a 0.5-ml microfuge tube, and the tip of the micropipette with cell attached was broken into 10 μ l of sample buffer containing 1 mg/ml leupeptin.

When gels of cardiac myocytes were run, the entire 10 μ l of sample buffer was loaded into a lane. Acrylamide concentrations were 9.5% (20:1 acrylamide/bis-acrylamide) in the separating gel and 3.5% (200:1 acrylamide/bis-acrylamide) in the stacking gel. Once the gels were stained and dried they were scanned with a laser scanning densitometer (Giulian et al., 1983). The relationship between C-protein concentration and staining intensity was determined and was found to be linear for the range of amounts of C-protein in the muscle samples (i.e., 5–50 ng/sample). To quantitate changes in protein content, the areas under the peaks corresponding to C-protein, LC₂, and TnC were calculated as fractions of the area under the LC₁ peak (Moss et al., 1983a).

Hill Analysis and Statistics

Tension-pCa relationships were characterized by Hill plot analysis (reviewed by Dahlquist, 1978). Separate straight lines were fit to the transformed data above and below 0.5 P_o , since in most cases the lower part of the tension-pCa relationship was much steeper than the upper part, making it impossible to fit the data with a single straight line (see Cornish-Bowden and Koshland, 1975), as observed previously in skeletal muscle (Moss et al., 1983b). Data were fit by least squares regression using the Hill equation:

$$\log [P_r / (1 - P_r)] = n (\log [Ca^{2+}]) + \log k \quad (1)$$

where P_r is tension as a fraction of P_o (or P/P_o), n is the Hill coefficient, and k is the intercept of the fitted line with the x-axis. Hill coefficients obtained from this analysis were designated n_1 for $P/P_o > 0.5$ and n_2 for $P/P_o < 0.5$. Using the constants derived from the Hill analysis, curves were drawn by computer onto the upper and lower portions of the tension-pCa relationship using the equation:

$$P/P_o = [Ca^{2+}]^n / (k^n + [Ca^{2+}]^n) \quad (2)$$

The curves were made to intersect 1.0 P/P_o at pCa 4.5.

A two-way analysis of variance was used to test whether C-protein or Ca^{2+} concentration significantly affected isometric tension (BMDP statistical software package; BMDP Statistical Software Inc., Los Angeles, CA). A level of $p < 0.05$ was chosen as indicating significance. When significant interactions were found, a Bonferroni *t*-test was used to determine the significance of the mean difference. Unless noted, all values are reported as the mean \pm SEM.

RESULTS

SDS-PAGE of Control and Partially C-Protein-Extracted Cardiac Myocytes and Skeletal Muscle Fibers

Cardiac myocytes and segments of skeletal muscle fibers were analyzed by SDS-PAGE to determine the extent of C-protein extraction and to determine whether there was

nonspecific extraction of other myofilament proteins. The gels in Fig. 1A show cardiac (lanes 3 and 4) and skeletal (lanes 5 and 6) muscle samples both before (lanes 3 and 5) and after (lanes 4 and 6) partial extraction of C-protein. From densitometric scans of these gels (Fig. 1B), the main difference between control and treated myocytes was the decrease in C-protein content following extraction. In nine different untreated myocytes, the C-protein/LC₁ ratio was 0.33 ± 0.05 , while in nine myocytes after the extraction procedure, the ratio was 0.10 ± 0.02 . This indicates that on average 70% of the C-protein was extracted from the cardiac myocytes. An average of $60 \pm 7\%$ ($n = 11$) of the C-protein was extracted from all the skeletal muscle fibers studied. There was no apparent loss of LC₂ from the cardiac cells during the extraction procedure, and the content of LC₂ in skeletal muscle fibers appeared to increase somewhat after the C-protein extraction procedure. The TnC content of both the cardiac and skeletal preparations increased after the extraction procedures. These increases were not unexpected, since both preparations were bathed at various times in purified TnC, and LC₂ was present in the skeletal muscle C-protein extracting solution (see Methods). Presumably, the apparent increases in TnC and LC₂ represent nonspecific binding of these proteins within the myofilaments. Examination of the remaining contractile proteins revealed no significant differences between control and extracted samples.

Alterations in Tension Following Partial Extraction of C-Protein

Photomicrographs of a cardiac myocyte in relaxing solution and during maximal activation, both before and after partial extraction of C-protein, are shown in Fig. 2. Typically, the cells were not visibly altered after the extraction procedure. Sarcomere lengths measured along the entire length of the cell in each of these photographs varied by $< 0.05 \mu\text{m}$. On average (six myocytes), the sarcomere length of control cells while relaxed was $2.18 \pm 0.04 \mu\text{m}$ and during maximal activation was $2.24 \pm 0.04 \mu\text{m}$, indicating that the compliance of the attachments of the cells to the apparatus was low.

Fig. 3 shows slow time-base records of isometric tension obtained from an individual myocyte during activations at two different pCa values both before and after partial extraction of C-protein. This cell developed 1.40 mg of active tension during maximal activation at pCa 4.5. The average values and ranges of maximum tension, sarcomere length, cell length, and cell width are presented in Table I for the cardiac myocytes used in the course of this study. Mean maximum tension was similar to values obtained previously by Fabiato and Fabiato (1975) from rat skinned cardiac cells. Because we were unable to obtain measurements of cell depth, force could not be normalized to cross-sectional area.

Effects on tension as a result of partial extraction of C-protein are shown in Fig. 3B. Maximum tension was not markedly affected by C-protein extraction, while tensions at submaximal concentrations of Ca²⁺ increased significantly. Cumulative tension-pCa relationships from 17 myocytes are shown in Fig. 4. On average, after partial extraction of C-protein, maximally activated tension (pCa 4.5) fell to $90.0 \pm 2.0\%$ of control ($n = 17$). This loss of maximum tension appears to be a nonspecific decline in tension-generating capabilities of some cells rather than a specific effect of C-protein extraction, since three cells lost no tension after extraction (e.g., Fig. 3B).

