The Effect of Altered Temperature on
Ca\(^{2+}\)-sensitive Force in Permeabilized
Myocardium and Skeletal Muscle

*Evidence for Force Dependence of Thin Filament
Activation*

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ABSTRACT The effect of changes in temperature on the calcium sensitivity of
tension development was examined in permeabilized cellular preparations of rat
ventricle and rabbit psoas muscle. Maximum force and Ca\(^{2+}\) sensitivity of force
development increased with temperature in both muscle types. Cardiac muscle was
more sensitive to changes in temperature than skeletal muscle in the range
10–15°C. It was postulated that the level of thin filament activation may be
decreased by cooling. To investigate this possibility, troponin C (TnC) was partially
extracted from both muscle types, thus decreasing the level of thin filament
activation independent of temperature and, at least in skeletal muscle fibers,
decreasing cooperative activation of the thin filament as well. TnC extraction from
cardiac muscle reduced the calcium sensitivity of tension less than did extraction of
TnC from skeletal muscle. In skeletal muscle the midpoint shift of the tension–pCa
curve with altered temperature was greater after TnC extraction than in control
fibers. Calcium sensitivity of tension development was proportional to the maxi-
mum tension generated in cardiac or skeletal muscle under all conditions studied.
Based on these results, we conclude that (a) maximum tension-generating capability
and calcium sensitivity of tension development are related, perhaps causally, in fast
skeletal and cardiac muscles, and (b) thin filament activation is less cooperative in
cardiac muscle than in skeletal muscle, which explains the differential sensitivity of
the two fiber types to temperature and TnC extraction. Reducing thin filament
cooperativity in skeletal muscle by TnC extraction results in a response to
temperature similar to that of control cardiac cells. This study provides evidence
that force levels in striated muscle influence the calcium binding affinity of TnC.
INTRODUCTION

Several investigators have observed changes in calcium sensitivity of tension development with altered temperature in permeabilized preparations of striated muscle. However, the direction of this effect, either to increase or decrease Ca\(^{2+}\) sensitivity, was not consistent among these studies. Brandt and Hibberd (1976) reported that increased temperature (20–29°C) caused an increase in Ca\(^{2+}\) sensitivity of tension in ventricular muscle. A similar effect of temperature (5–20°C) on calcium-sensitive tension was observed in crayfish fibers (Orentlicher et al., 1977). Ashley and Moisescu (1977), on the other hand, found no change in the Ca\(^{2+}\) sensitivity of tension in barnacle muscle when temperature was increased (4–20°C). Stephenson and Williams (1981) reported a decrease in Ca\(^{2+}\)-sensitive tension in fast twitch skeletal muscle fibers with increased temperature (3–35°C), although they found no shift in the tension–pCa relation of slow twitch fibers in the same temperature range. An apparent decrease in Ca\(^{2+}\) sensitivity with increased temperature has also been reported by Godt and Lindley (1982) in frog skeletal fibers (4–22°C), Fabiato (1985) in canine cardiac Purkinje cells (12–20°C), and Goldman et al. (1987) in rabbit psoas fibers (8–30°C). Recently, Harrison and Bers (1989) published results that agree with the early findings of Brandt and Hibberd (1976), showing an increase in Ca\(^{2+}\) sensitivity of tension in rabbit myocardium with increased temperature (1–36°C). We have undertaken studies of tension as a function of temperature and Ca\(^{2+}\) concentration in permeabilized fast twitch skeletal muscle fibers and cardiac myocytes in an attempt to reconcile these conflicting results.

Temperature-dependent alterations in Ca\(^{2+}\) sensitivity of tension development may result from changes in affinity of troponin C (TnC) for Ca\(^{2+}\) secondary to changes in developed force or in numbers of cross bridges bound to thin filaments. Bremel and Weber (1972) were the first to show an effect of bound cross bridges to increase the affinity of TnC for Ca\(^{2+}\) in vitro, using a preparation of myosin S1 and regulated thin filaments. Several subsequent studies demonstrated alterations in Ca\(^{2+}\) sensitivity of isometric tension with changes in muscle length in both cardiac and skeletal muscles (Endo, 1972; Fabiato and Fabiato, 1978; Hibberd and Jewell, 1982; Moss et al., 1983). It was not clear whether changes in Ca\(^{2+}\) sensitivity were a function of sarcomere length per se, or were due to alterations in force that accompanied the length changes. Recently it was suggested that changes in force alone can affect Ca\(^{2+}\) binding by TnC (Gordon and Ridgway, 1987; Hofmann and Fuchs, 1987; Allen and Kentish, 1988); coincident force changes could thus account for the changes in Ca\(^{2+}\) sensitivity of isometric tension observed when length is altered, although this has been disputed (Stephenson and Wendt, 1984). In a series of studies, Gordon and Ridgway (1987) and Ridgway and Gordon (1984, 1990) attempted to discern whether changes in the affinity of TnC for calcium with altered force were due to changes in cross bridge number or changes in force per crossbridge. They found that altering either of these variables could produce a significant modulation of the aequorin light signal generated by a cell. In this report we present evidence for a relationship between the Ca\(^{2+}\) sensitivity of force development and maximum active tension, probed in single muscle cells; maximum tension was altered by varying temperature and TnC content. We propose that these
effects are not due to changes in cross-bridge number, but rather depend on the total force developed by active cross-bridges.

Although single skeletal muscle fibers have often been used in studies of this type, analogous cardiac preparations for measurements of mechanical properties have been used less frequently. Fabiato and Fabiato (1975) were the first to obtain tension measurements from single skinned cardiac cells, which adhere tightly to glass microneedles for measurement of tension. The technique used by these investigators to mechanically skin ventricular cells is elegant and effective but technically difficult to perform and has not been adopted by other groups. Other reports of mechanical measurements on single cells using various attachment techniques have since appeared (De Clerck et al., 1977; Brady et al., 1979; Tarr et al., 1981), although none of these techniques has been widely used. We describe in this report a technique for low compliance attachment of a mechanically isolated, small diameter preparation of chemically skinned mammalian myocardium. The preparation is relatively easy to isolate and attach to the apparatus and permits rapid and reproducible force measurements. Our preparation allows rigorous control of such variables as sarcomere length and intracellular concentrations of substances of interest, while eliminating the confounding factors of long diffusion distances and parallel elasticity due to connective tissue present in larger preparations.

METHODS

Skinned Fiber Preparation and Apparatus

Female Sprague-Dawley rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40–50 mg/kg). The heart was removed and the ventricles were isolated and cut into 5-mm pieces. The myocardium was minced in relaxing solution (described below), placed in a blender, and further disrupted at low speed for 6 s. 6 ml of the resulting suspension was centrifuged at 120 g for 30 s and the resulting pellet was resuspended in 12 ml of relaxing solution.

An experimental chamber similar to one previously described (Moss, 1979) was mounted on an inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Trough volume was reduced to 200 µl to accommodate the much smaller cardiac myocytes and a fourth trough was added for use as an attachment chamber. The cell attachment is similar to that described by P. A. Hofmann, H. C. Hartzel, and R. L. Moss (manuscript submitted for publication). A borosilicate glass micropipette (o.d. 500–400 µm; Glass Co. of America, Bargaintown, NJ) was pulled to a tip diameter of 4 µm (model P-80/PC Flaming Brown Micropipette Puller; Sutter Instrument Co., San Rafael, CA) and inserted into the output tube of a force transducer (model 406; Cambridge Technology, Cambridge, MA) having a sensitivity of 200 mV/mg and a resonant frequency of 100 Hz. The pipette was held in position by a paraffin seal. A second pipette (o.d. 1 mm; Vitro Dynamics Inc., Rockaway, NJ) was pulled to the same tip diameter and then glued into a 4.5-cm length of stainless steel tubing (o.d. 1.7 mm). This pipette assembly was inserted into a small block of aluminum, which in turn was attached to a piezoelectric translator (Physik Instrumente GmbH & Co., Waldbronn, Germany) able to complete 0–60-µm length steps in <1 ms. Voltage command signals from an interval generator (World Precision Instruments, Inc., New Haven, CT) were input to a bipolar operational power supply/amplifier (Kepco, Inc., Flushing, NY), which was used to drive the piezoelectric translator. The translator was calibrated by applying different voltages and photographing the translator micropipette on the same film frame both before and during the
voltage pulse. The resulting photomicrograph showed the movement resulting from each applied voltage. The movement was linear at 0.05 μm/V. Both the force transducer and the translator were mounted on three-way positioners to allow precise positioning of the pipettes. Force and length signals were recorded on a digital oscilloscope (model NIC-310; Nicolet Instrument Corp., Madison, WI) and then stored on magnetic disk for later analysis. The entire experimental chamber was cooled by three thermoelectric devices (Cambion Thermoelectric Devices; Midland-Ross Corp., Cambridge, MA) which in turn were cooled by a circulating water heat sink. Temperature was monitored continuously with a probe inserted into the stainless steel trough assembly, 3 mm from the middle experimental trough. Photomicrographs were obtained from either a Nikon 35mm camera adapted for the Zeiss microscope or a Polaroid camera back-mounted above the microscope eyepiece.

