Biophysical Journal Volume 68 April 1995 1430–1442

Myosin Binding-Induced Cooperative Activation of the Thin Filament in Cardiac Myocytes and Skeletal Muscle Fibers

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ABSTRACT Myosin binding-induced activation of the thin filament was examined in isolated cardiac myocytes and single slow and fast skeletal muscle fibers. The number of cross-bridge attachments was increased by stepwise lowering of the [MgATP] in the Ca^{2+}-free solution bathing the preparations. The extent of thin filament activation was determined by monitoring steady-state isometric tension at each MgATP concentration. As pMgATP (where pMgATP is $-\log$[MgATP]) was increased from 3.0 to 8.0, isometric tension increased to a peak value in the pMgATP range of 5.0–5.4. The steepness of the tension-pMgATP relationship, between the region of the curve where tension was zero and the peak tension, is hypothesized to be due to myosin-induced cooperative activation of the thin filament. Results showed that the steepness of the tension-pMgATP relationship was markedly greater in cardiac as compared with either slow or fast skeletal muscle fibers. The steeper slope in cardiac myocytes provides evidence of greater myosin binding-induced cooperative activation of the thin filament in cardiac as compared with skeletal muscle, at least under these experimental conditions of nominal free Ca^{2+}. Cooperative activation is also evident in the tension-pCa relation, and is dependent upon thin filament molecular interactions, which require the presence of troponin C. Thus, it was determined whether myosin-based cooperative activation of the thin filament also requires troponin C. Partial extraction of troponin C reduced the steepness of the tension-pMgATP relationship, with the effect being significantly greater in cardiac than in skeletal muscle. After partial extraction of troponin C, muscle type differences in the steepness of the tension-pMgATP relationship were no longer apparent, and reconstitution with purified troponin C restored the muscle lineage differences. These results suggest that, in the absence of Ca^{2+}, myosin-mediated activation of the thin filament is greater in cardiac than in skeletal muscle, and this apparent cooperativity requires the presence of troponin C on thin filament regulatory strands.

INTRODUCTION

In striated muscle, Ca^{2+}-activated contraction is regulated by the thin filament proteins troponin and tropomyosin. At low concentrations of cytosolic free Ca^{2+} ($<10^{-7}$ M), contraction is inhibited by the thin filament proteins troponin and tropomyosin. Contraction is initiated by an increase in the concentration of Ca^{2+} to about 10^{-5} M. The increased Ca^{2+} in the cytoplasm binds to troponin, which initiates a series of conformational changes among the regulatory protein components of the thin filament, culminating in a movement of tropomyosin to a position favorable to the formation of strongly bound, force-generating cross-bridges. It is now apparent that the thin filament transition from the relaxed state to the fully activated state requires Ca^{2+} binding to troponin as well as myosin binding to actin (Bremel and Weber, 1972; Güth and Potter, 1987; Zot and Potter, 1989; Swartz and Moss, 1992).

Thin filament activation is a cooperative process that involves molecular interactions among functional groups along the thin filament (Brandt et al., 1984; Moss et al., 1985, 1986). A functional group is defined structurally as at least seven contiguous actin monomers, one tropomyosin, and one whole troponin complex (Bremel and Weber, 1972). The activation of a functional group by Ca^{2+} binding to troponin, and/or cross-bridge binding to actin, influences the activation state of neighboring functional groups. Molecular cooperativity in muscle fibers is evident in the steepness of the relationship between tension development and the cytosolic concentration of Ca^{2+}. Indeed, Hill coefficients ranging from about 4 to 8 have been derived from tension-pCa data obtained from fast skeletal muscle fibers (Brandt et al., 1984; Metzger and Moss, 1987). Because there are only two low affinity, regulatory Ca^{2+} binding sites per fast skeletal troponin C molecule, Hill coefficients greater than 2 are usually taken as evidence of cooperative molecular interactions that span neighboring functional groups along the thin filament. The existence of thin filament molecular cooperativity is further supported by experiments in which the steepness of the tension-pCa relationship is reduced after partial extraction of troponin C from the thin filament (Brandt et al., 1984; Moss et al., 1985).

Cooperative activation of the thin filament is also mediated by cross-bridge binding to actin. Both noncycling (rigor, ATP-free) and cycling (ATP-bound) cross-bridges enhance the affinity of troponin C for Ca^{2+}. Thus, the Ca^{2+} sensitivity of tension is increased in skinned fibers upon lowering the concentration of the substrate MgATP in the activating solutions (Brandt et al., 1972; Godt, 1974). Güth and Potter (1987) found an increase in the fluorescence of dansylaziridine labeled troponin C in rabbit psoas fibers due to both Ca^{2+} binding to troponin C and cross-bridge binding to the thin filament. Further, Gordon and Ridgway (1990) demonstrated the appearance of an extra Ca^{2+} signal in voltage-clamped barnacle single muscle fibers resulting from a step release in muscle length during the declining phase of the Ca^{2+} transient. This extra Ca^{2+} appears to be released from

Received for publication 25 April 1994 and in final form 19 January 1995.
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0006-3495/95/04/1430/13 $2.00

the regulatory Ca^{2+} binding sites of troponin C due to a decrease in the number of attached cross-bridges during shortening. Taken together, these studies indicate that thin filament activation involves reciprocal interactions between Ca^{2+} binding to troponin C and cross-bridge binding to actin.

Insight into the role of cross-bridge binding in the activation of the thin filament is provided by experiments in which the concentration of MgATP is incrementally lowered in muscle fibers in the absence of Ca^{2+} (Brandt et al., 1990). The steepness of the ascending region of the tension-pMgATP relationship (i.e., region from about 4 mM MgATP to the reduced substrate concentration that gives maximum tension) reflects cooperative activation of the thin filament induced by cross-bridge binding. Brandt et al. (1990) reported a reduction in the steepness of the tension-pMgATP relationship due to extraction of troponin C in skeletal muscle fibers, an effect similar to the reduction of Ca^{2+}-induced cooperativity inferred from the reduction in the steepness of the tension-pCa relationship after extraction of troponin C (Brandt et al., 1984; Moss et al., 1985).

There is comparatively little information available on the mechanisms underlying cooperative activation of the thin filament in cardiac muscle. Biochemical studies indicate the existence of cooperative binding of Ca^{2+} to cardiac thin filaments even in the absence of added myosin (Tobacman and Sawyer, 1990). To account for this finding, there must be significant near-neighbor molecular interactions along the cardiac thin filament because there is only one low affinity, regulatory Ca^{2+} binding site in cardiac troponin C (Pan and Solaro, 1987).

The present study examines cooperative activation of the cardiac thin filament by attached cross-bridges in the absence of added Ca^{2+}. Comparative analysis of cardiac and skeletal muscle fiber data demonstrate marked activation of the cardiac thin filament by attached cross-bridges, at least under conditions in which Ca^{2+} is absent.

MATERIALS AND METHODS

Muscle preparations

Cardiac myocyte preparation

Ventricles were isolated from whole hearts of female Sprague-Dawley rats and minced into small fragments (about 3 mm^3) in relaxing solution (see below). The minced tissue was homogenized for about 6 s at low speed in a Waring blender (Sweitzer and Moss, 1990). The resulting cell suspension was centrifuged at 120 × *g* for 1 min, and the pellet was resuspended in 10 ml of relaxing solution containing the protease inhibitor leupeptin (0.1%, final concentration).

The isolation procedure permeabilized the cardiac myocytes because exposure of the cells to 0.2% Triton X-100 for up to 30 min did not alter maximum force or the tension-pCa relationship (Sweitzer and Moss, 1990) (present study, data not shown). Because brief exposure to Triton improved resolution of the striation pattern, cells were exposed to Triton X-100 for 15–30 s before collecting mechanical data.

Skinned single skeletal muscle fibers

Fast-twitch skeletal muscle fibers were obtained from psoas muscles of adult male New Zealand rabbits as detailed previously (Metzger et al., 1989). These muscle fibers are histochemically defined as type IIb fibers and contain fast isoforms of contractile and regulatory proteins as determined previously (Metzger and Moss, 1991). Slow skeletal fibers were obtained from the soleus muscles of adult female rats. These fibers express the slow isoforms of contractile and regulatory proteins (Metzger and Moss, 1987). Bundles of approximately 50 fibers were dissected from each muscle while in relaxing solution (below) and were tied with surgical silk to glass capillary tubes. Bundles were stored for up to three weeks at −20°C in relaxing solution containing 50% (v/v) glycerol.

Experimental chamber and apparatus

Cardiac myocyte preparation

An experimental chamber similar to one used previously (Metzger et al., 1989) was constructed for attaching cardiac myocytes to the recording apparatus and for the activation and relaxation of the isolated cell. The chamber was positioned on an anti-vibration table to isolate the experimental apparatus from building noise. Further, to eliminate acoustical noise, a plexiglass enclosure with a sliding front door was constructed and positioned over the experimental apparatus. The temperature of the experimental chamber was controlled to 15°C (±0.1°C).