Of greater interest was the consistently observed increase in tension in the range of pCa's from 5.7 to 6.5. These increases were unaffected by subsequently bathing the myocytes in TnC, Tn, or LC₂. Also, the slope of the tension-pCa relation was reduced after C-protein extraction. After partial extraction of C-protein, both n_1 and n_2 were reduced and the Hill plot could be reasonably well fit by a single straight line (Fig. 4 B).

To establish that the effects observed in Fig. 4 were specifically due to extraction of C-protein, purified C-protein was recombined into several myocytes that had previ-

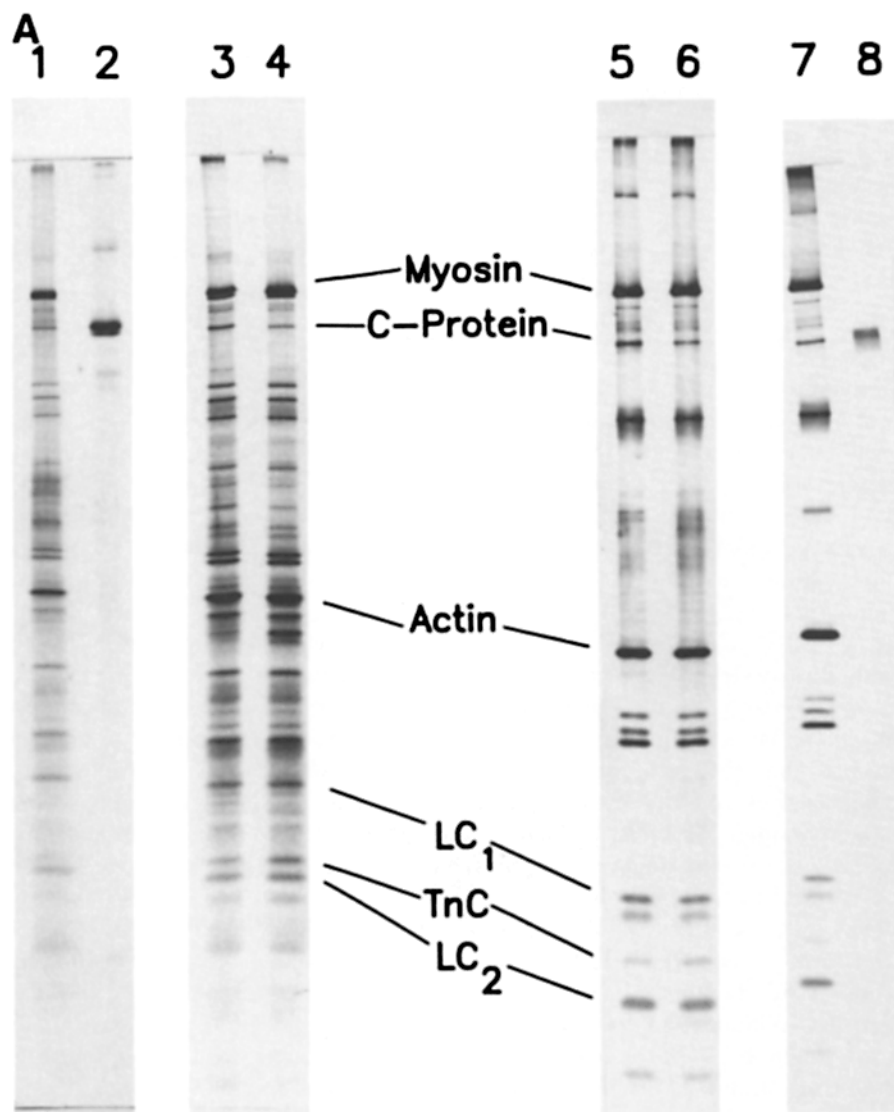


FIGURE 1

ously been extracted. Fig. 5 presents data obtained from one myocyte after the readdition protocol. The changes in the slope of the tension-pCa relation and in pCa_{50} (i.e., the pCa at which relative tension was half-maximal) due to C-protein extraction were fully reversed by recombination of C-protein (Fig. 5). Similar results were obtained in two additional myocytes.