To attach the cardiac preparation to the apparatus, the fourth trough was lowered vertically and a coverslip containing a drop of silicone adhesive (Dow Corning Corp., Midland, MI) and a drop of cell suspension was placed over the trough. Once the myocytes were chosen by viewing the drop through the microscope, the two micropipette tips were dipped into the adhesive and then lowered onto each end of the chosen preparation. After the adhesive cured for 45 min, the micropipettes were lifted from the coverslip, thereby suspending the attached myocardium between the transducer and translator. The myocytes were then transferred to a chamber containing low Ca²⁺ relaxing solution and sarcomere length was adjusted to ~2.30 μm, estimated with an eyepiece micrometer. A photomicrograph was then taken and exact sarcomere length was determined; sarcomere length was further adjusted if necessary. It was found that the preparations were permeabilized after isolation in the blender, since tension-pCa relations obtained immediately after isolation were not altered by soaking the attached cell in 0.2% Ultrapure Triton X-100 (Pierce Chemical Co., Rockford, IL) for times up to 30 min. Because a brief soak in Triton improved resolution of the sarcomere pattern, all cells in this study were soaked in 0.2% Triton X-100 solution for 15 s in advance of any mechanical measurements and were thus chemically skinned.

Rabbit single psoas fibers were obtained and attached to the apparatus as described previously (Moss, 1979). In brief, bundles of glycerinated psoas fibers were skinned in a 0.5% solution of Brij-58 in relaxing solution. A single fiber was dissected free and then attached between a force transducer and a torque motor by tying it into metal troughs with fine suture. Relaxed sarcomere length was adjusted to ~2.60 μm.

Solutions

The Ca²⁺-free relaxing solution used during the blender treatment contained (in mM): 100 KCl, 2 EGTA, 1 MgCl₂, 4 Na₂ATP, and 10 imidazole at pH 7.0. Relaxing and activating solutions used during experimental measurements contained (in mM): 7 EGTA, 1 free Mg²⁺, 20 imidazole, 14.5 creatine phosphate, and sufficient KCl to adjust ionic strength to 180 mM. The fibers were activated in solutions containing concentrations of free Ca²⁺ between 10⁻⁹ (relaxing solution) and 10⁻⁴.5 M (maximal activation), which are reported as pCa (−log[Ca²⁺]). Solution recipes for each experimental temperature were obtained using the computer program of Fabiato (1988), which takes into account changes in species association with temperature. By accounting for altered association of complexes with temperature, added Na₂ATP, MgCl₂, and CaCl₂ were adjusted so that free Mg²⁺ was 1 mM, free MgATP was 4 mM, and free Ca²⁺ was the desired value in all solutions. Solutions were then prepared at 10, 15, and 22°C, and solution pH was adjusted to 7.0 at each temperature. The pH meter was calibrated to correct for changes in the ionization of water with temperature. Exact solution composition is detailed in Table I. Creatine phosphokinase was not added to solutions used in these experiments. The endogenous creatine phosphokinase level in the cardiac myocytes was high enough to continually replenish the ATP supply during an experiment, as indicated by a
failure of added creatine kinase to alter maximum tension (mean tension after addition of creatine kinase was 96 ± 7% control maximum tension, n = 5). Previous reports also indicate that endogenous creatine kinase is not lost from cardiac bundles after skinning (Ventura-Clapier et al., 1987). For the experiments similar to those of Goldman et al. (1987), relaxing and activating solutions contained (in mM): 20 EGTA, 15 β-glycerophosphate, 1 free Mg, 20 creatine phosphate, 5.5 ATP, and KCl to adjust ionic strength to 200 mM. CaCl₂ was added to yield pCa's from 6.5 to 4.5. Solutions were made at 20°C and pH was adjusted to 7.1. Since the temperature jump protocol used by Goldman et al. (1987) did not allow them to change solutions when temperature was changed, the solutions used in the experiments described here were made at 20°C and then used at 10, 20, and 30°C.

### Table I

**Summary of Solution Contents at Each Temperature**

|          | 22°C | 15°C | 10°C |
|----------|------|------|------|
|          | pCa 9.0 | pCa 4.5 | pCa 9.0 | pCa 4.5 | pCa 9.0 | pCa 4.5 |
| EGTA     | 7.0 | 7.0 | 7.0 | 7.0 | 7.0 | 7.0 |
| Imidazole| 20.0 | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 |
| MgCl₂    | 5.46 | 5.27 | 5.42 | 5.26 | 5.39 | 5.26 |
| KCl      | 77.6 | 62.7 | 79.2 | 64.0 | 79.5 | 64.7 |
| CaCl₂    | 0.017 | 7.0 | 0.016 | 7.01 | 0.016 | 7.02 |
| Phosphocreatine | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 |
| ATP      | 4.65 | 4.71 | 4.74 | 4.81 | 4.82 | 4.90 |
| KOH      | 10.6 | 26.3 | 6.5 | 22.5 | 4.8 | 20.0 |

All values are millimolar. ATP and phosphocreatine were added as sodium salts. EGTA was added as a potassium salt. Imidazole contributed ~5 mM to the ionic strength. Solutions of intermediate pCa values were obtained by mixing the pCa 4.5 and pCa 9.0 solutions according to the following equation. For 10 ml:

\[
\text{volume of pCa 4.5} = \frac{([\text{Ca}^{2+}]_{\text{initial}} \times 10) - ([\text{Ca}^{2+}]_{\text{final}} \times 10)}{([\text{Ca}_{\text{total}}]_{4.5} - [\text{Ca}_{\text{total}}]_{9.0})}
\]

pCa 9.0 solution was then added to generate a total volume of 10 ml.

**Experimental Protocol**

After attachment of myocytes, the chamber was cooled to the experimental temperature. When more than one temperature was used during an experiment, the initial temperature was chosen at random. After activation steady tension was allowed to develop, at which time a slack step was introduced by triggering a movement of the piezoelectric device. The slack steps in experiments described here were ~20% of cell length. The preparations were transferred back to relaxing solution before return of the translator to its original position. The difference between steady developed tension and the tension baseline obtained immediately after the slack step was measured as total tension. Active tension was calculated as the difference between total tension and resting tension measured with the myocytes in relaxing solution. Tensions (P) at submaximally activating levels of Ca²⁺ were expressed as a fraction of the tension obtained during maximal activation at pCa 4.5 (P₀). Every third or fourth contraction was performed at pCa 4.5 to assess any decline in fiber performance (Moss, 1979). When maximal tension in a fiber declined more than 20% during a control tension–pCa series, the data were not used. Submaximal tensions measured at different temperatures or after TrPC
extraction were expressed as a fraction of $P_o$ obtained under the same conditions. Lines were fit to the upper and lower portions of the tension–pCa curves using the Hill equation. Because the Hill coefficients were different for the upper and lower portions, the midrange of each curve was fit to the data by eye. Cumulative Hill plots were used to determine the midpoint of the tension–pCa curve (pCa$_{50}$) for each condition; therefore, these values are given without standard errors.