The cardiac myocyte attachment procedure was modified from Sweitzer and Moss (1990) and involved pulling borosilicate glass micropipettes to tip diameters of about 1 μm. The micropipettes were then inserted into an output tube of a force transducer (Model 403A, Cambridge Technology, Cambridge, MA; sensitivity, 1–5 μg; 1–99% response time, 1 ms; resonant frequency, ~1 kHz, as determined experimentally by attaching a natural rubber fiber between the micropipettes) and a piezoelectric translator (Model P-173, Physik Instruments, Waldbronn, Germany; nominal expansion, 40 μm at 1000 V; resonant frequency, 6.2 kHz) or Cambridge Technology 6350 optical scanner (moving-coil galvanometer). Micropipettes were secured in place with a paraffin seal. The force transducer and piezoelectric translator/Cambridge motor were attached to three-way positioners to allow exact positioning of the micropipettes. The cardiac myocyte attachment procedure involved placing a drop of the myocyte suspension onto a coverslip that was placed over the first trough in the experimental chamber. The position of the trough was lowered by about 1 mm with respect to the neighboring troughs so that when the cell was transferred to the relaxing and activating troughs it was completely submersed in solution. The tips of the micropipettes were broken (O.D. ≈ 5 μm), coated with a silicone adhesive (Dow Chemical, Midland MI) and then positioned over an individual cardiac myocyte that was selected based on rod-shaped appearance, clear sarcomere pattern, and dimensions consistent with those of an isolated single cardiac myocyte. One micropipette was lowered onto an end of the cardiac myocyte, and gentle downward pressure was applied that caused the silicone to envelop the top surface and sides of the cardiac myocyte. The second micropipette was attached to the other end of the cardiac myocyte. After about 10–30 min, the silicone had cured sufficiently (i.e., tack-free) to make a strong attachment and the preparation was carefully raised up from the coverslip. Next, the underside of each attachment was coated with silicone using a third micropipette to ensure that silicone surrounded the entire cross section of the micropipette tip-cardiac myocyte interface.

The average dimensions of the attached cardiac myocyte were length 70 μm (measured as the distance between the two silicone attachment points) and width 20 μm (Fig. 1). For comparison, enzymatically isolated cardiac myocytes from rats average about 120 μm in length and 20 μm in width (Stemmer et al., 1992). Thus, the attached cardiac myocyte preparation used in this study has the dimensions of a segment of a single isolated cardiac myocyte. Sarcomere length was set at 2.2 μm.

Skinned single skeletal muscle fibers

The attachment procedure involved placing the ends of the fiber in troughs that were fashioned from 29-gauge stainless steel tubing (Moss, 1979; Metzger et al., 1989). The fiber was secured in place by overlaying a 1-mm section of 5-0 monofilament nylon suture over the end of the fiber. The monofilament pin was then tied down using 10-0 nylon. Using this attachment procedure, end compliance averages about 1–4% of fiber length as

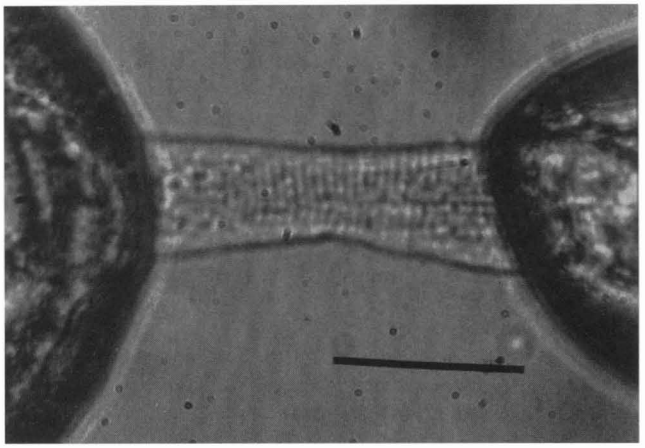

FIGURE 1 Photomicrograph of a cardiac myocyte attached to the glass micropipettes. Sarcomere length was 2.2 μm. Calibration bar = 50 μm.

judged by the y-intercept in plots of length change versus duration of unloaded shortening (i.e., slack-test plots; Metzger and Moss, 1988). Single fibers were mounted between a force transducer (model 400A; noise level at the output equivalent to 1 mg-wt peak-to-peak) and a high performance moving-coil galvanometer (model 6350; Cambridge Technology). Sarcomere length was set at 2.50–2.60 μm. Complete details of the mounting procedure and experimental setup have been reported elsewhere (Metzger et al., 1989).

Solutions

Relaxing and Ca^{2+}-activating solutions

Relaxing and activating solutions contained (in mmol l^{-1}): EGTA, 7; free Mg^{2+}, 1; MgATP, 4; creatine phosphate, 14.5; imidazole, 20; and sufficient KCl to yield a total ionic strength of 180 mmol l^{-1}. Solution pH was adjusted to 7.00 with KOH. The pCa (i.e., $-\log[Ca^{2+}]$) of the relaxing solution was 9.0, whereas the pCa of the solution for maximal activation was 4.5. The computer program of A. Fabiato (1988) was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex, using the stability constants listed by Godt and Lindley (1982). The apparent stability constant for Ca^{2+}-EGTA was corrected for ionic strength, pH, and experimental temperature (Fabiato, 1988).

Solutions for determining the Ca^{2+}-free, tension-pMgATP relationship

Solutions contained (in mmol l^{-1}): EGTA, 7; free Mg^{2+}, 1; MgATP, varied from pMgATP 3.0 to 8.0, using 8.699×10^3 M^{-1} for the apparent stability constant for MgATP at pH 7.00 and 15°C; imidazole, 20; and sufficient KCl to yield a total ionic strength of 180 mmol l^{-1}. Creatine phosphate (CP) was usually not added to these solutions in an attempt to compare our findings with those of Brandt et al. (1990), who did not include CP in their solutions. In two control experiments, CP (14.5 mM) and CP + creatine phosphokinase (150 units/ml) were added to the solutions and the results obtained were found to be comparable with those without added CP or CPK (see Appendix). Solution pH was adjusted to 7.00 with KOH; free Ca^{2+} was set at pCa 9.0. Stock solutions with pMgATP set at 3.0 and 8.0 were mixed to make intermediate pMgATP solutions.

Protocols to determine the tension-pMgATP relationship

The cardiac myocyte preparation was transferred from relaxing to activating solution (pCa 4.5) before determination of the tension-pMgATP relationship (Fig. 2). Typically, the preparation was transferred to Ca^{2+}-free solutions in which pMgATP was set at 3.0–4.0, which is a sufficiently high ATP concentration to relax fully the cardiac myocyte. The cardiac myocyte was then cycled through solutions in which pMgATP was raised by about 0.1–0.5 pMgATP unit per solution change until pMgATP equalled 8.0. In the steep region of the curve, the increments were 0.1 pMgATP unit so that about 4–5 points were obtained between the tension minimum and maximum. To obtain isometric tension at each pMgATP the preparation was rapidly (<1 ms) shortened and then re-lengthened (about 20% of overall length) to obtain the tension baseline. Resting tension at pMgATP 3.0 was then subtracted from each value.

Protocols for determination of the tension-pMgATP relationship in slow and fast skinned single skeletal muscle fibers were similar to those used for the cardiac myocyte preparation. However, in some skeletal fiber experiments solution changes were accomplished by injecting solutions into the bottom of the experimental chamber (Fig. 4). Constant solution volume was maintained in these experiments by positioning a section of polyethylene tubing (O.D. 0.5 mm) over the top of the chamber, which in turn was connected to a constant vacuum source so that the solution level was unaltered during infusion of the new solution. The flow-through chamber was used to eliminate possible effects on rigor tension due to passing the fiber through the solution/air interface. To ensure complete exchange of the solutions, 1–2 ml of solution was typically infused into the 200-μl chamber.

FIGURE 2 Slow time-base records of isometric tension during the experimental protocol to determine the tension-pMgATP relationship in a cardiac myocyte. a. The cardiac myocyte was transferred (*arrow*) from relaxing to activating solution (pCa 4.5) resulting in maximum isometric tension. To determine the total tension the cardiac myocyte was first slackened to obtain a force baseline (*b*) (not resolved on slow time-base recording), then re-extended and transferred to a pMgATP 2.4 relaxing solution (*c*). The cardiac myocyte was then exposed to solutions in which the substrate MgATP was incrementally lowered. The pMgATP were as follows: (*d*) 4.0; (*e*) 5.0; (*f*) 5.2; (*g*) 5.4; (*h*) 5.6; (*i*) 6.0; (*j*) 8.0. Determination of the steepness of the tension-pMgATP relationship required that the pMgATP be incremented by about 0.1 pMgATP units in the relaxed to activated region of the curve (e.g., Fig. 3). Because of altered surface tension surrounding the recording micropipettes upon changing solutions, a small (5–10 μm) and rapid release and re-extension in cardiac myocyte length was performed in order to obtain the tension baseline at each pMgATP (not seen on slow time-base records).