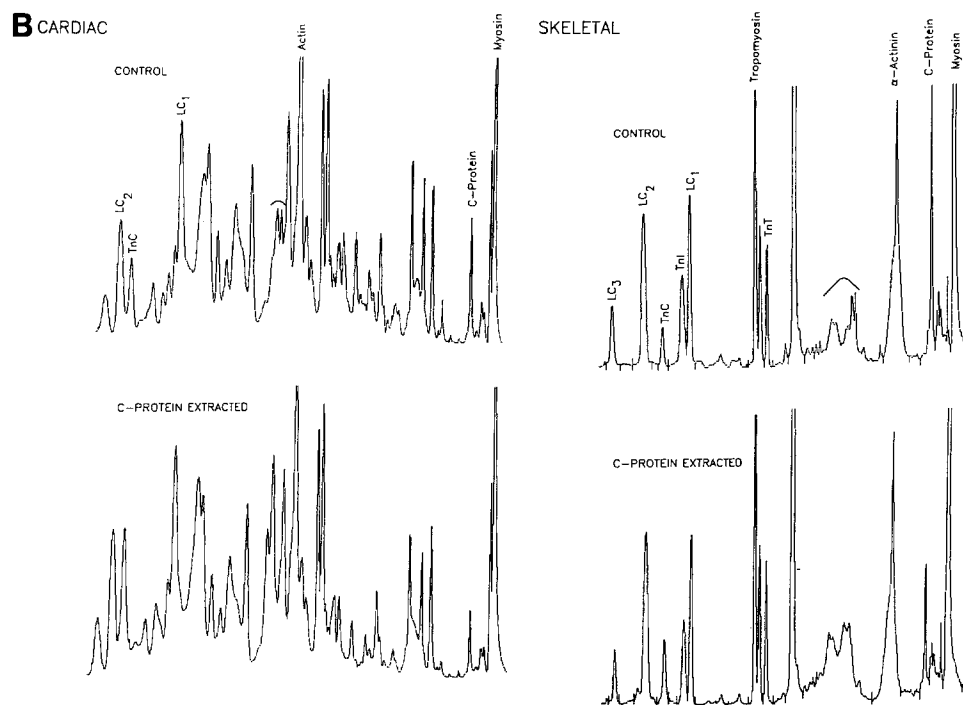


FIGURE 1. SDS-PAGE of cardiac myocytes and segments of skeletal muscle fibers both before and after protocol to extract C-protein. (A) SDS-polyacrylamide gels. Lane 1 is a control cardiac myocyte, while lane 2 contains rat cardiac purified C-protein (20 ng) loaded on the same gel. Lanes 3 and 4 are cardiac myocytes obtained from the same heart, while lanes 5 and 6 are segments from a single skeletal muscle fiber. Samples for gel analysis were collected before treatment (lanes 3 and 5) and after partial extraction of C-protein (lanes 4 and 6). C-protein content was reduced by 58% in lane 4 and 62% in lane 6 relative to the corresponding control samples. Lane 7 is a control skeletal muscle fiber segment, while lane 8 contains rabbit skeletal C-protein (30 ng) loaded on the same gel. (B) Densitometric scans of gel lanes 3–6 from A. The brackets indicate peaks corresponding to nonmuscle proteins, possibly skin proteins introduced during handling of the samples.

Control experiments were done to test whether experimental artifacts might account for any of the effects observed upon extraction of C-protein. First, in order to determine whether there was nonspecific decay of contractile properties during extraction, the duration and temperature of extraction were simulated by bathing myocytes in relaxing solution for 1 h at 22°C. No significant effect on the

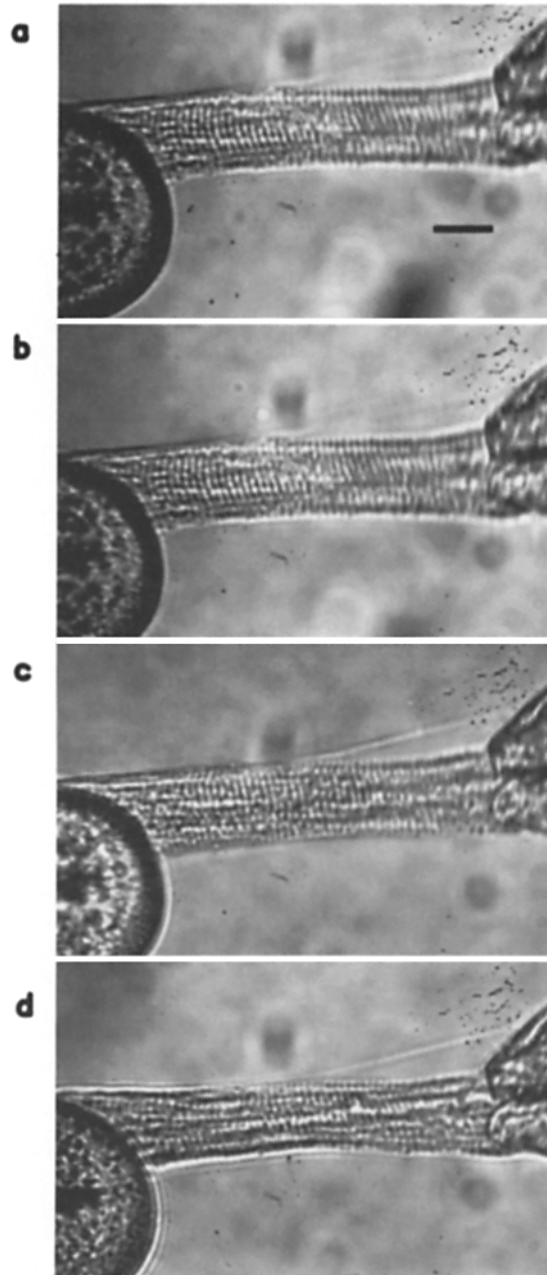


FIGURE 2. Light photomicrographs of a single cardiac myocyte (103189-2) while relaxed and during activation at pCa 4.5. Photomicrographs of the control myocyte at (a) pCa 9.0 and (b) pCa 4.5 or after partial extraction of C-protein at (c) pCa 9.0 and (d) pCa 4.5 showed no changes in sarcomere length or overall appearance. The calibration bar in *a* represents 25 μm . Cell length, measured between pipette tips, was 143 μm . Sarcomere length was $2.30 \pm 0.02 \mu\text{m}$ in all photomicrographs.

tension-pCa relation was observed. Second, to approximate the add-back protocol, C-protein-extracted myocytes were bathed in relaxing solution for 2 h to determine whether the tension-pCa relation would recover its control form in the absence of C-protein. We found that the Ca^{2+} sensitivity of tension decreased during this procedure, an effect thought to be due to dissociation of TnC. To minimize this

effect, we added 10 mM $MgCl_2$ to the relaxing solution in order to stabilize TnC binding to the thin filament (Zot and Potter, 1982). Addition of $MgCl_2$ prevented recovery of the tension-pCa relationship in the absence of C-protein and so was included in the C-protein add-back solution. Third, control myocytes were bathed for 1 h in a solution containing 0.26 mg/ml C-protein and 0.20 mg/ml troponin to determine whether an otherwise untreated cell would bind C-protein and thereby alter muscle function. No change in the tension-pCa relationship was observed under these conditions.