TnC was extracted in varying amounts from cardiac myocytes using a solution containing 5 mM EDTA, 10 mM HEPES, and 500 μM trifluoperazine dihydrochloride (TFP; Smith, Kline and French Laboratories, Philadelphia, PA) at 15°C for various amounts of time as described by Metzger et al. (1989). A tension–pCa relationship was then determined at 15°C. The maximal tension at 10°C after TnC extraction was so small that measurement of a tension–pCa relationship was not feasible at this temperature. TnC was extracted from psoas fibers using a modification of the method of Cox et al. (1981) as described by Moss et al. (1985), and tension–pCa relationship were then determined at both 15 and 10°C. To restore full Ca$^{2+}$ sensitivity of tension development, TnC-extracted psoas fibers were bathed in relaxing solution containing 0.25–0.5 mg/ml TnC purified from rabbit skeletal muscle (Moss et al., 1985). TnC-extracted cardiac myocytes were bathed in relaxing solution containing 0.25 mg/ml purified bovine cardiac TnC and 10 mM Mg$^{2+}$. Fibers were exposed to this solution for short periods of time (10 s for psoas, 3 s for cardiac myocytes) and then returned to a relaxing solution with no TnC added. Preparations were cycled through this procedure an average of three times until the isometric force at pCa 4.5 was restored to control levels or until successive cycles did not further augment force.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis**

After each experiment the myocytes were detached from one of the two micropipettes by lifting one of the tips from the experimental chamber. The myocytes, attached to the micropipette still in the chamber, were lifted from the solution and the pipette tip was crushed in a 0.5-ml microfuge tube containing 10 μl SDS sample buffer (Giulian et al., 1983) and 1 mg/ml leupeptin (Sigma Chemical Co., St. Louis, MO) to prevent proteolysis during storage. The tube was kept at −80°C for subsequent analysis of the contractile and regulatory protein content by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The method described by Giulian et al. (1983) has been modified for use on embryonic skeletal (Reiser et al., 1988) and cardiac muscle cells. Psoas fiber segments were saved and analyzed by SDS-PAGE as detailed elsewhere (Moss et al., 1985).

**Statistical Analysis**

Data were tested using analysis of variance (ANOVA) to determine whether there were significant changes in tension with the various alterations in experimental conditions. When the ANOVA showed significant variation in tension or pCa$_{50}$ with a change in experimental conditions, a Bonferroni $t$ test was used to determine $P$ values. A level of $P < 0.05$ was chosen as significant. All values are reported as mean ± SEM unless otherwise noted.

**RESULTS**

**Mechanical Characterization of the Permeabilized Myocardial Preparation**

The myocyte suspension obtained after blender treatment contained a mixture of single cardiac cell fragments, cells in series joined by intercalated discs, and larger
groups of several cells both in series and in parallel with one another. Experiments were done on small pieces of myocardium, estimated to be from one to four cells. Light photomicrographs of an attached myocardial preparation are shown in Fig. 1. Comparison of A (pCa 9.0) and B (pCa 5.8, 34% maximum tension) shows that the striation pattern remained visible during activation. While the preparation was not completely isometric (see discussion of oscillatory contractions below) the changes in sarcomere length with activation were minimal, indicating a very low level of end compliance as a result of the adhesive attachment. C shows the same cell during maximum activation under conditions in which length increased due to lateral movement of one of the micropipettes. The increase in length was accompanied by an increase to a new sarcomere length, which accounts completely for the increased cell length; this is evidence of the very low compliance of the attachment to the apparatus.

Table II is a summary of the characteristics of 24 rat myocardial preparations studied at 15°C. The values obtained are in good agreement with those obtained by others, particularly the pCa90 (Hibberd and Jewell, 1982; Harrison et al., 1988). The attached myocytes were distinctly rectangular in cross section, with the width of each preparation much greater than its depth. Because the depth of field of our apparatus was >12 μm, it was not possible to routinely measure the depth of the myocytes, so that we have no estimates of force per cross-sectional area. Our absolute tension values were nearly double those obtained by Fabiato and Fabiato (1975), and our preparations were, on average, twice as wide as the cells in their study. The average width of our myocytes was similar to that measured in enzymatically isolated myocytes in our laboratory (27.0 ± 6.0 μm, n = 6) and by others (Haworth et al., 1987). The preparations were quite stable at 15°C, with maximum tension (P0) increasing an average of 3% during measurement of a tension–pCa relationship (~12 activations). Experimental protocols lasting 4 h were routinely carried out when temperatures of 15°C and below were used. At 22°C the preparations were less stable, with performance deteriorating as a result of activation; an average loss of 7.5% P0 was seen during measurement of the tension–pCa relationship (approximately eight activations) at this temperature.

The cardiac tension–pCa curve at 15°C is compared with the well-characterized relationship in rabbit psoas fibers under the same conditions in Fig. 2. Tension development in myocardium was much less sensitive to Ca2+ than in psoas fibers, due at least in part to the fact that the experiments on myocardium were done at a shorter sarcomere length. Changing sarcomere length has been shown, in both skeletal and cardiac muscle, to change both the pCa90 and the steepness of the tension–pCa relation (Hibberd and Jewell, 1982; Moss et al., 1983; Allen and Moss, 1987). The data were linearized to the Hill plot form for more convenient comparison (Fig. 2 B). Hill plots from psoas and cardiac muscles were best fit by two straight lines as described previously (Moss et al., 1983). Differences in the shape of the tension–pCa curves are indicated by differences in the slope of the lower portion of the Hill plots (nH). Included on this graph for further comparison is the Hill plot of cumulative data from TnC extracted psoas fibers (see below).
FIGURE 1. Photomicrographs of a rat cardiac myocyte preparation attached for force measurements. (A) Cell 82691 in relaxing solution (pCa 9.0). Resting tension = 4% P_o; cell length = 101 μm; sarcomere length (SL) = 2.32 μm. (B) The same cell during activation at pCa 5.8. Force = 2.56 μN, 33.6% P_o; cell length = 105 μm; SL = 2.32 μm. (C) The same cell shown during maximal activation (pCa 4.5). Force = 7.66 μN; cell length = 112.8 μm; SL = 2.55 μm. The bar represents 20 μm.
Effect of Temperature on Ca\(^{2+}\) Sensitivity

Original tension records from a rat myocardial preparation activated at four Ca\(^{2+}\) concentrations at both 10 and 15°C are shown in Fig. 3. Tension at pCa 4.5 represents maximum activation as this tension value was similar to that observed at pCa 4.0. The records illustrate that (a) absolute force increases with increasing temperature at any given Ca\(^{2+}\) level, and (b) the Ca\(^{2+}\) sensitivity of tension increases as temperature rises. These changes were observed regardless of the order in which the temperatures were tested, and forces at any given temperature and Ca\(^{2+}\) concentration were highly reproducible in an individual preparation. The cumulative tension–pCa data for rat cardiac myocytes are shown in Fig. 3 B. For the three temperatures studied, relative force at a particular Ca\(^{2+}\) concentration increased as temperature was raised, leading to a left shift of the normalized tension–pCa curve. The pCa\(_{50}\) increased significantly with temperature, from pCa 5.36 ± 0.03 at 10°C to pCa 5.68 ± 0.02 at 15°C and pCa 5.84 ± 0.03 at 22°C. The magnitude of the temperature-dependent shift was less in the higher temperature range; this may in part reflect a loss of sarcomere length uniformity at the higher temperature, which may itself affect Ca\(^{2+}\) sensitivity. A qualitatively similar shift of the tension–pCa relation was observed in several canine ventricular preparations studied at 15°C (n = 4) or 22°C (n = 3).

The effects of temperature on maximum tension and the Ca\(^{2+}\) sensitivity of tension development were also studied in rabbit skinned psoas muscle. When temperature was increased from 10 to 15°C, maximum force rose. As summarized in Fig. 4, skeletal muscle showed a left shift of the tension–pCa relation at the higher temperature which was similar to that in cardiac muscle, although less pronounced. This effect was significant only at low levels of Ca\(^{2+}\). In neither muscle type was a significant change in resting tension observed with changes in temperature from 10 to 15°C.

Cardiac muscle thus appears to be more sensitive to changes in temperature in the range between 10 and 15°C than skeletal muscle, exhibiting both a greater increase.