Earlier experiments showed that trypan blue-containing relaxing solution was completely washed out after injection of 2–3 vol of a trypan blue-free relaxing solution.

Troponin C extraction and reconstitution protocol

Troponin C was specifically extracted from the troponin complex using a solution containing 5 mM EDTA, 10 mM HEPES, and 500 μM trifluoperazine dihydrochloride (TFP; Smith, Kline and French Laboratories, Philadelphia, PA) as described previously (Metzger et al. 1989; Metzger and Moss, 1991). The composition of the extraction solution was derived from Cox et al. (1981) and is used here in modified form with the addition of TFP (Metzger et al., 1989). Importantly, after extraction, the preparations were washed multiple times in relaxing solution to completely remove TFP. Repeated washes to remove TFP were critical because it has been shown that Ca^{2+}-activated contraction is altered in the presence of TFP (Kurebayashi and Ogawa, 1988). In earlier experiments in which troponin C was extracted and subsequently reconstituted with skeletal troponin C in psoas fibers, the pre- and postreconstitution tension-pCa relationships and maximum tension values were similar (Metzger et al., 1993).

Skeletal fibers were reconstituted with purified skeletal troponin C (0.1–0.3 mg/ml) by incubating the extracted fiber for 10–20 s in a troponin C-containing relaxing solution (Metzger et al., 1989). Repeated exposures to the troponin C solution were continued until tension at pCa 4.5 reached a plateau. In skeletal fibers, the total time of exposure to the troponin C-containing solution was approximately 2–3 min. Cardiac myocytes were reconstituted with purified cardiac troponin C (0.1 mg/ml) for extended time periods (1–2 h), after which tension at pCa 4.5 was determined as a functional test of the reconstitution protocol. The maximum tension in cardiac myocytes after reconstitution was 0.84 ± 0.03 P_o (n = 3). It is not known why longer soaks were required to reconstitute cardiac myocytes compared with skeletal fibers. Purified cardiac and skeletal troponin C were kindly provided by Drs. Richard Moss and Marion Greaser (University of Wisconsin, Madison, WI).

Determination of protein composition by gel electrophoresis

Each skeletal fiber segment was placed in a 0.5-ml microfuge tube containing SDS sample buffer (10 μl/mm segment length) and stored at −80°C for subsequent analysis of contractile and regulatory protein content by SDS-PAGE, and scanning densitometry, as described previously (Metzger and Moss, 1991). The gel electrophoresis procedure used a multiphasic buffer system that incorporated the following features: 1) acrylamide-Bis ratio of 200:1, 2) running gel buffer pH of 9.3, and 3) running gel buffer molarity of 0.75 M. The acrylamide content of the running gel was 12%. Gels were fixed with glutaraldehyde overnight, washed, silver-stained, and dried between mylar and cellophane sheets similar to that detailed previously (Giulian et al., 1983). Gels were analyzed by measuring the areas under the peaks corresponding to troponin C, and myosin light chains 1 and 2 using an LKB Ultrascan XL densitometer.

Curve fitting and statistics

Curve fitting

Curves were fit to data corresponding to the portion of the tension-pMgATP relationship where tension was fully relaxed (pMgATP 3–4) to the point where tension was maximal. For each preparation, there were about 10 data points used for curve fitting, with usually 4–5 data points defining the steep region of the curve. To fit the data, a modified version of the Hill equation was used:

$$\text{tension} = \text{maximum tension}/(1 + (\text{pMgATP}/K)^n).$$

Curve fitting used the Marquardt-Levenberg nonlinear least-squares fitting algorithm and was performed using commercially available software (NFIT). Statistics were calculated to determine the goodness of each fit. The SD for each fit is reported in the figure legends as ±SD. In this study, the r^2 averaged 0.994 ± 0.002. K is the concentration of MgATP that results

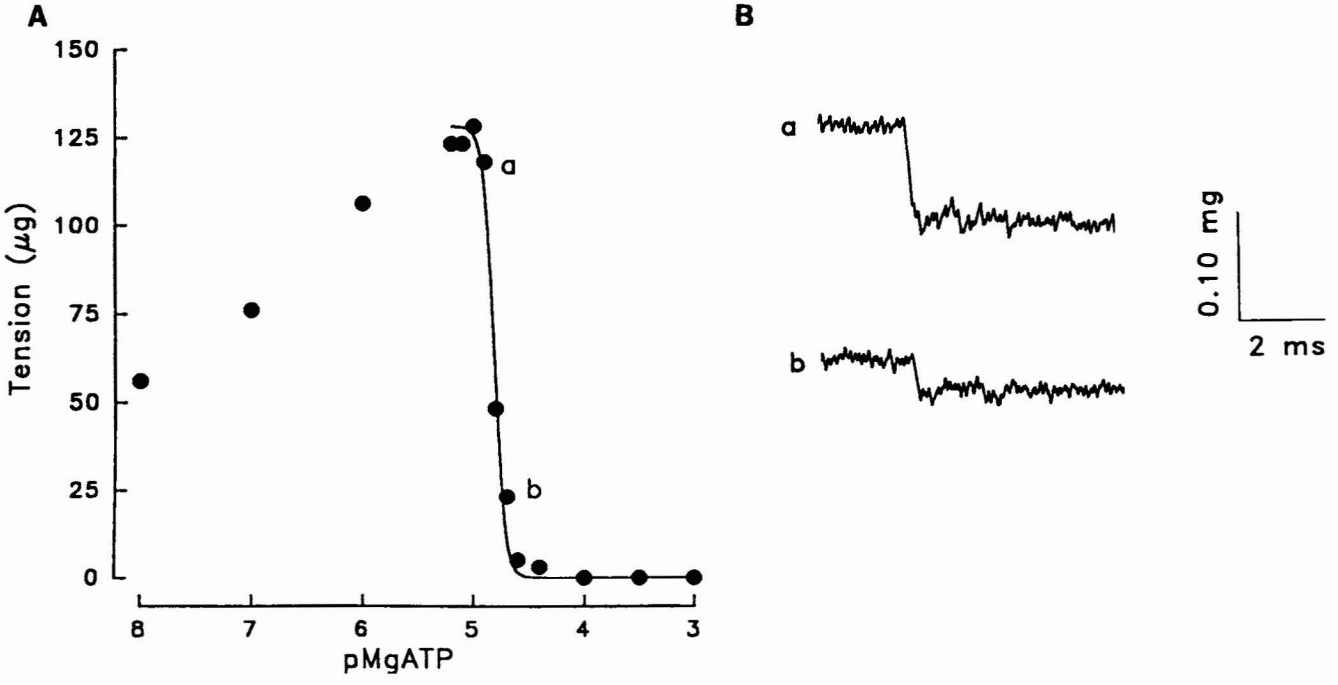

FIGURE 3 Tension-pMgATP relationship obtained from a cardiac myocyte. In *A*, the tension-pMgATP relationship is shown with the best-fit line corresponding to pMgATP data from pMgATP 3.0 to pMgATP 5.2, the substrate concentration where Ca^{2+}-independent tension was maximal. In *B*, fast time-base records of tension are shown with parts *a* and *b* corresponding to the points marked a and b in part A. In this experiment, $pMgATP_{50}$ was 4.81 and n was 9.0 (±1.6, SD of fit).

in half-maximal tension ($pMgATP_{50}$). The Hill coefficient, n, is the slope of the transition from the relaxed to full tension-generated state.

Statistics

When more then two data sets were compared ANOVA was used to determine whether significant differences exist between groups. When interactions among the groups were indicated by ANOVA, a Student's two-tailed t-test was used as a post hoc test to determine significant differences between two mean values using Bonferroni-corrected values for multiple comparisons. The mean value was derived from a sample size of 7–17 observations. A probability level of $p < 0.05$ was selected as indicating significance.

RESULTS

To determine the effects of bound cross-bridges on the activation mechanism of the thin filament, cardiac myocytes and skeletal muscle fibers were exposed to solutions in which the concentration of MgATP was varied from pMgATP 4.0 to 8.0 in the absence of added Ca^{2+}. Fig. 2 shows a representative slow time-base record of isometric tension obtained during the protocol to determine the tension-pMgATP relationship in a cardiac myocyte.