Results from experiments on skinned psoas fibers from which C-protein was partially extracted are shown in Fig. 6 A. The cumulative data from these six fibers

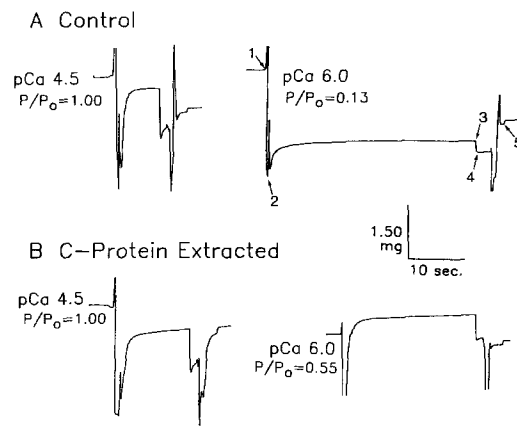


FIGURE 3. Original slow time base records of isometric tension from the same myocyte obtained before (A) and after (B) C-protein extraction. The numbered arrows indicate the major events in the protocol for activation and relaxation of the myocyte. Between arrows 1 and 2, the cell was moved from solution of pCa 9.0 to, in this case, a solution of pCa 6.0. Typically, zero-force baselines in different solutions were not identical due to surface tension

of the solution around the micropipettes, which varied with the location of the micropipette within the drop of solution. After entry of the micropipette into the activating solution (arrow 2), there was usually a transient increase in tension as the cell was moved to the center of the drop. Next, tension rose as solution crept around the micropipette and the cell contracted. Due to these artifacts, active tension was always measured as the difference between the plateau of tension (arrow 3) and the zero tension baseline obtained upon release of the cell to slack length (arrow 4), minus the passive tension obtained during similar measurements at pCa 9.0. The cell was then returned to solution of pCa 9.0 and re-extended to its original length (arrow 5). After C-protein extraction, tension at pCa 4.5 was 0.98 of control P_0 . Myocyte 8189; overall cell length, 74 μm ; sarcomere length, 2.23 μm .

indicate that there were significant increases in tension at pCa 6.1 and pCa 6.0 after partial extraction of C-protein. However, as in the cardiac myocytes, maximum tension at pCa 4.5 was not greatly affected by the extraction since tension at pCa 4.5 was 0.96 ± 0.03 of control P_0 . Also, the steepness of the tension-pCa relation decreased upon extraction of C-protein. Both n_1 and n_2 were reduced by extraction, but the effect on n_2 was greater (Fig. 6, legend).

The effects of C-protein extraction in skeletal muscle were completely reversed by readdition of C-protein (Fig. 6 B). Thus, the effects of the extraction procedure on the tension-pCa relation of skeletal muscle fibers were qualitatively similar to the

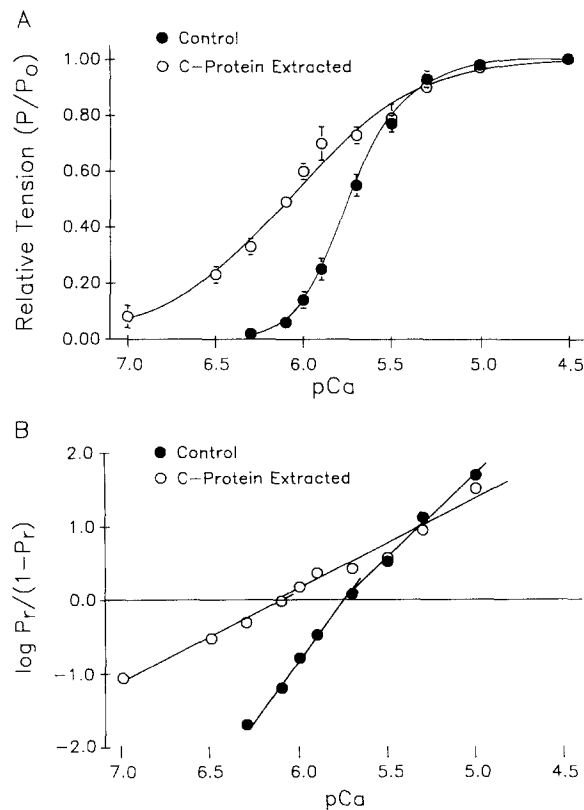


FIGURE 4. Tension-pCa data obtained before (●) and after (○) partial extraction of C-protein from rat ventricular myocytes. Symbols and error bars indicate means \pm SEM from 17 myocytes. (A) The data are plotted as relative tension-pCa relationships; (B) the same data have been transformed to yield a Hill plot. The pCa's for half-maximal tension (pCa₅₀) and the Hill coefficients (n_1 and n_2) were as follows:

	pCa ₅₀	n_1 ($P/P_o > 0.50$)	n_2 ($P/P_o < 0.50$)
Control	5.77 \pm 0.03	2.22 \pm 0.19	4.44 \pm 0.23
C-protein extracted	6.13 \pm 0.03	1.20 \pm 0.16	1.31 \pm 0.15

effects in cardiac myocytes and are likely to be due specifically to the loss of C-protein.

Effects on the Tension-pCa Relation Due to Sequential Extractions of TnC and C-Protein

To determine whether disruption of cooperative activation along the *thin* filament would alter the effects due to C-protein extraction, the effects of partial extraction of C-protein were examined in TnC-deficient myocytes. Initially, it was necessary to determine the effects upon the tension-pCa relationship of skinned cardiac myocytes

due solely to partial extraction of cardiac TnC (cTnC). Fig. 7A presents tension-pCa data from a partially cTnC-extracted myocyte to which purified cTnC was subsequently readded. The pCa_{50} decreased from 5.71 before extraction to 5.47 after extraction and recovered to pCa 5.70 after readdition of cTnC. Similar data were obtained in two additional myocytes (data not shown). Such recovery is similar to that shown by Babu et al. (1987) on hamster trabeculae.

