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The Control of Myocardial Contraction with Skeletal Fast Muscle Troponin C*

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The present study describes experiments on the myocardial trabeculae from the right ventricle of Syrian hamsters whose troponin C (TnC) moiety was exchanged with heterologous TnC from fast skeletal muscle of the rabbit. These experiments were designed to help define the role of the various classes of Ca^{2+}-binding sites on TnC in setting the characteristic sensitivities for activations of cardiac and skeletal muscles. Thin trabeculae were skinned and about 75% of their troponin C extracted by chemical treatment. Tension development on activations by Ca^{2+} and Sr^{2+} was found to be nearly fully blocked in such TnC extracted preparations. Troponin C contents and the ability to develop tension on activations by Ca^{2+} and Sr^{2+} was permanently restored after incubation with 2–6 mg/ml purified TnC from either rabbit fast-twitch skeletal muscle (STnC) or the heart (CTnC, cardiac troponin C). The native (skinned) cardiac muscle is characteristically about 5 times more sensitive to activation by Sr^{2+} than fast muscle, but the STnC-loaded trabeculae gave response like fast muscle. Attempts were also made to exchange the TnC in psoas (fast-twitch muscle) fibers, but unlike cardiac muscle tension response of the maximally extracted psoas fibers could be restored only with homologous STnC. CTnC was effective in partially extracted fibers, even though the uptake of CTnC was complete in the maximally extracted fibers. The results in this study establish that troponin C subunit is the key in setting the characteristic sensitivity for tension control in the myocardium above that in the skeletal muscle. Since a major difference between skeletal and cardiac TnCs is that one of the trigger sites (site I, residues 28–40 from the N terminus) is modified in CTnC and has reduced affinity for Ca^{2+} binding, the possibility is raised that this site has a modulatory effect on activation in different tissues and limits the effectiveness of CTnC in skeletal fibers.

The thin filament-linked regulation of contraction in vertebrate striated muscles is initiated by the binding of Ca^{2+} to a special class of sites (Ca^{2+}-specific) on the troponin C (TnC)[1] moiety (Leavis and Gergely, 1984). This produces dynamic conformational changes in distant regions of TnC and thereby modulates the interactions between the various regulatory components to promote activation (Ebashi and Endo, 1968; Grabarek *et al.*, 1986). However, the detailed nature and pathways for the transfer of information of conformational changes to each of the components is still being worked out, and the question has remained whether the Ca^{2+} binding to the particular sites is also the final determinant of the actual activation characteristics in different tissues. To address this question, in the present study, we have adapted the extraction procedures for troponin C, recently worked out for myofibrils and skinned fibers of skeletal muscle (Cox *et al.*, 1981; Brandt *et al.*, 1984; Babu *et al.*, 1986), to the myocardium. The extracted moiety could be replaced with troponin Cs from skeletal and cardiac muscles and their influence on the contractile properties are studied.

TnC from the fast-twitch skeletal muscle is quite similar to the subunit from cardiac muscle in its molecular weight and both have two separate classes of Ca^{2+}-binding sites (Ca^{2+}-specific and Ca^{2+}-Mg^{2+}). Ca^{2+}-specific sites are putatively the trigger sites during activation. However, the amino acid sequence of one of the Ca^{2+}-specific sites in cardiac muscle (site I, residues 28–40 from the N terminus) indicates several amino acid replacements (van Eerd and Takahashi; 1975) and a tremendous reduction in its Ca^{2+}-binding affinity. STnC binds 4 mol of Ca^{2+} (2 mol to the low affinity Ca^{2+}-specific sites I and II, 2 mol to the high affinity Ca^{2+}-Mg^{2+} sites III and IV), but CTnC thus binds only 3 mol and has only one trigger site in the physiological range (Holroyde *et al.*, 1980). Thus the ability to exchange CTnC for STnC offered the possibility of studying the functional role(s) of the Ca^{2+}-specific sites. The relationships between isometric tension development and free Ca^{2+} for cardiac and skeletal muscles appear to be quite similar; but with Sr^{2+}, which can replace Ca^{2+} in physiological (Donaldson and Kerrick, 1975; Kitazawa, 1976; Gulati and Babu, 1985a) and biochemical (Ebashi and Endo, 1968) studies, major differences are seen in sensitivity of the two tissues for tension developments (Kitazawa, 1976) and actomyosin ATPase activities (Ebashi and Endo, 1968). These differences in the Sr^{2+} activations of skeletal and cardiac muscles are exploited here in combination with TnC exchange in the myocardium both to investigate the role