A tension-pMgATP relationship obtained from a cardiac myocyte is shown in Fig. 3. The tension data are fit using a

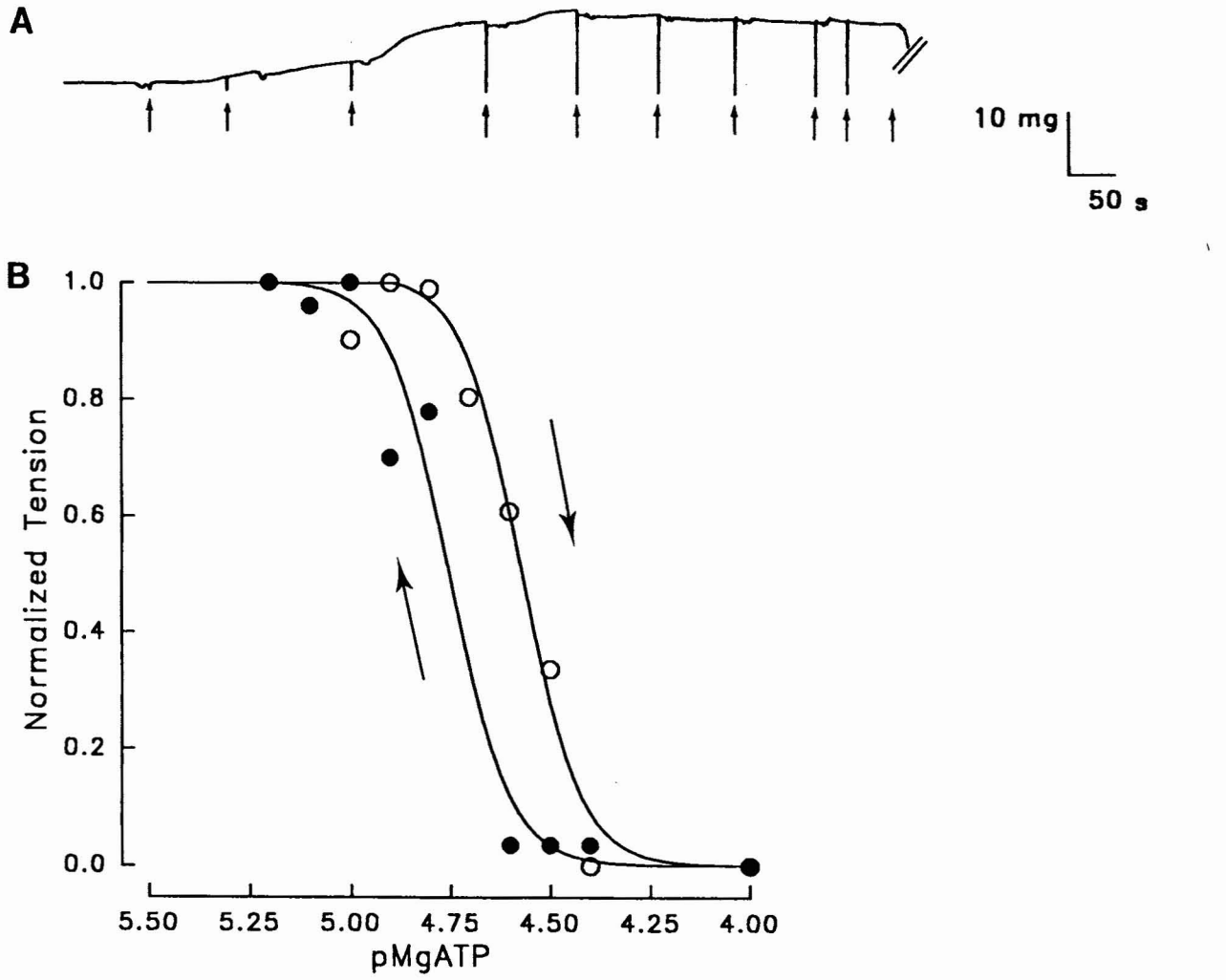

FIGURE 4 Determination of tension-pMgATP relationship in a single psoas fiber. (*A*) Slow time-base record of isometric tension under conditions in which the concentration of MgATP was incrementally lowered from 4 mM to nominal MgATP (i.e., pMgATP of 8.0). Arrows mark time-points where muscle length was rapidly released and re-extended to obtain tension baseline (downward tension transients shown do not reach baseline on these slow time-base records). At the first arrow, tension baseline was obtained and the solution bathing the fiber was changed from 4.0 to 4.5 pMgATP by using the solution injection technique described in Results. Immediately after each subsequent determination of tension baseline (*arrows*), the pMgATP of the solution was changed to 4.7, 4.8, 4.9, 5, 5.1, 7, 8, and finally 3.0, which fully relaxed the fiber (record truncated). (*B*) Tension-pMgATP relationship in a single skinned psoas fiber. The tension-pMgATP relationships are shown with the best-fit line corresponding to data from pMgATP 3.0 to the pMgATP that gave maximum tension. The filled circles were collected by incrementally increasing pMgATP (decreasing [MgATP]) from a starting pMgATP of 3.0, and the open circles were collected from the same preparation by lowering pMgATP starting at pMgATP 8.0. Data are normalized to the maximum tension value obtained in each experiment to allow direct comparison of the shape and position of the tension-pMgATP relationships. Tension data at pMgATP > 5.25 are not shown to indicate more clearly differences in the position of the tension-pMgATP relationship. n and $pMgATP_{50}$ were: 5.8 (±1.6 SD), 4.75 for filled circles, and 5.9 (±0.9 SD), 4.57 for open circles, respectively.

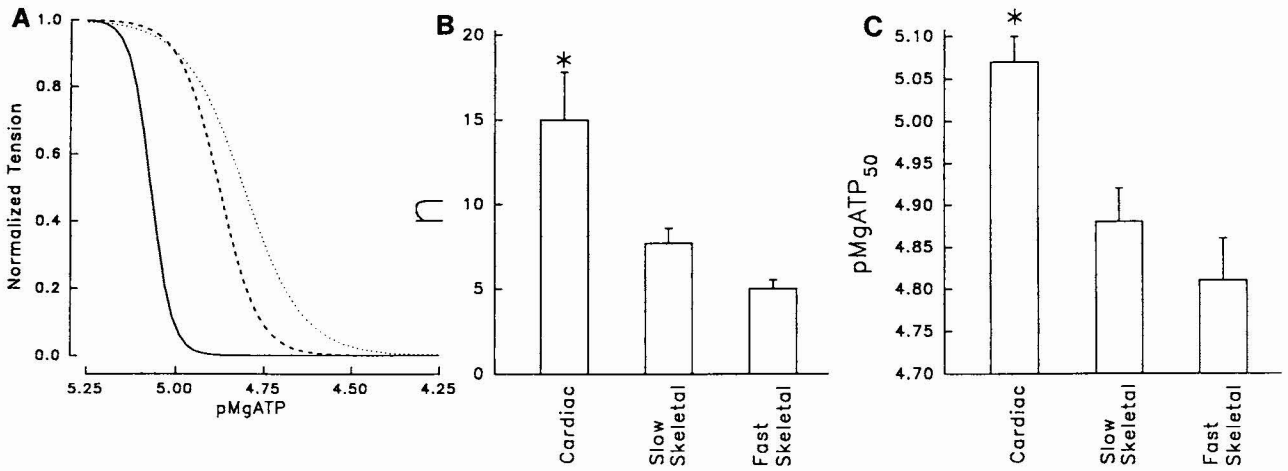

FIGURE 5 Summary of the tension-pMgATP relationships obtained from cardiac myocytes, and fast and slow skeletal muscle fibers. (*A*) Computer generated tension-pMgATP relationships using average n and $pMgATP_{50}$ for cardiac myocytes (——), slow soleus skeletal muscle single fibers (- - - -), and fast psoas skeletal muscle single fibers (· · · · ·); curves are normalized to the maximum rigor tension (1.0). The steep region of the tension-pMgATP relationship is shown to permit comparison of the steepness and position of the relationship among the striated muscle types. (*B*) Summary of tension-pMgATP-derived Hill coefficients (n). Asterisk indicates cardiac value significantly greater than slow and fast skeletal values. (*C*) Summary of tension-pMgATP-derived $pMgATP_{50}$s. Asterisk indicates cardiac value significantly greater than slow and fast skeletal values. Values are mean ± SEM, n = 7–17.

modified form of the Hill equation to determine the concentration of MgATP at which isometric tension is half-maximal ($pMgATP_{50}$), and to derive the Hill coefficient, n, which reports the steepness of the tension-pMgATP relationship in the transition region between the relaxed state and the activated state where rigor tension is maximal. The steepness (n) of this transition region is thought to reflect cooperative activation of the thin filament regulatory system by bound cross-bridges (Brandt et al., 1990).

The finding that the tension-pMgATP relationship is generally biphasic in cardiac and skeletal muscles is in agreement with previous findings. (Reuben et al., 1971; Brandt et al., 1972, 1990; Fabiato and Fabiato, 1975; Ferenczi et al., 1984; Goldman et al., 1984; Moss and Haworth, 1984). The tension-pMgATP relationship typically consists of three distinct pMgATP regions: 1) from 2 to 4, where tension is relaxed; 2) from about 4 to 5 where tension ascends; and 3) from about 5 and greater where tension is either maintained (e.g., Fig. 7) or descends (Fig. 3).