### Table II

| Summary of Rat Myocyte Characteristics at 15°C |
|------------------------------------------------|
| Mean ± SD | High value | Low value |
|-----------|------------|-----------|
| Cell length, µm | 177.0 ± 39.3 | 246.0 | 99.2 |
| Sarcomere length, µm | 2.31 ± 0.06 | 2.46 | 2.21 |
| Width, µm | 24.1 ± 6.2 | 35.0 | 15.4 |
| Maximal force, µN | 9.02 ± 3.33 | 14.70 | 2.80 |
| Rest tension, µN | 0.59 ± 0.39 | 2.06 | 0.20 |
| RT/P\(_m\) % | 7.10 ± 2.92 | 15.70 | 2.60 |
| pCa\(_{50}\) | 5.86 ± 0.08 | 5.77 | 5.39 |

Cell length, sarcomere length, and width were measured by photomicroscopy. The calcium concentration at which the fiber generated half-maximal force (pCa\(_{50}\)) was determined using Hill plot analysis. N, Newtons; RT, resting tension; P\(_m\), maximal force. Mean values were obtained from 23 or 24 cells.
FIGURE 2. Plots of the dependence of tension on pCa in rat cardiac myocytes (△) and rabbit psoas fibers (○) at 15°C. (A) Normalized tension–pCa curves. SL = 2.31 ± 0.06 μm (mean ± SD, cardiac) and 2.54 ± 0.08 μm (skeletal). (B) The data are shown after transformation to the linearized Hill plot form. A Hill plot of tension data from TnC-extracted psoas fibers (○) has been added for comparison. Table III summarizes the pCa₅₀ and Hill coefficient data obtained from the Hill plots.

|                          | pCa₅₀ | n₁  | n₂  |
|--------------------------|-------|-----|-----|
| Cardiac cells (n = 24)   | 5.68  | 1.85| 3.47|
| Psoas fibers (n = 9)     | 6.09  | 1.93| 5.13|
| TnC-extracted (n = 6) psoas fibers | 5.81  | 1.78| 3.79|

in maximum tension and a greater increase in Ca²⁺ sensitivity as temperature was raised, although the changes were significant in both muscle types. As temperature was increased from 10 to 15°C, maximum tension increased by an average of 174 ± 24% in cardiac muscle (n = 10), while maximum tension in skeletal muscle increased by 31 ± 3% in this same range (n = 9). The pCa₅₀ for cardiac muscle shifted leftward 0.32 pCa units (from 5.68 to 5.36) as temperature was increased from 10 to 15°C, while the shift in skeletal muscle was only 0.09 pCa units (from 6.09 to 6.00).
FIGURE 3. The effect of temperature on calcium sensitivity in cardiac muscle. (A) Original slow time base recordings of isometric tension from cardiac cell 40891 at 15 and 10°C during exposure to a variety of Ca\(^{2+}\) concentrations. Maximum force at 10°C was 42.4% of that at 15°C. Table IV lists the relative tension value for each condition. The numbered arrows in the record at pCa 6.0 and 15°C denote points in the activation sequence. At 1 a transient occurs as the cell is switched from the chamber containing relaxing solution to that containing the activating solution. At 2, after steady tension has developed, a slack step is initiated, the termination establishing the zero tension baseline (3). A transient marks the transfer of the cell back to the chamber containing relaxing solution, where the fiber is reextended (4). The arrows marked with asterisks in the record at pCa 5.7 and 15°C label an offset in the transducer output during activation. The break in the record at pCa 6.0 and 10°C represents 173 s.

(B) A plot of the cumulative tension-pCa data for rat cardiac myocytes at 22°C (X), 15°C (●), and 10°C (Δ). All preparations studied at 10°C were also tested at 15°C. Those tested at 22°C were not studied at any other temperature. The pCa's are 5.84 (22°C), 5.68 (15°C), and 5.36 (10°C).

| TABLE IV | Relative Tension Values for Each Condition in Fig. 3 |
|----------|------------------------------------------------------|
|          | pCa 4.5 | pCa 5.4 | pCa 5.7 | pCa 6.0 |
| 15°C     | 1.00    | 0.772   | 0.567   | 0.054   |
| 10°C     | 1.00    | 0.707   | 0.140   | 0.009   |
Because the shift in skeletal muscle was opposite that found previously (Stephen-
son and Williams, 1981; Goldman et al., 1987), and because differences in solution
composition might account for this disparity, we attempted to reproduce as closely as
possible the solutions used by Goldman et al. (1987) and then measure the Ca\(^{2+}\)
sensitivity of force development in psoas fibers at 10, 20, and 30°C. We did not use
temperature jumps when conducting these experiments, but rather reached a
steady-state experimental temperature before making any measurements. The data
from one fiber are shown in Fig. 5. We were unable to make any measurements at
30°C, as the fibers tore during maximum activation at this temperature. At 20°C,
after an initial measurement at pCa 4.5, measurements of tension could only be
made at low Ca\(^{2+}\) levels. As Ca\(^{2+}\) concentration was increased at the higher
temperatures, a decrease in sarcomere length uniformity could be seen as movement
within the fiber during the activation, and if several high calcium measurements were
attempted the fiber usually tore. Nevertheless, from the data at low Ca\(^{2+}\) levels it was

apparent that there was a small increase in Ca\(^{2+}\) sensitivity as temperature was raised
from 10 to 20°C (Fig. 5). Qualitatively similar results were obtained from two other
fibers.

**Effect of TnC Extraction on the Tension–pCa Relationship in Cardiac Myocytes**

To investigate possible roles of thin filament cooperativity in the temperature
dependence of Ca\(^{2+}\) sensitivity, TnC was partially extracted from cardiac myocytes.
It was first necessary to characterize the effect of TnC extraction alone on regulation
of tension in the myocyte preparation. Original tension records from one myocyte
preparation during maximal and submaximal activations both before and after TnC
extraction are shown in Fig. 6 A. A trace obtained during maximum activation after
TnC readdition is also shown. In most myocardial preparations (n = 9) the absolute
and relative tensions at all Ca\(^{2+}\) levels decreased after TnC extraction, as shown here.
However, in four preparations, despite a 30–70% fall in maximum tension after
partial extraction of TnC, there was no change in Ca sensitivity. A summary of the data from all 13 myocyte preparations studied is shown in Fig. 6B. The right shift seen in the tension–pCa relation after partial extraction of TnC is similar to that seen previously in skeletal muscle (Brandt et al., 1984; Moss et al., 1985). With readdition of bovine cardiac TnC, Ca²⁺ sensitivity returned to control levels. However, despite the return to normal Ca²⁺ sensitivity, the average tension recovery after TnC readdition was only to 66.2 ± 7.8% P₀ (n = 5; range, 43–87%).

We attempted to quantify the extraction of TnC from the myocytes by gel electrophoresis. While we were able to identify the TnC band on the gels, this migrated very close to LC₂ and the two could not be completely resolved by densitometric scanning. We were therefore unable to determine the actual relationship between loss of maximum force and the amount of TnC lost from a cell. Because of this, we have compared only the %P₀ between cardiac and skeletal muscle after extraction, assuming that the relationship between force and level of TnC extraction in cardiac muscle is roughly the same as that in skeletal muscle (Moss et al., 1985).

After partial extraction of TnC from cardiac myocytes using a solution containing TFP, some recovery of tension toward P₀ was invariably seen. This recovery was presumably due to cytosolic retention and subsequent rebinding of endogenous TnC following the extraction soak. To minimize recovery, after extraction and before any activation each preparation was washed in a pCa 9.0 solution four times for a total of 8 min. Recovery of >20% P₀ during measurement of tension–pCa relationships after extraction (but before TnC readdition) led to elimination of the data from the study (n = 4). Among the remaining preparations (n = 13) the mean tension at pCa 4.5 immediately after extraction was 35 ± 17% P₀ (mean ± SD), and recovery up to a mean value of 44 ± 17% P₀ was seen before TnC readdition. There did not appear to be any change in the Ca²⁺ sensitivity of force development associated with this tension recovery. In addition, tension at very low Ca²⁺ (pCa > 6.0) was slightly
FIGURE 6. Effects of TnC extraction on tension in cardiac myocyte preparations. (A) Original slow time base recordings of isometric tension from preparation 60992 at various pCa's under control, TnC-extracted, and TnC-recombined conditions. The maximum force after TnC extraction was 39.90 ± 16.82% P₀, with recovery to 79.90% P₀ after recombination with purified bovine cardiac TnC. Table V lists the relative tension value for each condition. The break in the TnC-extracted record at pCa 5.7 represents 34 s, that in the control record at pCa 5.8 represents 51 s, and that in the TnC-extracted record at pCa 5.8 is 22 s. Cumulative tension–pCa data from all rat cardiac myocyte preparations studied are shown in B. Normalized data are shown for control (●, n = 24), TnC-extracted (○, n = 13), and TnC-recombined preparations (Δ, n = 5). Maximum force after TnC extraction was 39.90 ± 16.82% P₀ (mean ± SD). Maximum force after recombination was 66.20 ± 17.34% P₀. The pCa₅₀'s were 5.68 (control), 5.50 (TnC-extracted), and 5.66 (TnC-recombined). All measurements were made at 15°C.