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[1] The abbreviations used are: TnC, troponin C; TnI, troponin I; TnT, troponin T; STnC, skeletal fast-twitch muscle TnC; CTnC, cardiac TnC; LCs, light chains (C-LC1 and C-LC2 of cardiac myosin and S-LC1, S-LC2 and S-LC3 of fast skeletal myosin); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; p*K*, p*Ca* or p*Sr* for half-maximal activation; P_0, tension made in p*Ca*4 (180 mM ionic strength) by the native preparations.

of the Ca^{2+}-specific sites in defining the activation characteristics in skeletal and cardiac muscles and thereby to gain further insights into the nature of the conformational changes in TnC moiety needed to initiate muscular contraction. The present study with myocardium seemed particularly worthwhile because recent similar attempts, in the converse experiment using skeletal muscle with CTnC, gave variable results and led to conclusions diametrically opposite of each other (Kerrick *et al.*, 1985; Moss *et al.*, 1986). Several new studies of TnC exchange were also made with skeletal fibers in an effort to seek plausible explanations for these differences.

Our results at near physiological ionic strength (180–190 mM) demonstrate the possibility of significant plasticity in the activation mechanism of the cardiac muscle contractile apparatus. On the basis of these findings the differences in the half-maximal activations for tension generation by divalent metal ions in different tissues could be positively assigned to the possible variations in the properties of the regulatory sites of TnC subunits. In addition, the modification of a trigger site (I) in cardiac TnC appeared to be critical for the tension control in skeletal muscle in 180 mM salt.

MATERIALS AND METHODS

Fiber Preparations—Skinned preparation of adult (6–9-month old) Syrian hamsters (Strain RB, Dr. M. J. Sole, University of Toronto) consisted of 80–150 μm (width) by 1–3 mm (length) trabeculae from the right ventricle. The skeletal single fiber preparation (30–80 μm wide) was from the psoas muscle. Skinning was accomplished by 30-min treatment at 10 °C with 0.5% Lubrol-WX detergent in 140 mM KCl, 10 mM imidazole, 5 mM $MgCl_2$, 5 mM ATP, 5 mM EGTA, 5 mM creatine phosphate. The relaxing and activating solutions contained about 100 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 5 mM ATP, 20 mM creatine phosphate and 250 units/ml of creatine phosphokinase, and either EGTA or Ca-EGTA, Sr-EGTA. The pH of each solution was adjusted to 7.00 ± 0.01 at the appropriate temperature. The ionic strength of the solutions was kept between 180–190 mM, which is close to the physiological range.[2] Free Mg^{2+} was kept at 1 mM, which is also close to the *in vivo* value (Gupta and Moore, 1981; Baylor *et al.*, 1982).

Preparation of TnC-extracted Fibers—To achieve TnC extraction from both skeletal and cardiac preparations, they were first transferred from the relaxing solution at 4 °C to a rigor solution containing 20 mM imidazole, 165 mM KCl, 2.5 mM EGTA, 2.5 mM EDTA, and pH 7.0. The temperature was raised to 30 °C and, after 5 min, the preparations were placed in the extracting solution (5 mM EDTA, 10 mM imidazole, and pH 7.2) at 30 °C (Babu *et al.*, 1986). The preparations were returned to the relaxing solution and checked for tension response in *p*Ca4 (20 °C), at intervals of 5 min in the extraction solutions; extraction was stopped when the tension was down to 0–10% of the native fiber. This is referred to as "maximal" extraction in the present study. Extraction time for the trabeculae in the present study was 20–50 min, and for the skeletal fibers from 5–30 min. In a few cases, the skeletal fiber preparation was extracted at 4 °C and this is pointed out in the text when applicable. Reconstitution was attempted by 30–120-min incubation with 2–6 mg/ml TnC in the relaxing solution at 15–20 °C. We made activations at 20 °C in *p*Ca4 and *p*Sr4 except where indicated. The sarcomere length was adjusted at 2.2 (and in a few cases at 2.5 μm) for psoas fibers and 2.2 μm for the trabeculae, using laser diffraction, and was monitored throughout the experiment.