Fig. 4 shows two tension-pMgATP relationships obtained from a single psoas fiber that were constructed by incrementally increasing pMgATP or decreasing pMgATP. For experiments in which pMgATP was incrementally increased, the preparation was first relaxed in the pMgATP range 3.0–4.0. As pMgATP was increased, there was a transition to peak rigor tension with a derived $pMgATP_{50}$ of 4.75 and an n of 5.8. In the same fiber, a second tension-pMgATP relationship was obtained by starting at pMgATP 8.0 and then lowering pMgATP in a stepwise manner to 3.0. Under these conditions, tension reached a peak value at pMgATP 4.9, began to decline starting at about pMgATP 4.8, and was zero at pMgATP 4.0. The $pMgATP_{50}$ was 4.57, and n was 5.9. For pMgATP > 5.25, rigor tension was similar in the two plots (these data are not shown in the plot to permit clear determination of the altered position of the tension-pMgATP relationship; Fig. 4). In general, the main difference in tension-pMgATP relationships constructed by increasing or decreasing pMgATP, was that $pMgATP_{50}$s were greater in plots constructed by increasing pMgATP. Thus, the position of the tension-pMgATP relationship was shifted to the right in plots constructed by decreasing pMgATP in comparison to plots constructed by increasing pMgATP, indicating a greater concentration of the substrate MgATP was needed to yield half-maximum rigor tension. Similar findings were obtained in cardiac myocytes (data not shown). The basis of the differences in the position of the tension-pMgATP relationship due to increasing or decreasing pMgATP could indicate that once the thin filament is activated by rigor cross-bridges, comparatively fewer attachments may be required to sustain a given level of submaximal thin filament activation. Alternatively, there may be more strongly bound cross-bridges when a pMgATP is approached from rigor rather than the relaxed state. A possible test of this hypothesis would require obtaining instantaneous stiffness measurements to determine quantitatively the number of cross-bridge attachments at each substrate concentration.

A comparison of tension-pMgATP relationships between cardiac, slow, and fast muscle is shown in Fig. 5. For all comparisons of the tension-pMgATP relationships, only results obtained by incrementally increasing pMgATP were used. These findings indicate marked differences in tension-pMgATP relationships among the various striated muscle types. The steepness of the tension-pMgATP relationship was greater in cardiac myocytes (n = 15.0 ± 3.0) compared with either fast (5.0 ± 0.5) or slow skeletal muscle fibers (7.7 ± 0.9). In addition, the concentration of MgATP had to be reduced to a significantly greater extent in cardiac compared with skeletal fibers to achieve half-maximum rigor tension.

There were no significant differences in tension-pMgATP-derived n or $pMgATP_{50}$ between slow or fast skeletal muscle fibers.

To gain mechanistic insight toward the basis of cooperative activation of the thin filament due to cross-bridge binding, troponin C was partially extracted from the striated muscle preparations (Figs. 6–9). The rationale for these experiments is that partial extraction of troponin C alters cooperative activation of the thin filament in striated muscle under conditions in which Ca^{2+} serves as the activating ligand (Brandt et al., 1984; Moss et al., 1985). In addition, partial extraction of troponin C has been demonstrated to decrease the steepness of the tension-pMgATP relationship in psoas skeletal muscle fibers (Brandt et al., 1990). Thus, it appears that both Ca^{2+}- and cross-bridge-mediated cooperative activation of the thin filament are reduced by troponin C extraction in fast skeletal muscles.

The effects of troponin C extraction on both Ca^{2+}- and cross-bridge-activated tension in cardiac myocytes were determined. After partial extraction of troponin C, maximum Ca^{2+}-activated tension was reduced and there was a reduction in the steepness and a rightward shift in the tension-pCa relationship (data not shown), results that are in agreement with an earlier study (Sweitzer and Moss, 1990).

To show that the extraction protocol was specific for troponin C, pools of 10–20 cardiac myocytes were collected and run on gels. Densitometric analysis of the gels indicates that the extraction protocol was specific for removal of troponin C (Fig. 6). In the example shown (Fig. 6), 58% of endogenous troponin C was extracted. This value was obtained by determining the areas under the troponin C and LC_2 peaks and dividing the extracted troponin C/LC_2 ratio by the control troponin C/LC_2 ratio. Similar results were obtained by scaling data to the LC_1 peak because the LC_2/LC_1 ratio was essentially unchanged by extraction, a result similar to that obtained previously in skeletal fibers (Metzger and Moss, 1991). In this comparison, the extracted LC_2/LC_1 ratio divided by control LC_2/LC_1 ratio equalled 1.08. Similar findings were obtained in four additional side-by-side comparisons between control and extracted cardiac myocytes.

After partial extraction of troponin C, there was a marked decrease in the steepness of the tension-pMgATP relationship in cardiac myocytes. For the cardiac myocyte shown in Fig. 7, the Hill coefficient was reduced from 14.0 to 2.6 after extraction. The maximum Ca^{2+}-activated tension was 0.59 P_o after extraction, whereas maximum rigor tension (after extraction/before extraction) was 1.08. Thus, rigor tension was little changed by extraction, whereas Ca^{2+}-activated tension was markedly reduced. After incubation of the extracted myocyte with purified cardiac troponin C, maximum Ca^{2+}-activated tension increased to 0.88 P_o. Importantly, reconstitution with cardiac troponin C increased n to 11.0, a value

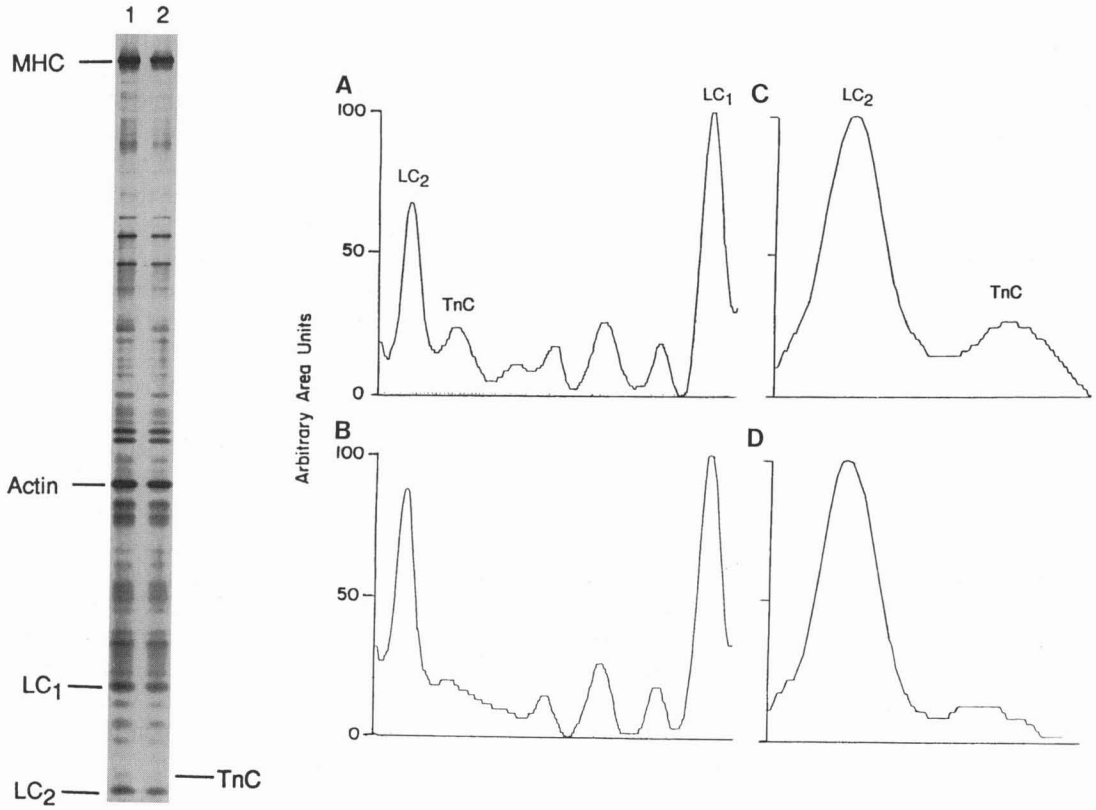

FIGURE 6 Sodium dodecyl sulfate polyacrylamide gel and densitometric scans obtained from isolated cardiac myocytes before and after the procedure to extract troponin C. (Part 1) SDS-polyacrylamide gel. Lane 1 is control, and lane 2 is troponin C extracted. Twenty cardiac myocytes were loaded per lane. Myocytes were collected with borosilicate micropipettes as detailed previously (Metzger et al., 1993). (Part 2) Densitometric scans of the lower portion the SDS gel shown in part 1. *A* and *C* are control, and *B* and *D* are troponin C-extracted. *C* and *D* were plotted on an expanded *x* axis for increased resolution.