| Table V | Relative Tension Values for Each Condition in Fig. 6 |
|---------|-----------------------------------------------------|
| pCa 4.5 | pCa 5.7 | pCa 5.8 |
| Control (n = 24) | 1.00 | 0.581 | 0.328 |
| TnC-extracted (n = 13) | 1.00 | 0.254 | 0.177 |
| TnC-recombined (n = 5) | 1.00 | 0.474* | 0.308* |

*Original records not shown.
elevated after TnC extraction of cardiac myocytes, and did not return to control levels after TnC recombination. This may indicate that the TFP extraction procedure in cardiac myocytes is removing another protein in addition to TnC. One possibility is that LC2 may be partially extracted by this protocol, although this has not been observed in TnC extractions of skeletal muscle (Metzger et al., 1989). Extraction of LC2 has been shown to increase the calcium sensitivity of tension at low levels of free Ca2+ in skeletal muscle fibers (Hofmann et al., 1990).

**Oscillatory Contractions of Cardiac Myocytes at Intermediate Levels of Activation**

An intriguing phenomenon frequently seen in the cardiac myocyte preparations under our experimental conditions is illustrated in Fig. 6 A. The control trace at pCa 5.7 shows an irregular tension development. This corresponded to oscillations in sarcomere length, which propagated along the length of the cell when it was activated at Ca concentrations around the pCa50. This phenomenon has been reported previously (Fabiato and Fabiato, 1978), and in agreement with that study we observed oscillations only during mid-range Ca2+ activations; they were not observed at either full or very low levels of activation. Also, we were unable to eliminate the activity by further treatment with detergent or by using drugs that interfere with sarcoplasmic reticulum function, such as 10 μm ryanodine (data not shown). Interestingly, we found that with partial extraction of endogenous TnC the oscillations were eliminated. Oscillations were not seen in extracted fibers either at absolute tensions or at relative tensions equivalent to those at which the oscillations occurred in control fibers. Further, in one fiber resumption of the oscillatory activity was seen upon readdition of TnC. This indicates that the oscillatory contractions in cardiac muscle may be due to phasic cooperative responses within the thin filament at intermediate levels of activation, which is consistent with the suggestion of Fabiato and Fabiato (1978) that local changes in length or cross-bridge number may alter the tension developed in individual sarcomeres, leading to oscillations in sarcomere length.

**Effect of TnC Extraction on the Tension–pCa Relationship at 10 and 15°C in Rabbit Psoas Muscle**

Although the rationale for TnC extraction was to investigate possible mechanisms of the temperature effect, this experiment proved to be quite difficult in cardiac myocytes. Absolute forces developed by TnC-extracted myocytes at 10°C were so small that measuring a tension–pCa relationship was not feasible. The experiment was done instead on rabbit skinned psoas fibers, since these fibers develop sufficient tension at 10°C to obtain force measurements at low Ca2+ concentrations after TnC extraction. Results of these experiments are summarized in Fig. 7. TnC extraction amplified the temperature-induced shift of the tension–pCa relation in skeletal muscle fibers. In control fibers, increasing the temperature from 10 to 15°C shifted the pCa50 to the left by an average of 0.09 pCa units, while in TnC extracted fibers the same temperature change caused a leftward shift of 0.19 pCa units. After TnC readdition the temperature-induced left shift was 0.11 pCa units, a value similar to that obtained in control fibers.

The tension–pCa relationship in skeletal muscle appears to be more sensitive than that in cardiac muscle to extraction of TnC. Mean relative tension after TnC
extraction from both muscles in this study was nearly identical, with a mean maximum tension value of 40 ± 17% $P_o$ in cardiac muscle and 41 ± 12% $P_o$ in skeletal muscle after extraction. The resultant shift in pCa$_{50}$ at 15°C was rightward and significant in both cases: 0.18 pCa units in cardiac muscle (from 5.68 to 5.50, $P < 0.05$) and 0.28 pCa units in skeletal muscle (from 6.09 to 5.81, $P < 0.001$). After extraction of ~50% of the native TnC, the slope of the lower portion of the Hill plot of psoas muscle was very similar to that found in control cardiac myocytes (Fig. 2 B).

Since both TnC extraction and reduced temperature resulted in a decrease in absolute force and a decrease in Ca$^{2+}$ sensitivity of force, data were graphed as shown in Fig. 8. In this figure, pCa$_{50}$ for a given condition was plotted versus maximum force obtained in the same fiber under the same condition, as a percentage of the largest force obtained in the fiber (i.e., in all fibers this was the control $P_o$ at 15°C). Fig. 8 A shows all the data from psoas fibers graphed in this way and demonstrates that the Ca$^{2+}$ concentration for half-maximal activation varies with maximum tension for all conditions studied ($P < 0.01$). Linear regression analysis of the data indicates that 85% of the variation in pCa$_{50}$ can be accounted for by coincident variation in tension. Data from cardiac myocytes are shown in Fig. 8 B. Change in pCa$_{50}$ with maximum tension is discernable here as well, although it is less distinct than in skeletal muscle. While variation in pCa$_{50}$ with tension is still significant, only 48% of the variation in pCa$_{50}$ can be attributed to changes in maximal tension.

**DISCUSSION**

**Examination of Previous Results and Analysis of Discrepancies**

The effect of elevated temperature to increase maximum tetanic force in skeletal muscle is well established (Ranatunga and Wylie, 1983; Kössler and Küchler, 1987;
Asmussen and Gaunitz, 1989). By contrast, previous studies of the effect of temperature on the Ca\(^{2+}\) sensitivity of isometric tension have come to conflicting conclusions and none of these earlier reports proposed a mechanism for the reported effects. Unfortunately, the basis for a temperature effect is not apparent from results of calorimetric measurements of enthalpy changes on Ca\(^{2+}\) binding to isolated cardiac and skeletal TnC molecules. Potter et al. (1977) first demonstrated that binding of Ca\(^{2+}\) to rabbit skeletal TnC in the absence of Mg\(^{2+}\) produces heat. This led to a prediction that the affinity of TnC for Ca\(^{2+}\) should decrease as temperature increased (Godt and Lindley, 1982). However, Yamada and Kometani (1982) showed that in the presence of 1 mM Mg\(^{2+}\), heat released upon Ca\(^{2+}\) binding to rabbit skeletal TnC did not change with temperature and heat released upon binding to cardiac TnC was increased only slightly by raising temperature (Kometani and Yamada, 1983). Using thermal denaturation, Jacobson et al. (1981) concluded that Ca\(^{2+}\) binding to cardiac TnC is not exothermic but rather an endothermic process. The differences in results remain unresolved. Calcium-binding properties of TnC change dramatically when the molecule is part of the whole troponin complex (Potter and Gergely, 1975; Holroyde et al., 1980), so that extrapolation of calorimetric data from isolated TnC to intact muscle may in any case be inappropriate. Clarification of the effect of temperature to alter activation of striated muscle is of interest, both because it can shed light on mechanisms of activation and because many experiments, particularly on permeabilized fibers, are done at temperatures well below physiological because of instability of preparations at higher temperatures.