Selection of Fast-Twitch Fibers—Hamster psoas is a mixed muscle (80% dark (fast-twitch) and 20% light (slow-twitch)) by histochemical staining at alkaline (9.7) pH for ATPase.[3] Thus to assure that only fast-twitch fibers were employed in this study, we selected the fibers for experiments by the tension response to *p*Sr5 activation, as described earlier (Babu *et al.*, 1986). Fast fibers gave nearly zero force in *p*Sr5 and slow fibers gave full force about equal to that in *p*Ca4. Such slow fibers were discarded. Fast-twitch fibers had unloaded shortening velocity in the range 4.5–9 lengths/s (20 °C), by slack-test (Gulati and Babu, 1985b).

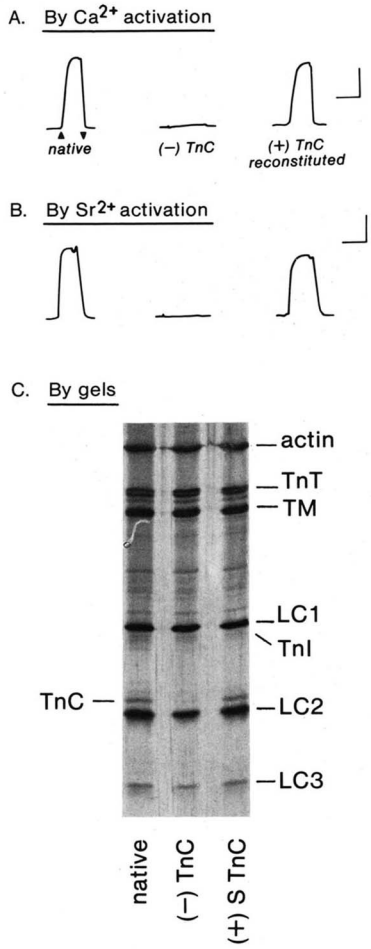

FIG. 1. **TnC extraction and reconstitution of fast-twitch skeletal muscle fibers from a psoas muscle.** Activations were made by *p*Ca4 (*A*) or pSr4 (*B*). First trace in each set shows the tension response prior to extraction, 2nd trace after extraction, and 3rd trace after reloading. Note nearly complete elimination of tension development by the extracted fiber, indicating effectively full TnC extraction. (*horizontal bar*, 10 s; *vertical bar*, 50 kN/m^2). (*C*) 15% SDS-PAGE runs on three skinned fiber segments: control (*native*), TnC-extracted, STnC-loaded. Silver stained. The identification of TnI, TnT, and tropomyosin (TM) bands was similar to Schachat *et al.* (1985).

Mechanical Set-up—The attachment of the skinned preparations and the force transducer and the servo-motor were the same as described before (Gulati and Babu, 1985b), and the reaction solutions during the experiments were contained in thermoelectrically controlled chambers similar to those described by Gulati and Podolsky (1978).

Troponin C Purification—Purified troponin Cs used in the studies for reconstituting the extracted preparations were made according to the method of Szynkiewicz *et al.* (1985) from rabbit heart and psoas muscle. The final peak from the column was dialyzed against an actin-polymerization buffer overnight, and any contaminating actin was removed by centrifugation at 100,000 × *g* for 90 min.

Experimental Protocol—Fig. 1 shows the typical protocol on hamster muscles. The protocol was first established on psoas fiber before applying to the trabeculae. Two sets of tension traces on fibers are given. Activations were by Ca^{2+} (*p*Ca4) and Sr^{2+} (*p*Sr4). In each case the first trace is the maximal tension response (P_0) of the "native" skinned fiber. Both Sr^{2+} and Ca^{2+} tensions were eliminated after TnC extraction (2nd trace in each set, Fig. 1) and could be recovered

[2] Inspection of the known values for the major intracellular constituents contributing to ionic strength (Table 6.2 in Kernan, 1972) gives an estimate for the intracellular ionic strength for mammalian fibers as at least 170 mM (assuming full activity; Palmer and Gulati, 1976). Also, under maximal activations, skinned frog fibers were found to be more stable in 180–200 mM salt than 100–140 mM in the 0–25 °C temperature range (Thames *et al.*, 1974; Gulati and Podolsky, 1981; Gulati and Babu, 1985b).

[3] J. Spiro, A. Babu, and J. Gulati, unpublished data.

almost fully on loading with purified skeletal TnC (3rd trace in each set in Fig. 1). After the experiment, the fibers were subjected to SDS-PAGE (*panel C* in Fig. 1; see "Gel Electrophoresis" below for technical details), confirming both the loss of TnC on extraction and reconstitution following incubation with purified TnC.