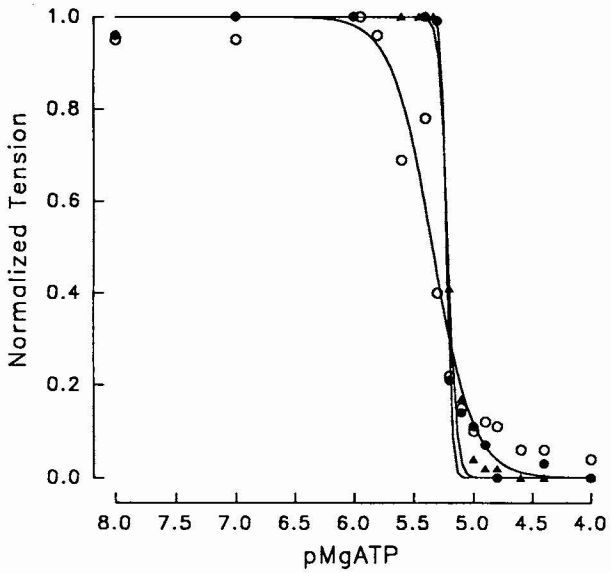

FIGURE 7 Effects on the tension-pMgATP relationship due to extraction and subsequent re-addition of troponin C in an isolated cardiac myocyte. Filled circles are data before extraction of troponin C, open circles are data from the same cardiac myocyte after extraction of troponin C, and filled triangles are data after reconstitution with purified cardiac troponin C. n and $pMgATP_{50}$ were 14.0 (±3.0 SD) and 5.22 for control, 2.6 (±0.5 SD) and 5.34 for troponin C-extracted, and 11.0 (±2.2 SD) and 5.21 after troponin C reconstitution, respectively. Curves were fit to the data in the transition from the relaxed to the maximum tension-generated state.

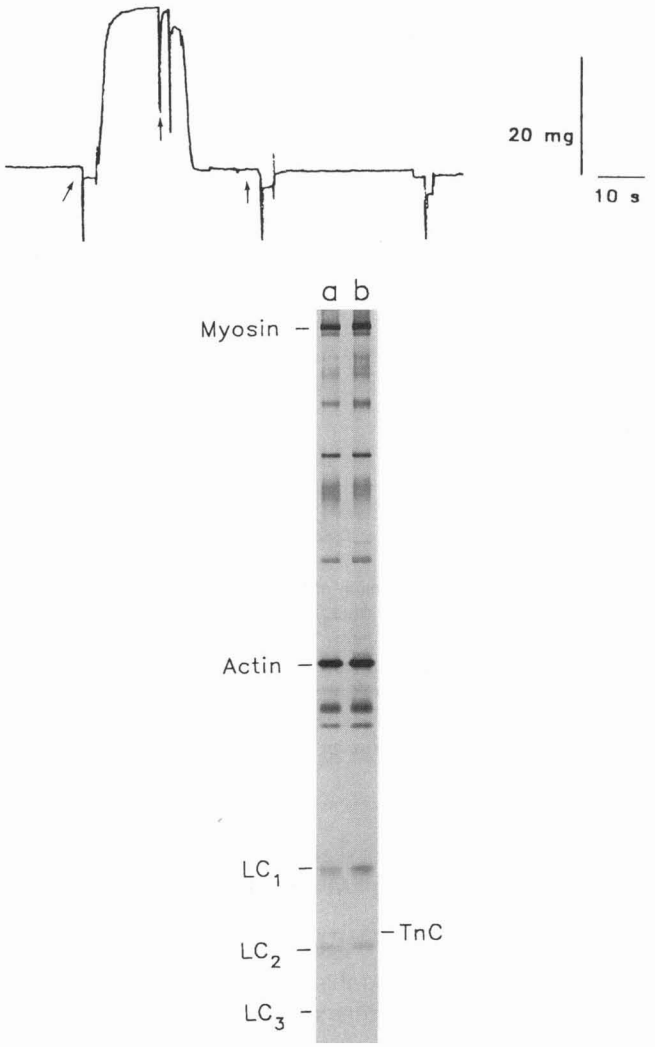

FIGURE 8 Effects on maximum Ca^{2+}-activated tension and protein composition due to extraction of troponin C in a single psoas fiber. (Part 1) Effects on maximum isometric tension (pCa 4.5) due to extraction of troponin C. At the first arrow, the solution bathing the fiber was changed from pCa 9.0 to 4.5. At the second arrow, muscle length was released to obtain tension baseline (not seen on slow time-base records), the solution was changed from pCa 4.5 to 9.0, and muscle length was re-extended to the pre-release value. The fiber was then exposed to the troponin C extraction solution. At the third arrow, the solution was changed from pCa 9.0 to 4.5. After extraction of troponin C, relative tension at pCa 4.5 was 0.02 P_o, where P_o is the tension at pCa 4.5 before extraction of troponin C. (Part 2) SDS-polyacrylamide gel obtained from segments of the same psoas fiber before (lane *a*) and after extraction of troponin C (lane *b*); results obtained from segments of a different fiber than that shown in part 1. To quantitate the extent of extraction of troponin C, the ratio troponin C/(LC_1 + LC_3) was determined for control and extracted fibers by measuring the areas under the peaks corresponding to these proteins using an LKB Ultrascan XL densitometer. The ratio obtained from the extracted fiber was then divided by the control value to determine the amount of troponin C extracted (Metzger and Moss, 1991). Calculated in this way, 80% of endogenous troponin C was extracted. As before (Metzger and Moss, 1991), the extraction procedure was specific for troponin C as the ratio of LC_2/(LC_1 + LC_3) was unaltered by the extraction procedure.

close to that obtained before extraction. Thus, after reconstitution with exogenous troponin C the shape and position of the tension-pMgATP plot were highly comparable with the control relationship (Fig. 7). Additional experiments demonstrated that this finding was reproducible. A summary of the troponin C extraction/reconstitution experiments is as follows: control n = 16.7 ± 2.7 (3); troponin C-extracted n = 3.8 ± 1.2 (3), and 0.49 ± 0.11 P_o; troponin C add-back n = 13.2 ± 2.2 (3), and 0.84 ± 0.03 P_o (this is a separate data set than is shown in Tables 1 and 2). Statistical analysis (see Materials and Methods) indicated that there was a significant increase in the Hill coefficient after troponin C reconstitution ($p < 0.05$). These findings provide good evidence that alterations in the tension-pMgATP relationship were due to extraction of troponin C and were not a nonspecific effect of the extraction protocol.

An effect of troponin C extraction to reduce the steepness of the tension-pMgATP relationship was also observed in slow skeletal fibers; however, in fast fibers the decrease in n due to partial extraction of troponin C (Fig. 9) was not statistically significant (Fig. 10; Table 1). It was also demonstrated in fast (Fig. 8) and slow (data not shown) skeletal fibers that the extraction protocol was specific for troponin C. Further, maximum Ca^{2+}-activated tension was restored upon reconstitution of skeletal fibers with exogenous purified troponin C (data not shown), as demonstrated previously (Metzger et al., 1989, 1993).

A summary of the effects on the tension-pMgATP relationship due to troponin C extraction in cardiac, slow, and fast fibers is shown in Fig. 10 and Table 1. There was a significantly greater effect on the steepness of the tension-pMgATP relationship due to troponin C extraction in cardiac

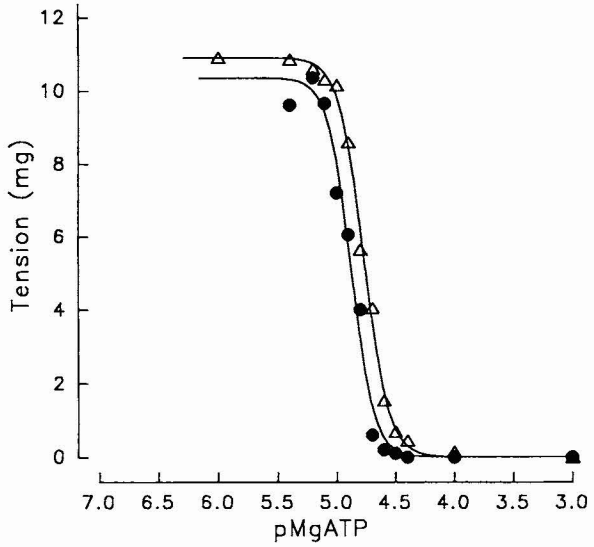

FIGURE 9 Effects on the tension-pMgATP relationship due to troponin C extraction in a single psoas fiber. Filled circles are control, and open triangles are troponin C extracted data. The n and $pMgATP_{50}$ were 4.5 (±0.6 SD) and 4.88 for control and 4.2 (±0.3) and 4.77 for troponin C-extracted data. After troponin C extraction force generation at pCa 4.5 was 0.08 P_o. In contrast, the relative rigor tension was 1.05 (i.e., maximum rigor tension after troponin C extraction/maximum rigor tension before troponin C extraction).

compared with slow or fast skeletal muscle fibers. In skeletal fibers, $pMgATP_{50}$ was significantly reduced by extraction of troponin C.

Extraction of troponin C had differential effects on Ca^{2+}- and cross-bridge-activated maximum isometric tension development. In all preparations studied, extraction of troponin C markedly reduced maximum Ca^{2+}-activated tension (Table 2). In contrast, extraction of troponin C had little effect on maximum rigor tension development in fast and slow skeletal fibers (Table 2). In all cardiac myocytes tested, maximum Ca^{2+}-activated tension was decreased to a greater extent than maximum rigor tension after troponin C extraction (Table 2).