**Figure 8.** Plots of pCa\(_{50}\) vs. maximum tension in skeletal (A) and cardiac (B) muscle. Each point is derived from the tension–pCa relationship for a single preparation under a given set of experimental conditions (e.g., TnC-extracted at 10°C). P/P\(_o\) is defined as the maximum tension for a given set of conditions divided by the maximum force in the same cell in the control condition at 15°C. Data for the control condition at 15°C are given as mean ± SD. ○, Control at 15°C; ▲, TnC-extracted at 15°C; ■, TnC-recombined at 15°C; ●, control at 10°C; △, TnC-extracted at 10°C; □, TnC-recombined at 10°C. (A) Skeletal muscle: slope = 0.561; r = 0.921; r\(^2\) = 0.848; (B) cardiac muscle: slope = 0.292; r = 0.691; r\(^2\) = 0.477.
Our results show that the Ca$^{2+}$ sensitivity of tension increases with increasing temperature in both cardiac and skeletal muscle (Figs. 3 and 4), in agreement with previous studies on permeabilized heart muscle (Brandt and Hibberd, 1976; Harrison and Bers, 1989). However, the observed shift in Ca$^{2+}$ sensitivity is opposite to that previously reported for skinned fast twitch skeletal muscles (Stephenson and Williams, 1981; Goldman et al., 1987) and canine cardiac Purkinje cells (Fabiato, 1985). To explore possible explanations for these discrepancies, an attempt was made to reproduce the solutions and experiments of Goldman and colleagues. Using our preparation with their solutions we measured Ca$^{2+}$ sensitivity of tension development at 10 and 20°C, pH 7.1, and ionic strength of 200 mM. Measurements at each temperature were done in the steady state. We were unable to verify their findings but instead obtained results qualitatively similar to our initial results at 10 and 15°C (compare Figs. 4 and 5). The fact that we observed significant sarcomere length nonuniformity at 20°C and were unable to obtain any data at all at 30°C points to one possible explanation for the inconsistency in the reported results. Changes in sarcomere length have been associated with changes in the Ca$^{2+}$ sensitivity of tension and the binding of Ca$^{2+}$ by TnC (Ekelund and Edman, 1982; Hibberd and Jewell, 1982; Hofmann and Fuchs, 1988). If significant sarcomere shortening occurred during an activation, Ca$^{2+}$ binding would be expected to decrease and the tension-pCa curve to shift right. Previous studies on skeletal muscle included measurements of force in skinned fibers at rather high temperatures (20–35°C) where, in our experience, the psoas preparation undergoes internal shortening or tears itself apart. Therefore, it would be very important in experiments of this type to use an attachment with as little compliance as possible, and to monitor sarcomere length throughout the experiment. Of course, some of the differences in the literature may be real, a reflection of differential temperature sensitivity of different tissues. However, in the absence of sarcomere length monitoring, one must consider that shortening of sarcomeres may explain a decreased calcium sensitivity seen after an increase in absolute force with increasing temperature. As demonstrated in Fig. 1 and in an earlier paper by Moss (1979), both our preparations have very low compliance, and sarcomere length at full activation is very close to that at rest. We consistently worked at lower temperatures where the striation pattern was stable. Also, the direction of the shift in Ca$^{2+}$ sensitivity that we observed when temperature was increased is opposite that expected with decreases in sarcomere length in a compliant preparation.

Probing Thin Filament Activation in Cardiac Myocytes by TnC Extraction

To explore the possible role of changes in thin filament cooperativity in response to altered temperature, we partially extracted TnC from our myocardial preparation. Decreases in maximum Ca$^{2+}$-activated force after extraction of TnC from cardiac myocytes probably reflect a decrease in the number of actin binding sites available to myosin during Ca$^{2+}$ activation. In addition, Ca$^{2+}$ sensitivity of tension was reduced after removal of TnC, similar to previous results from skeletal muscle (Brandt et al., 1984; Moss et al., 1985). However, Ca$^{2+}$ sensitivity decreases less for extraction of TnC from cardiac muscle than after extraction to a similar relative tension in skeletal muscle. Extraction to ~40% $P_o$ in psoas fibers resulted in a right shift of pCa$_{50}$ by
0.28 pCa units, while extraction to the same relative $P_o$ in the cardiac cell preparation resulted in a right shift of only 0.18 pCa units (compare Figs. 6 and 7). This may indicate that intact thin filaments in heart muscle are less cooperative than those in skeletal muscle, a finding which would be expected based on the decreased steepness of the tension–pCa relation found when cardiac TnC is substituted into skeletal muscle fibers (Moss et al., 1986). This conclusion is supported by the finding that the tension–pCa curve after recombination with TnC is not significantly different from the control curve in cardiac muscle, even when tension has recovered to just 66% $P_o$. In skeletal muscle, even a slight TnC deficiency results in a decrease in the Ca$^{2+}$ sensitivity of tension development (Moss et al., 1985). Brandt et al. (1984, 1987) showed that removal of as little as 5% of the TnC from skeletal muscle fibers reduced cooperativity, demonstrated by a decreased slope of the Hill plot. This argues that cooperative activation of calcium binding to TnC may be communicated across longer distances in skeletal than in cardiac thin filaments.

It is possible that the lower level of thin filament cooperativity in cardiac muscle is due to the presence of only one low affinity Ca$^{2+}$ binding site on cardiac TnC compared with two on skeletal TnC. If this were the only type of thin filament cooperativity in cardiac muscle, then partial extraction of TnC should not affect the shape or position of the tension–pCa relation. However, when sufficient cardiac TnC is extracted, the tension–pCa relation becomes less steep and shifts right, implying that there is some intermolecular cooperativity between TnC molecules on the cardiac thin filament (see also Tobacman and Sawyer, 1990). Still, we cannot exclude a contribution of intramolecular events to the greater cooperativity seen in skeletal muscle.

$P_o$ sometimes failed to return to control levels with readdition of purified cardiac TnC to previously extracted myocytes. It is most likely that there is incomplete recombination of TnC into the thin filaments despite repeated soaks in purified TnC solution, perhaps due to differences between rat native cTnC and the bovine cTnC used for recombination. The basis for inconsistency of TnC recombination between preparations is unclear at present. Another possible explanation for the failure of $P_o$ to recover in cardiac muscle is nonspecific deterioration of the myocytes, leading to an inability to fully recover tension despite reincorporation of cTnC. Several lines of evidence suggest that this is not the case. Maximum tension in preparations that had not been extracted rarely dropped below 80% $P_o$ (1 in 47 studied), even in 5-h experiments where no TnC was added at any time during the protocol. Also, several TnC-extracted myocyte preparations did recover to >80% $P_o$ with readdition of TnC, and one recovered to 98% $P_o$.

### Possible Role of Thin Filament Cooperativity in the Force Response to Temperature

It would not be surprising if changing the temperature altered the activation level of the thin filament, since properties of myofibrillar proteins might change with temperature. Tropomyosin (Tm), for instance, becomes more rigid as temperature is lowered in the range of 35–12°C (Swenson and Stellwagen, 1989). The decrease in Ca$^{2+}$ sensitivity that we observed when temperature was lowered suggests that more rigid Tm is difficult for adjacent Tm to displace, thus reducing cooperative activation. The functional properties of other proteins may be affected as well. To
probe the mechanism of the effect of temperature to alter calcium sensitivity, we extracted TnC to disrupt the normal activation of the thin filament and then tested the effect of temperature on this less cooperative system. We found that psoas fibers deficient in TnC showed a greater temperature-induced change in Ca\(^{2+}\) sensitivity than seen in control fibers. This result implies that changes in cooperative activation were probably not responsible for the altered Ca\(^{2+}\) sensitivity. The larger shift of pCa\(_{50}\) after extraction was found to correlate with a larger loss in maximum force with decreased temperature after the extraction. From previous studies there is considerable evidence that force in muscle may influence the affinity of TnC for Ca\(^{2+}\). Gordon and Ridgway (1987) argue convincingly for a role of force in Ca\(^{2+}\) binding to TnC, showing that extra Ca\(^{2+}\) released to the myoplasm upon a step release in muscle length is proportional to the force change rather than the length change in their barnacle muscle preparation. This result was confirmed in ferret ventricular muscle by Allen and Kentish (1988). Hofmann and Fuchs (1987) have shown that reduction of force in bovine cardiac muscle by vanadate correlated with a decrease in Ca\(^{2+}\) bound to TnC as measured by Ca\(^{45}\) binding. A frequent problem with studies of this type is that length is altered in order to change tension, making interpretation difficult since length has also been implicated as a possible factor in modulating the affinity of TnC for Ca\(^{2+}\) (Stephenson and Wendt, 1984). In the work presented here, as in the work by Hofmann and Fuchs (1987), force was altered without changing sarcomere length. The striking relationship in Fig. 8 shows a strong influence of absolute force level, varied with temperature or partial extraction of TnC, on the Ca\(^{2+}\) sensitivity of tension development.