Ca^{2+} and Sr^{2+} Activations: Relation between Skeletal and Cardiac Muscles—Fig. 2 compares the *p*Ca-force and *p*Sr-force relationships for the hamster skeletal (at sarcomere length, 2.2 μm) and cardiac preparations (also 2.2 μm). The data and the computer fits of Hill's equations are indicated. Sensitivities for Ca^{2+} activations appear to overlap for the two tissues at these sarcomere lengths (left-hand plot) but the marked difference in the sensitivities of skeletal and cardiac muscles is brought out by Sr^{2+} activations. The native (skinned) cardiac preparation of the hamster is found to be about 5 times more sensitive to Sr^{2+} than the skeletal fiber (pK = 4.4 for psoas, 5.0 for the trabeculae). The disparity in the Sr^{2+} relationships between skeletal and cardiac muscles greatly facilitated testing of the effects of TnC exchange in these tissues.

Gel Electrophoresis—To establish the extraction procedure for trabeculae, cardiac bundles (5–10 times the sample size used for mechanical measurements) were used for initial gel runs. The treated bundles were dissolved in SDS sample application buffer with the addition of 6 M urea and analyzed by SDS-PAGE according to the method of Laemmli (1970). Fig. 3 shows the results. These gels (15%) were stained with Coomassie Brilliant Blue R250 and scanned on a Beckman DU-8 spectrophotometer at 584 nm to detect the peak maximal for apparent molecular weight determinations and for quantitation of troponin C. To correct for unequal loading of the gel lanes, these data were normalized to both the 38- and 26-kDa bands.

Densitometric scans of the lanes in these initial runs showed that 80–90% of the TnC had been extracted (the tension response of the extracted bundles (*p*Ca4) was 0.07 P_0). Proteins in the TnC band were the major ones extracted with the exception of one other band that had an apparent molecular weight 11,000. No effort was made to identify this low molecular weight component.

The gel runs (10 or 15%) of all other experimental fibers and trabeculae were silver stained for improved sensitivity (the expected TnC < 10 ng in our fiber segments) and scanned with LKB laser densitometer (Ultroscan XL). The gels were fixed in 5% glutaraldehyde overnight prior to staining; the solvents and the equipment (Protean II) were obtained from Bio-Rad. The tissue samples for the gels were carefully dissolved in SDS sample buffers (without urea) with ultrasonication using tapered ⅛-inch micro-tip (Branson Sonifier, Model 200). The fixed gels were thoroughly washed with double distilled (with a 3-stage Millipore Filter System) water prior to staining. To check for the resolution of silver staining, test gel lanes were run with two amounts of sample solution (20 and 40 μl) and TnC, TnI, LC1, LC2, and LC3 bands were found to be within 10% of

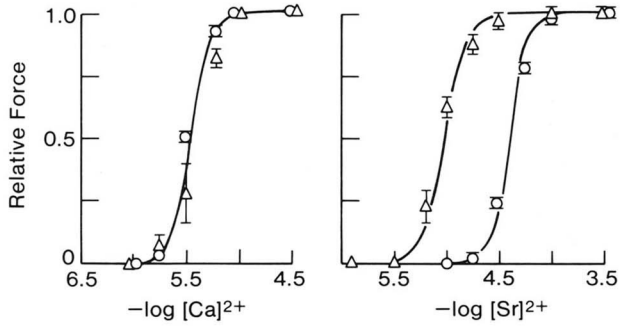

FIG. 2. **Comparison of activation characteristics for fast-twitch muscle fibers and cardiac muscles.** Number of skeletal fiber preparations was four and cardiac five, and the sarcomere length was 2.2 μm. At 2.5 μm, the activation curves for psoas fibers were shifted to the left by 0.15 units (not shown) and such shifts are consistent with results in the literature (Stephenson and Wendt, 1984). The data for both Ca^{2+} and Sr^{2+} activations are computer-fitted by Hill's equation: relative force = $[Me]^n/(K^n + [Me]^n)$, where *Me* is either Ca^{2+} or Sr^{2+}. Fitted parameters for Ca^{2+} (*left panel*): pK = 5.4, n = 4.7 for the combined data on psoas (*circles*) and trabeculae (*triangles*); for Sr^{2+} (*right panel*), pK = 4.4, n = 6.0 on psoas (*circles*); pK = 5.0, n = 4.4 on trabeculae (*triangles*). Note the marked disparity in Sr^{2+} activations of the two preparations, and there was close similarity in our Hill's coefficient (n, for Ca^{2+}) on hamster trabeculae with Kentish *et al.* (1986) on rat.