DISCUSSION

The main findings of this study are as follows. 1) The steepness of the tension-pMgATP relationship is markedly greater in cardiac as compared with either fast or slow skeletal muscle. 2) The steepness of the tension-pMgATP relationship is reduced to a greater extent in cardiac than skeletal muscle after partial extraction of troponin C, eliminating muscle type differences in steepness. 3) Muscle type differences in the shape of the tension-pMgATP relationship are restored after reconstitution of extracted preparations with purified troponin C. It has been proposed that the steepness of the tension-pMgATP relationship is due to cooperative activation of the thin filament by rigor cross-bridges (Brandt et al., 1990). In this context, the present results provide evidence of marked activation of cardiac thin filament by attached cross-bridges, at least in the absence of Ca^{2+}.

To facilitate discussion of these findings, a model of myosin binding-induced cooperative activation of the thin filament derived from Bremel and Weber (1972) is briefly presented below. Upon reduction in the concentration of MgATP, myosin cross-bridges that have no ATP bound will interact strongly with actin, forming a rigor-bound state even though no Ca^{2+} is present. These rigor cross-bridges, which are assumed to be distributed randomly along the thin filament, may provide sufficient activation of the thin filament to permit neighboring cross-bridges that have nucleotide bound to cyclically interact with actin. The increased cross-bridge attachment would lead to cooperative activation of the thin filament and would be manifested as an increase in Ca^{2+}-independent isometric tension (e.g., Fig. 3). An earlier finding that both the position and steepness of the tension-pMgATP and stiffness-pMgATP relationships are similar in skinned crayfish muscle fibers (Kawai and Brandt, 1976) gives support to this model.

Possible mechanism of myosin binding-induced cooperative activation of the thin filament

The mechanism underlying myosin binding-induced cooperative activation of the thin filament is not fully understood. Bremel and Weber (1972) demonstrated in reconstituted thin filaments that myosin binding to actin increases the affinity of troponin for Ca^{2+} and that neighboring actin molecules become activated even though Ca^{2+} is absent. It was proposed that rigor bond formation somehow alters the conformation of the Ca^{2+} regulatory proteins resident on the thin filament. The effect of myosin cross-bridges to alter the conformation of thin filament regulatory proteins has been demonstrated directly in skinned fiber experiments in which endogenous troponin C is extracted and replaced with a fluorescently labeled troponin C moiety that reports alterations in Ca^{2+} binding to the low affinity, regulatory sites of this molecule. Güth and Potter (1987) provided evidence that myosin cross-bridge binding to actin induces alterations in the conformation of troponin C in rabbit skinned psoas fibers and that cycling cross-bridges have a greater effect on troponin C than rigor cross-bridge attachments. Qualitatively similar results have been reported using rat cardiac muscle preparations (Hannon et al., 1992). In this context, the mechanism underlying the cross-bridge binding-induced cooperative transition from the relaxed to the activated state apparent in the tension-pMgATP relationship may be due to cycling cross-bridges that are permitted to interact with actin because of the presence of neighboring rigor attachments.

It has been shown that myosin subfragment 1 binding to reconstituted thin filaments in vitro is a cooperative process and that disruption of the head-to-tail interactions between adjoining tropomyosin molecules decreases, but does not completely eliminate, the cooperative binding of myosin to actin (Pan et al., 1989). These results suggest that the basis of cooperative activation of the thin filament involves interactions between near-neighbor functional groups, as proposed earlier by Murray and Weber (1980), and that this

TABLE 1 Summary of effects of troponin C extraction (TnC XT) on the Hill coefficient and $pMgATP_{50}$ derived from tension-pMgATP plots in cardiac myocytes, and slow and fast skeletal muscle fibers

	Hill coefficient		$pMgATP_{50}$	
	Control	TnC XT	Control	TnC XT
Cardiac myocyte	15.0 ± 3.0	4.0* ± 1.1	5.07 ± 0.03	5.01 ± 0.07
Slow skeletal	7.7 ± 0.9	5.0* ± 0.4	4.88 ± 0.04	4.79* ± 0.02
Fast skeletal	5.0 ± 0.5	4.0 ± 0.5	4.81 ± 0.05	4.68* ± 0.03

Values are mean ± SEM (n = 7–17). Asterisks indicate control significantly different than TnC XT value, $p < 0.05$.

TABLE 2 Summary of the effects of troponin C extraction on maximum Ca^{2+}-activated and maximum rigor activated isometric tension in cardiac myocytes, and slow and fast skeletal muscle fibers

	P/P_o		Relative rigor tension	
	Control	TnC XT	Control	TnC XT
Cardiac myocyte	1.00	0.11* ± 0.05	1.00	0.74* ± 0.09
Slow skeletal	1.00	0.28* ± 0.02	1.00	1.18 ± 0.14
Fast skeletal	1.00	0.11* ± 0.04	1.00	0.97 ± 0.09

Values are mean ± SEM (n = 7–17). Asterisks indicate control significantly different than TnC XT value, $p < 0.05$. P/P_o is the relative tension value obtained by dividing the tension at pCa 4.5 under control or TnC XT conditions by the control tension at pCa 4.5 in the same preparation. The relative rigor tension value was obtained by dividing the maximum rigor tension value in the control or TnC XT extracted state by the control maximum rigor tension value in the same preparation.

effect is mediated by the head-to-tail polymerization of tropomyosin as well as interactions within a functional group.

Differences between cardiac and skeletal muscle

The greater steepness of the tension-pMgATP relationship in cardiac as compared with skeletal muscle suggests that, in the absence of Ca^{2+}, cross-bridge binding induces greater cooperative activation of the thin filament in cardiac than skeletal muscle. The basis of this difference probably involves differential contractile gene expression in these muscle lineages (Nadal-Ginard and Mahdavi, 1989). Troponin C plays a central role in thin filament molecular cooperativity and is differentially expressed in cardiac and skeletal muscle. It is possible, therefore, that muscle type differences in tension-pMgATP plots are in part troponin C isoform-dependent. However, this explanation is not complete because cardiac and slow skeletal muscle express the same isoform of troponin C and yet display markedly different ascending limbs of the tension-pMgATP relationship (Fig. 5).

Cooperative activation of the thin filament induced by cross-bridge binding, in the absence of Ca^{2+}, differs qualitatively from cooperative activation induced by increased $[Ca^{2+}]$ at high [MgATP]. For example, Ca^{2+}-induced cooperativity, as manifested in the steepness of the tension-pCa relationship, is greater in fast skeletal muscle ($n \approx 4$–8; Brandt et al., 1984; Metzger and Moss, 1987) than cardiac myocytes ($n \approx 3$–5; Sweitzer and Moss, 1990; Metzger et al., 1993), and both of these muscle types demonstrate greater Ca^{2+}-induced cooperative activation of the thin filament than slow skeletal muscle ($n \approx 1$–2; Metzger and Moss, 1987). Thus, muscle type differences in cooperative activation of the thin filament depend on whether Ca^{2+} or cross-bridges serve as the activating ligand.

The steepness of the tension-pMgATP relationship is dependent on $[Ca^{2+}]$, such that as $[Ca^{2+}]$ is increased, the steepness of the tension-pMgATP relationship decreases markedly (Reuben et al., 1971; Brandt et. al., 1972). Thus, the effect of attached cross-bridges to activate cooperatively the thin filament is altered by Ca^{2+}. Recently, Brandt et al. (1990) presented a model of cooperative activation of the thin filament in which the effects due to multiple activating ligands were considered. The model predicts that the extent of cooperative activation induced by one activating ligand, for example, attached cross-bridges, will be reduced in the presence of a second activating ligand (i.e., Ca^{2+}). In general agreement with the model, the steepness of the tension-pCa relationship decreases in the presence of rigor cross-bridges (Brandt et al., 1990; but see Godt, 1974) or in the presence of a strong binding myosin analog (S1-NEM; Swartz and Moss, 1992).

Effects due to partial extraction of troponin C

Partial extraction of troponin C markedly reduced the steepness of the tension-pMgATP relationship in cardiac myocytes. This provides evidence that myosin binding-induced cooperative activation of the thin filament, as manifested by the steepness of the tension-pMgATP relationship, requires the presence of troponin C and that in its absence the integrity of the thin filament is altered, perhaps via a mechanism involving alterations in the molecular interactions that span neighboring functional groups in cardiac muscle.

The basis of the greater activating effect of attached cross-bridges in cardiac than skeletal muscle, in the absence of Ca^{2+}, could relate to differences in thin filament near-neighbor molecular cooperativity in these muscles. After extraction of troponin C in all fiber types, there remained significant residual cooperativity as manifested by Hill coefficients of about 4 derived from the tension-pMgATP relationship. This residual cooperativity apparently does not require fully intact near-neighbor molecular interactions because these interactions are expected to be markedly altered by extraction.