Fig. 7B presents data from a cardiac myocyte that was first partially cTnC extracted and then partially C-protein extracted. In this particular cell, pCa_{50} decreased from 5.67 in the control to 5.29 after cTnC extraction and then increased to pCa 5.76 with subsequent extraction of C-protein. C-protein extraction of

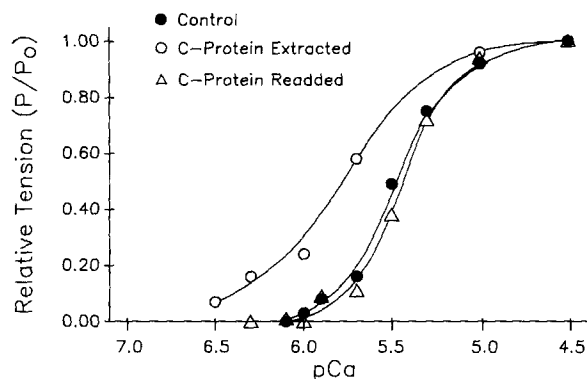


FIGURE 5. Tension-pCa relationships demonstrating the effects of rat cardiac C-protein readdition to a previously extracted cardiac myocyte. Data shown were obtained before extraction (●), after C-protein extraction (○), and finally, after readdition of C-protein (△). After C-protein extraction, tension at pCa 4.5 was 0.94 of control P_0 ; after C-protein readdition, maximum tension was 0.89 of control P_0 . Myocyte 2290; overall cell length, 95 μm ; sarcomere length, 2.11 μm . The pCa_{50} 's and Hill coefficients were as follows:

	pCa_{50}	$n_1 (P/P_0 > 0.50)$	$n_2 (P/P_0 < 0.50)$
Control	5.48	2.13	2.80
C-protein extracted	5.76	1.77	1.47
C-protein readded	5.42	2.53	2.72

TnC-deficient myocytes resulted in a mean leftward shift of pCa_{50} by 0.47 ± 0.08 pCa units in three cells compared with 0.36 ± 0.04 in non-TnC-extracted myocytes (Fig. 4). Thus, the direction and magnitude of this shift were not significantly different from those observed after extraction of C-protein from cTnC-replete myocytes. The steepness of the tension-pCa relation decreased after TnC extraction and decreased still further after extraction of C-protein.

Influence of Sarcomere Length on Effects of C-Protein Extraction

To determine whether the modulation of tension by C-protein involved interactions between C-protein and actin, psoas fibers were stretched to sarcomere lengths at

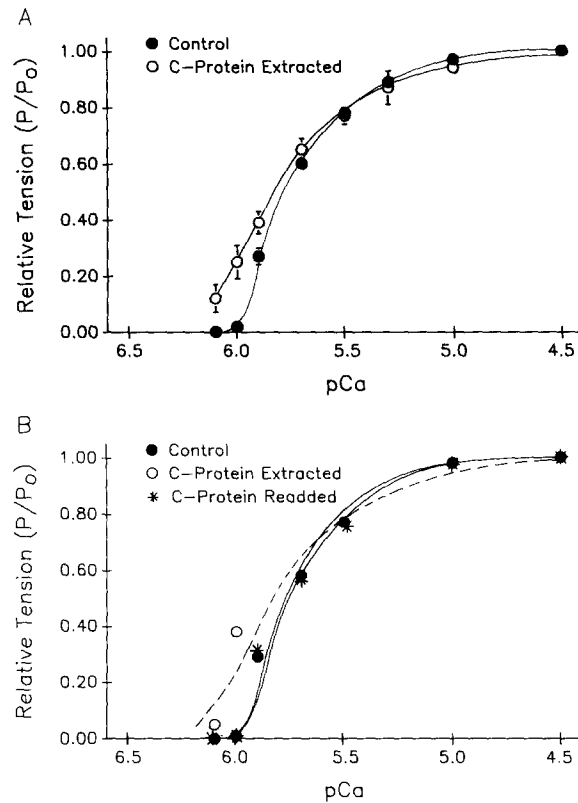


FIGURE 6. Tension-pCa relationships showing the effects of C-protein extraction on tension developed by rabbit psoas fibers. (A) Cumulative data obtained before (●) and after (○) partial extraction of C-protein. Data are from six fibers having a mean sarcomere length of $2.56 \pm 0.02 \mu\text{m}$ (\pm SEM). The pCa_{50} 's and Hill coefficients were as follows:

	pCa_{50}	$n_1 (P/P_0 > 0.50)$	$n_2 (P/P_0 < 0.50)$
Control	5.86 ± 0.01	1.87 ± 0.12	7.45 ± 0.80
C-protein extracted	5.88 ± 0.02	1.40 ± 0.13	3.83 ± 0.57

(B) Data from a C-protein extracted psoas fiber to which skeletal C-protein was subsequently readded. Data were obtained before (●) and after (○) partial extraction of C-protein, and after subsequent readdition of C-protein (*). We were unable to obtain complete tension-pCa relations for each condition, since fibers did not survive the large number of activations that were required by such a protocol. Thus, the leftward shift of the tension-pCa relation as a result of C-protein extraction was verified by tension measurements only at pCa 6.0, pCa 6.1, and pCa 4.5. For reference, the line fitted to the cumulative "C-protein extracted" data in A is replotted as a dashed line in B. After extraction, tension at pCa 4.5 was 0.99 of control P_0 ; while after C-protein readdition, maximum tension was 0.90 of control P_0 . Psoas fiber 81689; overall length, 2.47 mm; sarcomere length, 2.55 μm . Similar data were obtained from three additional fibers.