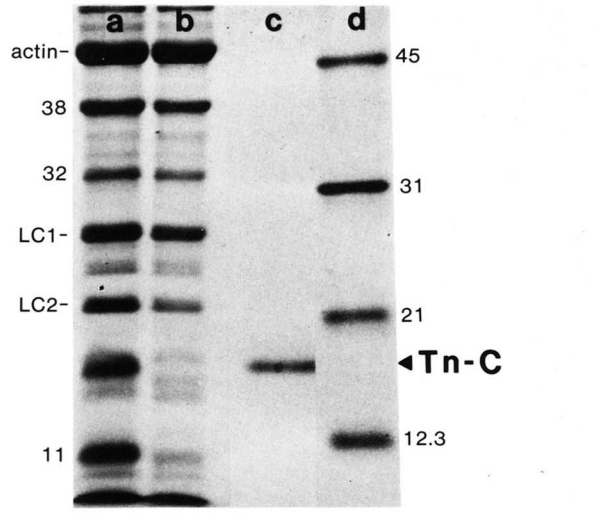

FIG. 3. **A composite of various lanes of 15% SDS-polyacrylamide gels from cardiac trabeculae, rabbit cardiac troponin C, and molecular weight standards.** *Lane a* is trabecular muscle and *lane b* another trabeculae extracted for troponin C. The purified troponin C used in the reconstitution experiments is shown in *lane c* with protein molecular weight standards in *lane d* of 45,000, 31,000, 21,000 and 12,300. Loading was different for *lanes a* and *b*. The bottom bands are close to the dyefront and contain material of 7 kDa or less. The 38-Da band probably contains TnT.

the expected values (normalization in these cases was generally to LC1 band, by area of the densitometer peak). The results of analysis from the experimental preparations are discussed below.

Statistics—All data are given as mean ± S.E. Curve fittings, wherever appropriate, were computed by the method of least squares on a microcomputer (Hewlett Packard-85).

RESULTS

Extraction of Troponin C from the Myocardium and Reconstitution—Fig. 4 shows the tension responses to Ca^{2+}-activations on two typical trabeculae preparations before and after exposure to the TnC extraction procedure. Like skeletal fibers, TnC was extracted until force was close to zero (middle traces in Fig. 4, *a* and *b*). The last two traces in Fig. 4, *a* and *b* show the reconstitution with purified cardiac and skeletal TnC, respectively. Nearly full recovery of the tension was found on the trabeculae with both types of TnC. Resting tension in the relaxing solution was not affected by TnC extraction.

The gel runs (silver stained) on these preparations in Fig. 4*c* show the presence and absence of cardiac TnC, and the pooled quantitative data from gel scans on successful skeletal and cardiac preparations is summarized in Table I. Fig. 4*c* clearly shows the restored CTnC band, but the presence of STnC is difficult to ascertain in the third lane as STnC runs in the same spot as cardiac LC2. These results show 1) that tension development in cardiac muscle drops to practically zero when 75% or more of the native TnC is extracted, 2) that nearly complete reconstitution of the trabeculae is made on incubation with purified TnC, and 3) that the loss of material other than TnC (*e.g.* the 11,000-dalton component seen in the runs in Fig. 3 and a possible loss of at most 10% C-LC2 in Table IB) during the extraction procedure was not important for tension generation by contractile apparatus in the cardiac muscle (S-LC2 loss was significant in psoas fibers, as explained under "Discussion"), and 4) that any differences in the skeletal and cardiac TnCs are not critical in the reconstituted myocardium to develop the *original level* of cardiac tension. Additional studies to determine the effects of various TnCs on the sensitivity to Sr^{2+} are given below.