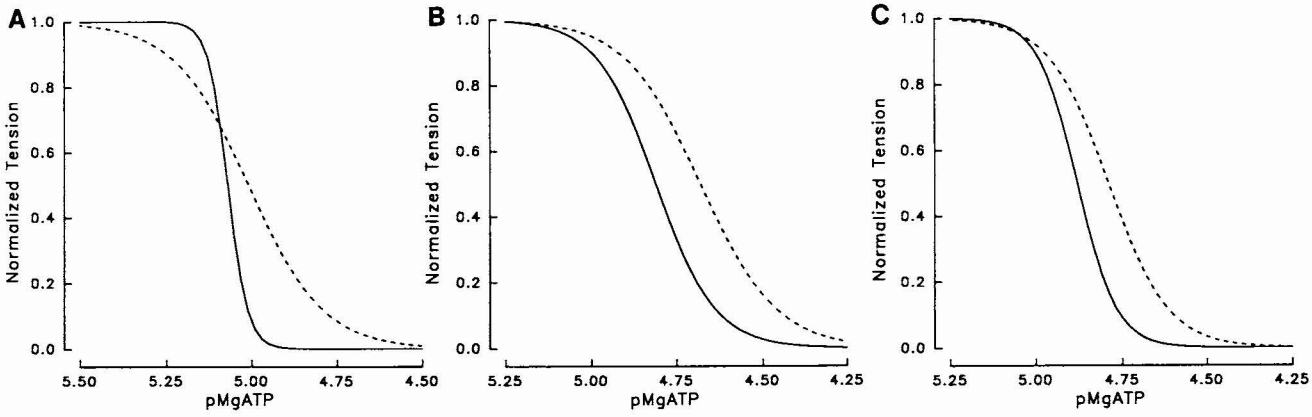

FIGURE 10 Summary of the effects of troponin C extraction on the tension-pMgATP relationships in cardiac myocytes (*A*), and fast (*B*) and slow (*C*) skeletal muscle fibers. Computer-generated lines were drawn using the average n and $pMgATP_{50}$ for control (——) and troponin C extracted (- - - -) data. In all records, maximum rigor tension was normalized to 1.0 to permit comparison of position and steepness of the curves. Note different x axis in *A*.

Another interesting finding of this study is that fiber-type differences in myosin binding-induced cooperative activation of the thin filament were eliminated upon extraction of troponin C. This was primarily due to an effect of extraction to markedly reduce n in cardiac myocytes because in skeletal fibers steepness was less affected by extraction of troponin C. This provides evidence that molecular interactions among neighboring functional groups in part determine the varied cooperative activation of cardiac and skeletal muscles, and that troponin C may affect the integrity of a regulatory strand to a greater extent in cardiac than skeletal muscle preparations.

In addressing the basis of cooperative activation of the thin filament by Ca^{2+}, Tobacman and Sawyer (1990) demonstrated in cardiac thin filaments that Ca^{2+} binds cooperatively to troponin and that this cooperativity was apparent even in the absence of added myosin. The underlying mechanism of cooperativity in the absence of myosin binding to cardiac thin filaments must be due to intermolecular effects that span neighboring troponin-tropomyosin functional groups along the filament. This is so because cardiac troponin C has only one low affinity, regulatory Ca^{2+} binding site (Pan and Solaro, 1987). Myosin-independent cooperative activation could be mediated by long range effects along a continuous troponin-tropomyosin strand or, alternatively, could be due to more local interactions, perhaps between troponin molecules on opposite sides of the thin filament (Tobacman and Sawyer, 1990).

SUMMARY

The results of this study indicate that cross-bridge binding markedly effects cooperative activation of the cardiac thin filament, at least in the absence of Ca^{2+}. Partial extraction of troponin C altered cross-bridge binding-induced cooperative activation, with the effect being greater in cardiac than skeletal muscle. These results suggest that, in the absence of added Ca^{2+}, myosin-mediated activation of the thin filament is greater in cardiac than skeletal muscle, and this apparent cooperativity requires the presence of troponin C on thin filament regulatory strands.

I thank Drs. Richard Moss, John Faulkner, and Margaret Westfall for helpful comments on earlier versions of this manuscript. I also thank Drs. Richard Moss and Marion Greaser for their kind gift of purified troponin C.
This work was supported in part by grants from National Institutes of Health, Whitaker Foundation, American Heart Association of Michigan, University of Michigan Office of Vice President for Research, and by a Rackham Faculty grant. J. M. Metzger is an Established Investigator of the American Heart Association.

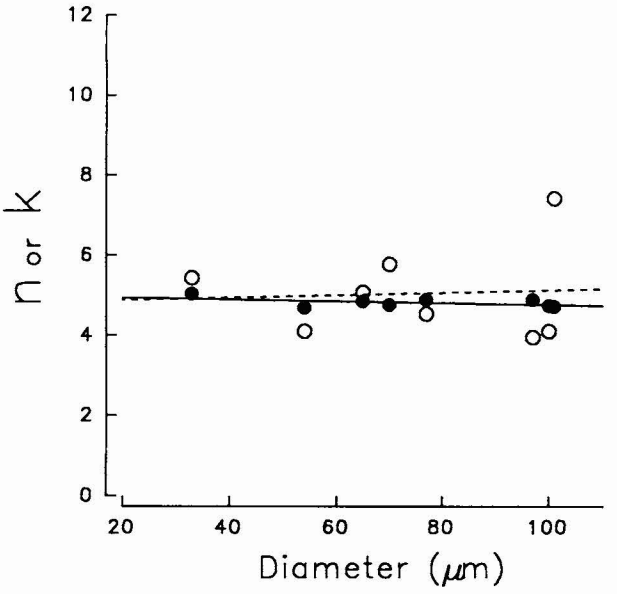

FIGURE 11 Summary of effects of fiber diameter on tension-pMgATP relationship-derived n (○, - - - -) and K ($pMgATP_{50}$; ●, ——) in psoas fibers. Best-fit linear regression analysis showed that the slopes of the straight lines fit to the data were not different from zero. Thus, over a wide range of fiber diameters, n and K were independent of diameter. Equation for best-fit line for n: $y = 0.003 \pm 0.019(x) + 4.8$, $r^2 = 0.004$. Equation for best-fit line for K: $y = -0.003 \pm 0.001(x) + 4.98$, $r^2 = 0.22$. Similar findings were obtained for soleus fibers (data not shown).

APPENDIX

Consideration of possible artifacts due to experimental solutions

In the present study, the concentration of free Mg^{2+} was set at 1 mM to ensure that the high affinity binding sites on troponin C would be occupied by divalent cations even at high pMgATP. Thus, varied MgATP concentrations were generated by altering total added ATP to the solutions. A second rationale for maintaining free Mg^{2+} constant is that alterations in free Mg^{2+} influence thin filament activation, at least under conditions where Ca^{2+} serves as the activating ligand (Metzger and Moss, 1992). By maintaining $[Mg^{2+}]$ constant, the question is raised that without an ATP regenerating system, gradients of ATP may form throughout the cross section of the preparation. However, based on the following discussion and experimental observations, it appears unlikely that functionally significant gradients of MgATP were formed in these experiments. A. V. Hill (1928) developed a set of equations that described the diffusion of oxygen and lactic acid in biological tissues. More recently, Godt (1974) adapted Hill formulations to describe the diffusion of ATP in single muscle fibers. Thus, the Hill equation can be adapted to the form:

$$\phi \approx C \times 4D/K \times r^2$$

where ϕ is the fraction of the muscle cross section (assuming muscle is a cylinder) in which ATP is present, C is the [MgATP], D is the diffusion constant for ATP in muscle, K is ATPase rate per unit cell volume, and r is muscle cell radius. Thus, if C is decreased and all else is kept constant ϕ would decrease, i.e., indicate large gradient of ATP across the cell cross section. However, as pointed out by Godt (1974), as C is decreasing so would K because K is highly sensitive to [ATP]. Indeed, shortening velocity decreases markedly with lowered [MgATP] (k_m = 0.47 mM; Ferenczi et al., 1984). In addition, because Ca^{2+} is absent in our solutions, the activation of cross-bridge cycling would be minimized. In this analysis, the effects of altered C and K may offset each other, in which case ϕ would be unchanged.

This possibility is supported in experiments in which the values for the steepness and position of the tension-pMgATP relationship were plotted as a function of cell radius (Fig. 11). Plots of n and $pMgATP_{50}$ versus psoas fiber diameter show that the slope of the straight line fit to the data was not significantly different than 0. For this calculation, data were used in which cell radius varied over a threefold range. From the equation above, a doubling of r should lead to a fourfold change in ϕ if all else is held constant. This provides evidence that gradients of ATP were not formed in these experiments.

In addition, in other experiments creatine phosphate (14.5 mM) and creatine phosphokinase (150 units/ml) were added to the Ca^{2+}-free pMgATP solutions, and results were found to be comparable with those obtained without added creatine phosphate. The average relative n and $pMgATP_{50}$ (i.e., value without buffering/value with buffering system) were 0.98 and 1.08, respectively. Finally, the values for tension-pMgATP-derived n obtained from psoas fibers (Table 1) are in good agreement with those reported earlier under conditions in which ATP gradients would not be expected to be formed (Brandt et al., 1990). Taken together, these findings provide evidence against the presence of significant gradients of MgATP across the fiber cross section.

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