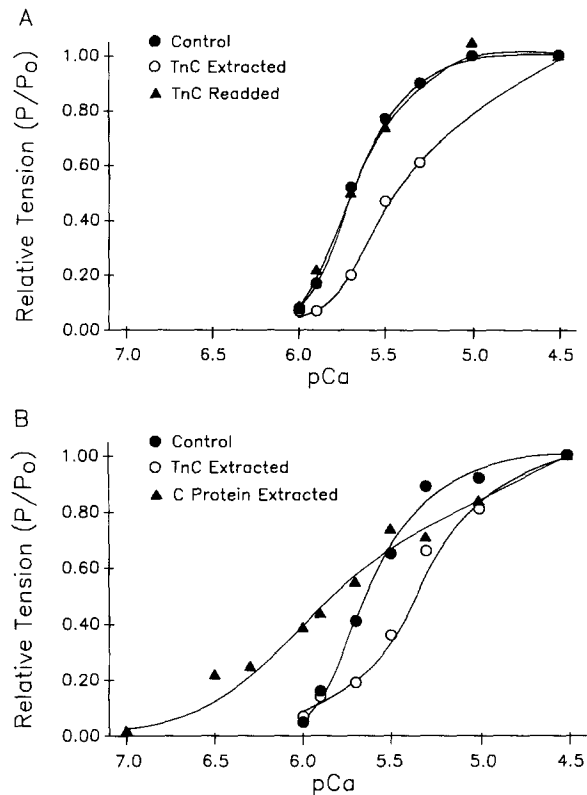


FIGURE 7. Plots of tension-pCa relations from two individual cardiac myocytes showing the effects of cTnC extraction. (A) Data from a myocyte before cTnC extraction (●), after extraction of cTnC (○), and finally, with readdition of cTnC (▲). After extraction, tension at pCa 4.5 was 0.49 of control P_0 ; with readdition, maximum tension returned to 0.75 of control P_0 . Myocyte 102589; overall cell length, 102 μm ; sarcomere length, 2.37 μm . (B) Data from a myocyte from which both troponin C and C-protein were extracted: control (●), after extraction of cTnC (○), and after the subsequent extraction of C-protein (▲). After extraction of cTnC, tension at pCa 4.5 was 0.40 of control P_0 ; after extraction of C-protein, tension was 0.37 control P_0 . Myocyte 71989; overall cell length, 81 μm ; sarcomere length, 2.27 μm . The $p\text{Ca}_{50}$ and Hill coefficients for the data in B were as follows:

	$p\text{Ca}_{50}$	$n_1 (P/P_0 > 0.50)$	$n_2 (P/P_0 < 0.50)$
Control	5.67	1.98	3.73
TnC extracted	5.29	1.13	1.37
C-protein extracted	5.91	0.67	1.00

which C-protein would presumably be physically unable to bind actin. According to immunocytochemical studies (Offer, 1972; Rome et al., 1973; Craig and Offer, 1976), at sarcomere lengths beyond $\sim 3.2 \mu\text{m}$, actin could only interact with the end-most portion of the thick filament, which is devoid of C-protein. Tension-pCa relations

were obtained from psoas fibers at an average sarcomere length of $3.57 \pm 0.04 \mu\text{m}$ ($n = 9$) both before and after partial extraction of C-protein (Fig. 8A). As was the case for fibers at sarcomere lengths around $2.5 \mu\text{m}$ (Fig. 6A), isometric tensions at pCa 6.1 and pCa 6.0 were significantly greater after C-protein extraction. Mean passive tensions in both control and partially C-protein-extracted skeletal muscle fibers at $3.57 \mu\text{m}$ were $0.26 \pm 0.02 P_o$. Maximum tension in fibers at long sarcomere lengths was $0.52 \pm 0.02 P/P_o$ of the maximum tension at sarcomere lengths of $2.56 \mu\text{m}$.

Tension data from Fig. 6A (sarcomere length of $2.56 \pm 0.02 \mu\text{m}$) and Fig. 8A (sarcomere length of $3.57 \pm 0.04 \mu\text{m}$) are replotted in Fig. 8B to compare the effects of C-protein extraction on tension-pCa relationships at the two lengths. Relative tension-pCa data from C-protein-extracted fibers were virtually identical at long and short lengths. Such comparisons would be better done in the same fiber at both sarcomere lengths; however, we were unable to complete all of the measurements in any one fiber. In additional experiments, we completed the entire length change and extraction protocol in the same fibers ($n = 3$) by performing tension measurements only at pCa's 4.5 and 6.0. The results of these experiments were similar to those obtained at pCa 6.0 in Fig. 8B.

The effects of C-protein extraction were also studied in cardiac myocytes stretched to sarcomere lengths greater than $3.3 \mu\text{m}$ (Fig. 9). In these cells also, partial extraction of C-protein resulted in increased tensions at high pCa and a decrease in the steepness of the tension-pCa relation. A comparison of these data to those obtained at the shorter sarcomere lengths (Fig. 4) suggests that the tension-pCa relations after C-protein extraction are similar. It must be noted that there were two major problems in making the measurements at long sarcomere lengths. In most myocytes, sustained high passive tension (for the cell in Fig. 9 passive tension was $50\% P_o$) frequently resulted in breakage of the cell between the points of attachment. Second, in many cases, sarcomere length was highly variable. For these reasons only 3 of 12 myocytes studied yielded interpretable data.

DISCUSSION

In this study, a ~ 60 – 70% extraction of C-protein from skinned cardiac myocytes and single skeletal muscle fibers resulted in a significant increase in isometric tension at submaximal levels of Ca^{2+} , and as assessed from the slope of the tension-pCa relationship, a decrease in cooperative activation of the myofilaments (Figs. 4 and 6). These effects were fully reversed in both muscles by recombination with purified C-protein (Figs. 5 and 6B). Experiments at different degrees of filament overlap in extracted fibers suggest that the modulation of isometric tension by C-protein extraction is not a result of binding of C-protein to actin (Figs. 8 and 9).

Specificity of the C-Protein Extraction Procedure

Offer et al. (1973) were the first to suggest that a solution containing phosphate and EDTA but with no ATP would effectively extract C-protein without a concomitant loss of myosin. Hartzell and Glass (1984) examined the usefulness of the phosphate/EDTA solution for extracting C-protein from cardiac myofibrils and found that the