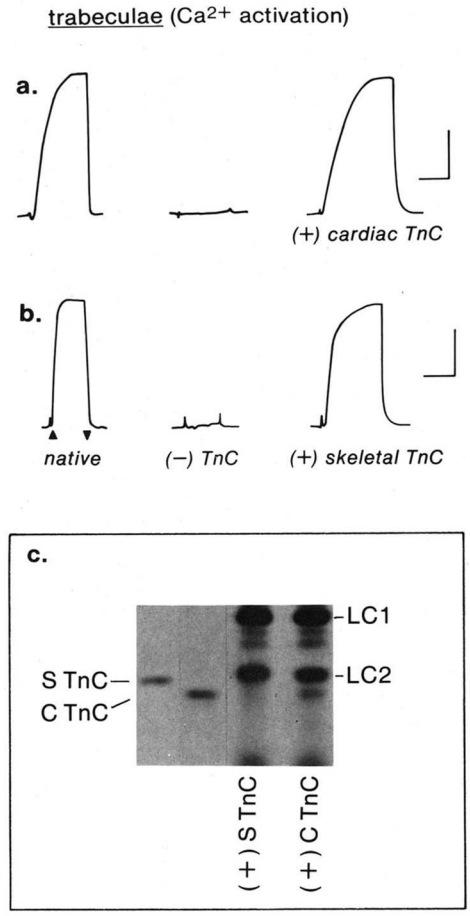

FIG. 4. **Trabeculae loaded with cardiac TnC (*a*) and skeletal TnC (*b*).** Note nearly full tension recovery (*p*Ca4) with either type of TnC. (*horizontal bar*, 20 s; *vertical bar*, 10 kN/m^2). *c*, 10% gels on CTnC- and STnC-loaded trabeculae (silver stained). The CTnC band was close to that in the native trabeculae when the data were normalized to the intensity of the LC1 band (see Table I). Also, note the near absence of CTnC in the 3rd lane.

Further evidence against the possibility of deleterious effects of the extraction procedure was derived from the stiffness measurements in rigor, since this parameter gives an index of the maximum possible number of cross-bridge attachments in the fiber. Table II compares the rigor stiffness of native and TnC-extracted preparations. For both the skeletal fiber and the myocardium, rigor stiffness was found to be unchanged by TnC extraction (Table II).

pCa-Force and pSr-Force Relationships of Reconstituted Fibers and Trabeculae: Loading with the Native-type (Homologous) TnC—Fig. 5 shows results on the reconstituted preparations over the entire activation range. The top two plots give the data (*circles*) on psoas fibers loaded with STnC following extraction. These experiments with psoas fibers serve as controls for the studies on trabeculae loaded with STnC (see below, Fig. 6). The lower plots in Fig. 5 (*triangles*) are on the trabeculae loaded with the homologous CTnC. The *solid lines* are transferred from Fig. 2 on native fibers and trabeculae, respectively, and they seem to adequately describe the data on both preparations extracted and reloaded with homologous TnCs.

TABLE II

Fiber stiffness in rigor

Stiffness was measured by stretching the fiber by 0.5% of the length in 750 μs. Temperature 5 °C. P_0 was measured with pCa4 prior to TnC extraction.

Stiffness	Native TnC	(−) TnC
$P_0/nm/hs$		
Psoas ($n = 5$)	0.19 ± 0.09	0.20 ± 0.07
Trabeculae ($n = 4$)	0.17 ± 0.04	0.17 ± 0.03

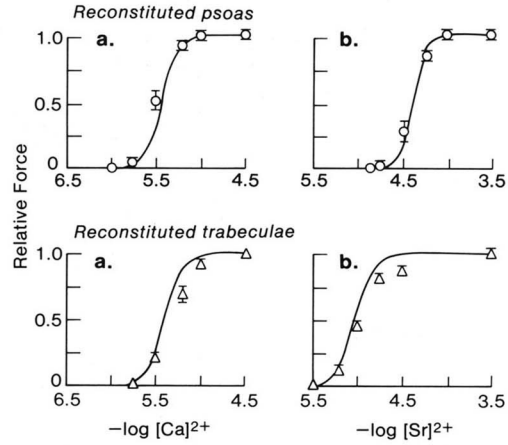

FIG. 5. **Activation characteristics of psoas and cardiac muscle preparations loaded with the homologous TnCs.** The *solid lines* are the same as determined on unextracted (native) preparations in Fig. 2.