**Explanation of Effects of Altered Temperature and TnC Extraction in Cardiac and Skeletal Muscle**

Maneuvers that reduce isometric force may do so in two ways. The number of tension-generating cross-bridges may decrease, or, alternatively, the mean force per cross-bridge may be reduced while the number of cross-bridges remains constant (this may be due either to a decrease in the proportion of strongly to weakly bound bridges, or to an actual drop in the mean force of each strongly bound cross-bridge). Partial extraction of TnC presumably reduces force by altering the number of actin binding sites available to myosin, thus reducing the number of tension-generating cross-bridges (Moss et al., 1985). The mechanism by which decreased temperature reduces maximum force has not yet been clearly explained. However, several groups (Ford et al., 1977; Bressler, 1981) have shown that the fall in stiffness accompanying a drop in temperature is not large enough to completely explain the decrease in tension. Their results indicate that a change in force per cross-bridge is at least partly responsible for the fall in tension as temperature is lowered, although the number of tension-generating cross-bridges may change as well. We have found (Fig. 8) that TnC extraction and reduced temperature similarly affect the calcium sensitivity of tension development in a manner linearly related to their effects on the maximum tension-generating capability of the muscle. If, indeed, TnC extraction reduces the number of bound cross-bridges while altered temperature primarily changes mean force per bound cross-bridge, then our data suggest that it is not the number of cross-bridges or mean force per cross-bridge, but rather overall force-generating
capability of the muscle, which alters calcium sensitivity of tension development, presumably by altering the affinity of TnC for Ca\(^{2+}\). The mechanism by which maximum force-generating capability of muscle modulates calcium sensitivity of tension development is unknown. It seems quite possible that both of these changes are manifestations of a third, as yet undetected, mechanism.

Despite the striking correlation between maximum force and pCa\(_{50}\), we cannot ignore the possible contribution of other factors to changes in calcium sensitivity seen in permeabilized fibers. It has been shown by several groups (Bremel and Weber, 1972; Grabarek et al., 1983) that the affinity of TnC for Ca\(^{2+}\) is increased in the presence of the S1 fragment of myosin. In isolated protein systems such as these, the thin filament presumably bears no force. In intact barnacle fibers Gordon and Ridgway (1987) suggest that cross-bridge number plays a more important role than force per cross-bridge in control of Ca\(^{2+}\) sensitivity. Using the skinned fiber preparation, we have implicated force as an important determinant of the Ca\(^{2+}\) sensitivity of tension development; however, we cannot separate out the effects due to S1 binding alone. The fact that the relationship between maximum force and pCa\(_{50}\) is the same when force is changed either by temperature (which alters both numbers of cross-bridges and force per cross-bridge) or by TnC extraction (which affects only numbers of cross-bridges) implies that both of these alterations are equally effective in altering calcium sensitivity of force development. Presumably the effects we observe are a combination of S1 binding effects and force effects on the affinity of Ca\(^{2+}\) binding to TnC.

Results reported here suggest that activation of the thin filament in cardiac muscle is less cooperative than activation in psoas muscle and that Ca\(^{2+}\) binding to TnC in both muscles is to some degree force dependent. We therefore propose the following model of activation. Cardiac muscle, due to its lower degree of molecular cooperativity within the thin filament, is less sensitive to TnC extraction than is the more highly cooperative skeletal muscle preparation. However, cardiac muscle exhibits a much larger change in Ca\(^{2+}\) sensitivity when temperature is dropped from 15 to 10°C than does skeletal muscle. This change in Ca\(^{2+}\) sensitivity reflects the differential sensitivity of force to temperature of the two muscles in this range, i.e., maximal force in cardiac muscle falls ~60% with this decrease in temperature, while tension in skeletal muscle falls only ~25% in this range. On this basis one would expect to see a much larger shift in the Ca\(^{2+}\) sensitivity of tension development in cardiac muscle. In skeletal muscle TnC extraction magnifies the effect of altered temperature on the position of the tension-pCa relationship, and this change in magnitude correlates well with a greater fall of maximum tension over this temperature range after removal of TnC. The greater effect of temperature on submaximal tension after extraction would be expected in a system with a reduced level of thin filament cooperativity. In fact, in this situation skeletal muscle behaves very much like cardiac muscle under control conditions (see Fig. 2 B).

**Conclusions**

Effects of temperature and TnC extraction to alter apparent Ca\(^{2+}\) sensitivity of force development are the result of a loss in tension which appears to reduce Ca\(^{2+}\) binding to TnC. In the less cooperative cardiac muscle, the change in Ca\(^{2+}\) sensitivity with
altered temperature is large. However, in this same muscle >30% of the TnC must be removed to alter the Ca$^{2+}$ sensitivity of thin filament activation. The highly cooperative thin filament of fast skeletal muscle, on the other hand, minimizes the effect of temperature. Only at low levels of Ca$^{2+}$, where very few cross-bridges are bound, is an effect of temperature on Ca$^{2+}$ sensitivity of force development seen. At these low levels of activation greater force development at higher temperatures has an effect on calcium–TnC interaction to increase binding affinity for Ca$^{2+}$. At slightly higher Ca$^{2+}$ concentrations the affinity of TnC for Ca$^{2+}$ is increased due to cooperative activation of the thin filament, and thus the temperature-induced increase in force no longer has a significant effect on Ca$^{2+}$ binding. When thin filament cooperativity in skeletal muscle is disrupted by TnC extraction, temperature becomes a more effective modulator of force and Ca$^{2+}$ sensitivity. This study also provides evidence that effects of force on Ca$^{2+}$ sensitivity of tension development are predominantly mediated by absolute force levels rather than by the number of cross-bridges or force per crossbridge. The magnitude of the effect of altered force on Ca$^{2+}$ sensitivity increases as the level of cooperativity within the thin filament decreases. Consistent with this idea, the degree of thin filament cooperativity is lowest in control cardiac myocytes and partially TnC-extracted skeletal muscle fibers, and these were the preparations that showed the greatest changes in pCa$_{50}$ as temperature was altered.

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REFERENCES

Allen, D. G., and J. C. Kentish. 1988. Calcium concentration in the myoplasm of skinned ferret ventricular muscle following changes in muscle length. *Journal of Physiology.* 407:489–503.

Allen, J. D., and R. L. Moss. 1987. Factors influencing the ascending limb of the sarcomere length-tension relationship in rabbit skinned muscle fibres. *Journal of Physiology.* 390:119–136.

Ashley, C. C., and D. G. Moiessou. 1977. Effect of changing the composition of the bathing solutions upon the isometric tension-pCa relationship in bundles of crustacean myofibrils. *Journal of Physiology.* 270:627–652.

Asnussen, G., and U. Gaunitz. 1989. Temperature effects on isometric contractions of slow and fast twitch muscles of various rodents—dependence on fibre type composition: a comparative study. *Biomedica Biochimica Acta.* 48:536–541.

Brady, A. J., S. T. Tan, and N. V. Ricchiuti. 1979. Contractile force measured in unskinned isolated adult rat heart fibres. *Nature.* 282:728–729.

Brandt, P. W., M. S. Diamond, B. Gluck, M. Kawai, and F. Schachat. 1984. Molecular basis of cooperativity in vertebrate muscle thin filaments. *Carlsberg Research Communications.* 49:155–167.

Brandt, P. W., M. S. Diamond, J. S. Rutchik, and F. H. Schachat. 1987. Cooperative interactions between troponin-tropomyosin units extend the length of the thin filament in skeletal muscle. *Journal of Molecular Biology.* 195:885–896.
Brandt, P. W., and M. G. Hibberd. 1976. Effect of temperature on the pCa-tension relation of skinned ventricular muscle of the cat. Journal of Physiology. 258:76P.

Bremel, R. D., and A. Weber. 1972. Cooperation within actin filament in vertebrate skeletal muscle. Nature New Biology. 238:97–101.