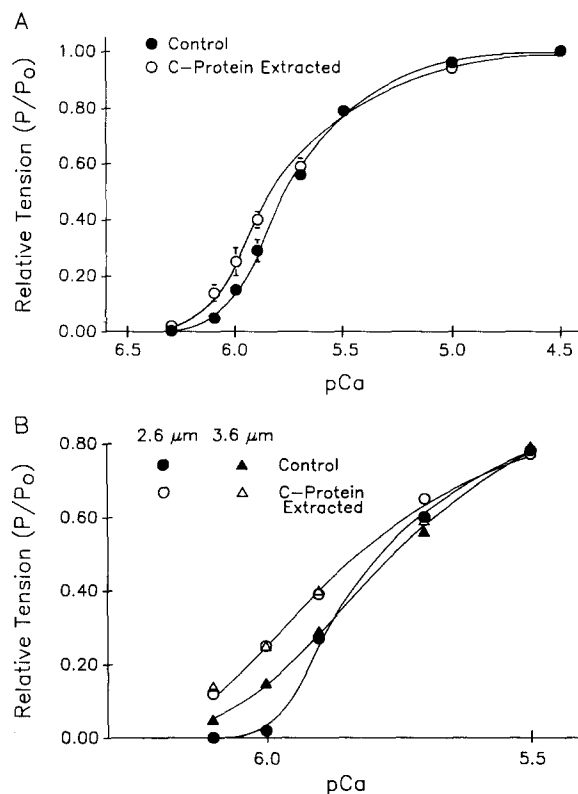


FIGURE 8. Tension-pCa relations demonstrating the effects of C-protein extraction on single skeletal fibers at optimum and long sarcomere lengths. (A) Cumulative data ($n = 9$) obtained at a mean sarcomere length of $3.57 \pm 0.04 \mu\text{m}$ both before (\bullet) and after (\circ) partial extraction of C-protein. After extraction, the tension at pCa 4.5 tension 0.90 ± 0.03 of control P_0 . The pCa_{50} and Hill coefficients for A were as follows:

	pCa_{50}	$n_1 (P/P_0 > 0.50)$	$n_2 (P/P_0 < 0.50)$
Control	5.83 ± 0.03	1.82 ± 0.13	5.28 ± 0.30
C-protein extracted	5.86 ± 0.02	1.62 ± 0.09	3.65 ± 0.30

(B) Comparison of tension pCa-data obtained at $2.5 \mu\text{m}$ and at $3.5 \mu\text{m}$. The data shown are simply a replot of the cumulative data between pCa 5.5 and pCa 6.1 from Fig. 6A (mean sarcomere length of $2.56 \pm 0.02 \mu\text{m}$) and from Fig. 8A. Error bars have been omitted for clarity.

extraction was specific for C-protein when pH was 5.6–6.0. Their phosphate/EDTA solution at pH 5.9, but with added troponin and, for skeletal muscle, added LC_2 , was used in the present study to extract C-protein from skinned cardiac myocytes and skeletal muscle fibers. The selectivity of the extraction procedure for C-protein was established in our preparations in two ways. First, SDS-PAGE of the muscle cells showed that of the myofibrillar proteins only C-protein was extracted (Fig. 1).

Second, readdition of purified C-protein fully restored the tension-pCa relations in extracted cardiac myocytes (Fig. 5) and skeletal muscle fibers (Fig. 6 B).

Isometric Tension and C-Protein Extraction

Previous studies of C-protein were mainly biochemical studies of myofibrillar proteins reconstituted in solution. Based on our finding of increased tension at submaximal pCa, we might predict that actomyosin ATPase activity would be elevated after the extraction of C-protein, but biochemical results to date are ambiguous. At ionic strengths less than 0.1 M, addition of C-protein to a solution of cardiac myosin and unregulated actin increased actin-activated myosin ATPase activity (Yamamoto and

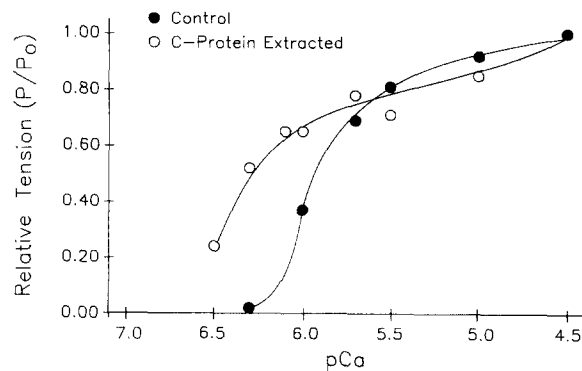


FIGURE 9. Tension-pCa relationships from a single cardiac myocyte demonstrating the effects of C-protein extraction at a sarcomere length of 3.51 μm . Data shown were obtained before (\bullet) and after (\circ) the partial extraction of C-protein. After extraction, tension at pCa 4.5 was 0.84 of control P_0 at the same sarcomere length. Resting tension in solution of pCa 9.0 was 0.50 ± 0.03 (mean \pm SEM, $n = 18$) of control P_0 measured at optimum sarcomere length. Myocyte 7689; overall cell length, 234 μm .

	pCa ₅₀	n_1 ($P/P_0 > 0.50$)	n_2 ($P/P_0 < 0.50$)
Control	5.95	1.05	4.87
C-protein extracted	6.31	0.69	2.65

Moos, 1983; Hartzell, 1985; Margossian, 1985). For skeletal muscle myosin and unregulated actin, the addition of C-protein significantly decreased ATPase activity at ionic strengths less than 0.1 M; however, at ionic strengths of 0.10–0.12 M, ATPase activity was increased (Offer et al., 1973; Moos and Feng, 1980; Yamamoto and Moos, 1983; Lim and Walsh, 1986). Further, Offer et al. (1973) found that Ca^{2+} did not alter the effects of C-protein on ATPase activity of skeletal muscle myosin in solution, while in the present study, the effects due to C-protein extraction were observed only at high pCa. This apparent discrepancy regarding the Ca^{2+} sensitivity of the effects of C-protein may be the result of differences in the preparations (i.e., proteins in solution vs. in a skinned myocytes preparation) in the two studies.

C-protein may have its effects upon isometric tension via an interaction with another protein such as myosin LC₂. In this regard, Margossian (1985) found that C-protein increased actomyosin ATPase activity, but only when LC₂ was bound to myosin. In previous work from this laboratory (Hofmann et al., 1990), partial extraction of LC₂ led to increased tension at high pCa, which is similar to the effect of C-protein extraction observed in the present study. An interaction between C-protein and LC₂ could provide an explanation for effects of C-protein extraction only at high pCa, since Ca²⁺ binding to LC₂ was previously suggested as a mechanism for increasing the probability of S-1 binding to actin (Moss et al., 1983*b*). Moss et al. (1983*b*) found that the tension-pCa relationship at short lengths (~2.5 μm) could best be described by two Hill coefficients, while the relationships at long lengths could in some cases be described by a single Hill coefficient. This suggests that at long lengths, where myosin heads are closely apposed to the thin filaments, activation is regulated only by the binding of Ca²⁺ to the thin filament, and Ca²⁺ modulation of S-1 binding is minimal (Moss et al., 1983*b*). Similarly, in cardiac myocytes, the removal of C-protein reduced n_2 to values near n_1 (Fig. 4*B*), which is consistent with the idea that C-protein in vivo limits the availability of S-1 to actin.