Bressler, B. H. 1981. Isometric contractile properties and instantaneous stiffness of amphibian skeletal muscle in the temperature range from 0 to 20°C. Canadian Journal of Pharmacology. 59:548–554.

Cox, J. A., M. Comte, and E. A. Stein. 1981. Calmodulin-free skeletal-muscle troponin C prepared in the absence of urea. Biochemical Journal. 195:205–211.

De Clerck, N. M., V. A. Claes, and D. I. Brutsaert. 1977. Force velocity relations of single cardiac muscle cells. Journal of General Physiology. 69:221–241.

Ekelund, M. C., and K. A. P. Edman. 1982. Shortening induced deactivation of skinned fibres of frog and mouse striated muscle. Acta Physiologica Scandinavica. 116:189–199.

Endo, M. 1972. Stretch-induced increase in activation of skinned muscle fibres by calcium. Nature New Biology. 237:211–213.

Fabiato, A. 1985. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. Journal of General Physiology. 85:247–289.

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

Fabiato, A., and F. Fabiato. 1975. Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. Journal of Physiology. 249:469–495.

Fabiato, A., and F. Fabiato. 1978. Myofilament-generated tension oscillations during partial calcium activation and activation dependence of the sarcomere length-tension relation of skinned cardiac cells. Journal of General Physiology. 72:667–699.

Ford, L. E., A. F. Huxley, and R. M. Simmons. 1977. Tension responses to sudden length change in stimulated frog muscle fibres near slack length. Journal of Physiology. 269:441–515.

Giulian, G. G., R. L. Moss, and M. Greaser. 1983. Improved methodology for analysis and quantitation of proteins on one-dimensional silver-stained slab gels. Analytical Biochemistry. 129:277–287.

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

Goldman, Y. E., J. A. McCray, and K. W. Ranatunga. 1987. Transient tension changes initiated by laser temperature jumps in rabbit psoas muscle fibres. Journal of Physiology. 392:71–95.

Gordon, A. M., and E. B. Ridgway. 1987. Extra calcium on shortening in barnacle muscle. Is the decrease in calcium binding related to decreased cross-bridge attachment, force, or length? Journal of General Physiology. 90:321–340.

Grabarek, Z., J. Grabarek, P. C. Lewis, and J. Gergely. 1983. Cooperative binding to the Ca2+-specific sites of troponin C in regulated actin and actomyosin. Journal of Biological Chemistry. 258:14098–14102.

Harrison, S. M., and D. M. Bers. 1989. Influence of temperature on the calcium sensitivity of the myofilaments of skinned ventricular muscle from the rabbit. Journal of General Physiology. 93:411–428.

Harrison, S. M., C. Lamont, and D. J. Miller. 1988. Hysteresis and the length dependence of calcium sensitivity in chemically skinned rat cardiac muscle. Journal of Physiology. 401:115–143.
Haworth, R. A., P. Griffin, B. Saleh, A. B. Coknur, and H. A. Berkoff. 1987. Contractile function of isolated young and adult rat heart cells. *American Journal of Physiology*. 253:H1484–H1491.

Hibberd, M. G., and B. R. Jewell. 1982. Calcium- and length-dependent force production in rat ventricular muscle. *Journal of Physiology*. 329:527–540.

Hofmann, P. A., and F. Fuchs. 1987. Evidence for a force-dependent component of calcium binding to cardiac troponin. *American Journal of Physiology*. 253:C541–C546.

Hofmann, P. A., and F. Fuchs. 1988. Bound calcium and force development in skinned cardiac muscle bundles: effect of sarcomere length. *Journal of Molecular and Cellular Cardiology*. 20:667–677.

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

Holroyde, M. J., S. P. Robertson, J. D. Johnson, R. J. Solaro, and J. D. Potter. 1980. The calcium and magnesium binding sites on cardiac troponin and their role in the regulation of myofibrillar adenosine triphosphatase. *Journal of Biological Chemistry*. 255:11688–11693.

Jacobson, A. L., G. Devin, and H. Braun. 1981. Thermal denaturation of beef cardiac troponin and its subunits with and without calcium ion. *Biochemistry*. 20:1694–1701.

Kometani, K., and K. Yamada. 1983. Enthalpy, entropy and heat capacity changes induced by binding of calcium ions to cardiac troponin C. *Biochemical and Biophysical Research Communications*. 114:162–167.

Kössler, F., and G. Küchler. 1987. Contractile properties of fast and slow twitch muscle of the rat at temperatures between 6 and 42°C. *Biomedica Biochimica Acta*. 46:815–822.

Metzger, J. M., M. L. Greaser, and R. L. Moss. 1989. Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. Implications for twitch potentiation in intact muscle. *Journal of General Physiology*. 93:855–883.

Moss, R. L. 1979. Sarcomere length-tension relations of frog skinned muscle fibers during calcium activation at short lengths. *Journal of Physiology*. 292:177–192.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1985. The effects of partial extraction of TnC upon the tension-pCa relationship in rabbit skinned skeletal muscle fibers. *Journal of General Physiology*. 86:585–600.

Moss, R. L., M. R. Lauer, G. G. Giulian, and M. L. Greaser. 1986. Altered Ca\(^{2+}\) dependence of tension development in skinned skeletal muscle fibers following modification of troponin by partial substitution with cardiac troponin C. *Journal of Biological Chemistry*. 261:6096–6099.

Moss, R. L., A. E. Swinford, and M. L. Greaser. 1983. Alterations in the Ca\(^{2+}\) sensitivity of tension development by single skeletal muscle fibers at stretched lengths. *Biophysical Journal*. 43:115–119.

Orentlicher, M., P. W. Brandt, and J. P. Reuben. 1977. Regulation of tension in skinned muscle fibers: effect of high concentrations of Mg-ATP. *American Journal of Physiology*. 233:C127–C134.

Potter, J. D., and J. Gergely. 1975. The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphatase. *Journal of Biological Chemistry*. 250:4628–4633.

Potter, J. D., F.-J. Hsu, and H. J. Pownall. 1977. Thermodynamics of Ca\(^{2+}\) binding to troponin-C. *Journal of Biological Chemistry*. 252:2452–2454.

Ranatunga, K. W., and S. R. Wylie. 1983. Temperature-dependent transitions in isometric contractions of rat muscle. *Journal of Physiology*. 399:87–95.

Reiser, P. J., M. L. Greaser, and R. L. Moss. 1988. Myosin heavy chain composition of single cells from avian slow skeletal muscle is strongly correlated with velocity of shortening during development. *Developmental Biology*. 129:400–407.
Ridgway, E. B., and A. M. Gordon. 1984. Muscle calcium transient. Effect of post-stimulus length changes in single fibers. *Journal of General Physiology.* 83:75–103.

Ridgway, E. B., and A. M. Gordon. 1990. Calcium binding and cross-bridge attachment: effects of stretch induced cross-bridge detachment and strain. *Biophysical Journal.* 57:547a. (Abstr.)

Stephenson, D. G., and I. R. Wendt. 1984. Length dependence of changes in sarcoplasmic calcium concentration and myofibrillar calcium sensitivity in striated muscle fibres. *Journal of Muscle Research and Cell Motility.* 5:243–272.

Stephenson, D. G., and D. A. Williams. 1981. Calcium-activated force responses in fast- and slow-twitch skinned muscle fibres of the rat at different temperatures. *Journal of Physiology.* 317:281–302.

Swenson, C. A., and N. C. Stellwagen. 1989. Flexibility of smooth and skeletal tropomyosins. *Biopolymers.* 28:955–963.

Tartt, M., J. W. Trank, and P. Leiffer. 1981. Characteristics of sarcomere shortening in single frog atrial cardiac cells during lightly loaded contractions. *Circulation Research.* 48:189–200.

Tobacman, L. S., and D. Sawyer. 1990. Calcium binds cooperatively to the regulatory sites of the cardiac thin filament. *Journal of Biological Chemistry.* 265:931–939.

Ventura-Clapier, R., H. Mekhfi, and G. Vassort. 1987. Role of creatine kinase in force development in chemically skinned rat cardiac muscle. *Journal of General Physiology.* 89:815–837.

Yamada, K., and K. Kometani. 1982. The changes in heat capacity and entropy of troponin C induced by calcium binding. *Journal of Biochemistry.* 92:1505–1517.