On the other hand, while it is known that C-protein binds to S-2 (Moos et al., 1975), we are unaware of evidence of direct interactions between C-protein and LC₂. Thus, the striking similarity of the effects on the tension-pCa relation due to extraction of the two proteins may simply indicate that both proteins independently modify the availability of S-1 for binding to actin. Hofmann et al. (1990) proposed that the extraction of LC₂ increased the probability of cross-bridge attachment due to greater flexibility of the myosin molecule subsequent to dissociation of LC₂ from the S1-S2 hinge region. Likewise, removal of C-protein may alter the packing of myosin molecules in the thick filament, thereby increasing the range of movement of the cross-bridge and increasing the probability of S-1 binding to actin.

Consideration of Possible C-Protein Binding to Actin

C-protein binds to F-actin in solution and to thin filaments in skeletal muscle myofibrils at an ionic strength of ~0.1 M (Moos et al., 1978; Moos, 1981). The binding of C-protein to regulated actin is Ca²⁺ dependent, increasing with increasing concentrations of Ca²⁺ (Moos, 1981; Yamamoto, 1986). The addition of C-protein to actin and myosin in solution increases solution turbidity, most likely due to increased formation of stable aggregates of thick and thin filaments (Hartzell, 1985). These results suggest that the effects of C-protein on actomyosin ATPase and tension could be due to competition between C-protein and myosin for binding sites on actin.

To test this possibility, skinned skeletal muscle fibers and cardiac myocytes were stretched to sarcomere lengths at which C-protein should be unable to bind actin. At these lengths, the tension-pCa relationships of untreated muscle cells were shifted to lower Ca²⁺ concentrations (Fig. 8), confirming earlier results (Endo, 1972; Moss et al., 1983*b*). After partial extraction of C-protein, submaximal tension increased further, but the increases were not as great as those seen at shorter sarcomere lengths (Fig. 8*B*). Still, the absolute positions of the tension-pCa relation after C-protein extraction were similar at long and short sarcomere lengths. This result argues that the shift in the tension-pCa relation observed after C-protein extraction at short lengths is not

the result of reduced binding of C-protein to actin. Otherwise, we would have expected the tension-pCa relation after C-protein extraction to overlay the control relation at long lengths. It is important to note, however, that the selection of a long sarcomere length in order to prevent binding of C-protein to actin was based on immunocytochemical data indicating that C-protein is not found on the terminal 0.3 μm of each end of the thick filament. A necessary caution is that antibody binding indicates the distribution of epitopes accessible to the antibodies, such that regions between and beyond the observed C-protein stripes may contain epitopes that are inaccessible to the antibody. Also, to our knowledge, there have been no localization studies of C-protein in cardiac muscle, and we have simply assumed that C-protein distribution in cardiac muscle is similar to that in psoas muscle.

The Role of C-Protein in Cooperative Activation

Since the activation of muscle contraction is a highly cooperative process (Brandt et al., 1980; El-Saleh et al., 1986), the effects of C-protein extraction to selectively increase tensions at submaximal concentrations of Ca^{2+} may involve cooperative mechanisms within the thin filaments. Ca^{2+} binding to TnC and the activation of the actomyosin ATPase by Ca^{2+} are enhanced by the binding of S-1 to actin (Bremel and Weber, 1972; Grabarek et al., 1983). The molecular cooperativity of activation is also evident in physiological results from skinned single fibers. The tension-pCa relationship of fast-twitch skeletal muscle is sigmoidal, but it is asymmetric in that the lower part of the curve ($P/P_0 < 0.5$) is steeper than the upper part (Moss et al., 1983b). Quantitation of the relationship by Hill plot analysis yields a Hill coefficient, n_1 , of ~ 2 for the upper part of the curve and a coefficient, n_2 , of 4 or more for the lower part of the curve in cardiac myocytes (Fig. 4). Molecular cooperativity within the thin filament has been inferred from changes in the tension-pCa relationship after partial extraction of TnC. Extraction of TnC causes a decrease in n_2 and a right-shift of the tension-pCa relation (Brandt et al., 1984; Moss et al., 1985), suggesting that inactive functional groups (a functional group is composed of one Tn, one Tm, and seven actin monomers) within the thin filament disrupt near-neighbor cooperative activation of adjacent functional groups. Thin filament cooperativity also appears to depend on the type of TnC that is present, which has been demonstrated by changes in the slope of the tension-pCa relationship by replacement of endogenous sTnC by cTnC in skinned skeletal muscle fibers (Moss et al., 1986) and cTnC by sTnC in skinned myocardium (Gulati et al., 1988).

It is unlikely that the effect of C-protein extraction on tension in either cardiac or skeletal muscle is by alteration of cooperative activation of the thin filament. Extraction of cTnC from cardiac myocytes resulted in disruption of thin filament cooperativity such that the tension-pCa relationship shifted to the right, i.e., the Ca^{2+} sensitivity of tension decreased (Fig. 7). Whether C-protein was extracted from cTnC-deficient or cTnC-replete myocytes, similar shifts of the pCa_{50} and decreases in the steepness of the tension-pCa relation were observed (compare Figs. 4 A and 7 B). This result argues that C-protein extraction does not affect cooperative, near-neighbor activation of functional groups within the thin filament. However, since binding of cross-bridges cooperatively increases the affinity of TnC for Ca^{2+} (Guth

and Potter, 1987; Hofmann and Fuchs, 1988), partial extraction of C-protein would be expected to increase the level of thin filament activation as a result of an increased availability of S-1 to actin.

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