Loading the Cardiac Muscle with Foreign TnC—The results in Fig. 4 of maximal Ca^{2+} activations had indicated that the myocardial preparation was able to develop the original tension level even when loaded with skeletal muscle TnC. Similar observations were made with *p*Sr4. To further examine the efficacy of the STnC in the myocardium, we next determined

TABLE I

Relative amounts of the various thick- and thin-filament subunits

A. Hamster psoas single-fiber segments

Fiber treatment[a]	STnC	CTnC	TnI	LC3	LC2	LC1
Native (skinned) (8)	0.26 ± 0.03		0.31 ± 0.01	0.26 ± 0.04	1.24 ± 0.11	1.0
(−) TnC (5)	0.07 ± 0.01		0.30 ± 0.03	0.26 ± 0.05	1.08 ± 0.07	1.0
(+) STnC (4)	0.27 ± 0.04		0.31 ± 0.03	0.31 ± 0.03	0.83 ± 0.06	1.0
(+) CTnC (4)	0.05 ± 0.03	0.27 ± 0.08	0.36 ± 0.01	0.24 ± 0.02	0.97 ± 0.11	1.0

B. Thin trabeculae segments

Treatment	CTnC	LC2	LC1
Native (skinned) (7)	0.32 ± 0.05	0.80 ± 0.07	1.0
(−) TnC (5)	0.07 ± 0.02	0.74 ± 0.05	1.0
(+) CTnC (3)	0.30 ± 0.04	0.81 ± 0.06	1.0

[a] Numbers in parentheses indicate the number of segments analyzed individually. These segments were the same on which tension measurements were made.

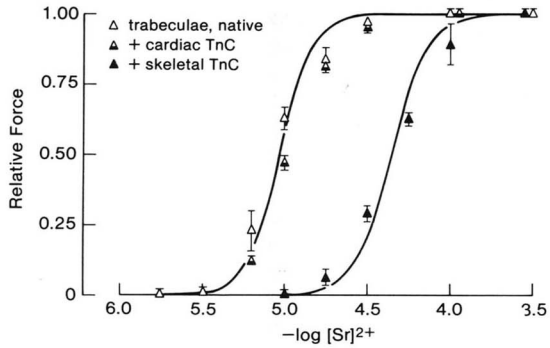

FIG. 6. **Loading of the trabeculae with foreign TnC to determine the role of the particular TnC in the tension control characteristics.** All results are shown at 2.2-μm sarcomere length. Note that results for the trabeculae reconstituted with the cardiac TnC overlap with the native preparation, but when reconstituted with the heterologous skeletal muscle TnC the results are shifted to the right, close to the skeletal muscle response.

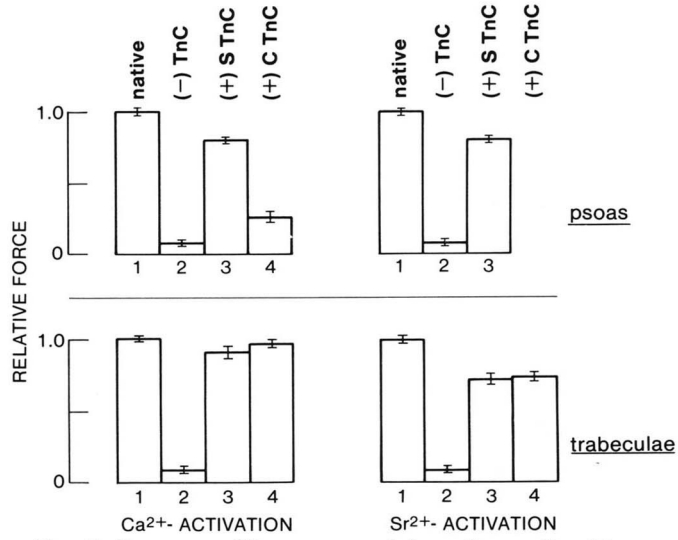

FIG. 7. **Summary.** The *upper panel* shows the results with psoas fibers and the *lower panel* with trabeculae. The *columns 1, 2, 3,* and *4* in the *lower panel* refer to the respective notations on the *top.* The *error bars* are standard error of the mean (all measurements were made on four samples); the *error bar* in *column 1* indicates the variation in tension in different cycles for the same fiber. The results for Sr^{2+} activation of CTnC-loaded psoas fiber were made on two fibers and they were similar to Ca^{2+}. Note that in this case cardiac TnC is less effective than the skeletal TnC. In contrast, tension in the trabeculae was equally well restored with cardiac-type and fast-muscle type TnC.

the entire *p*Sr-tension relationships. The data are given in Fig. 6. They show that the force response of the STnC-loaded myocardium was shifted to the right by 0.7 pSr units (compare *filled triangles* with *half-filled triangles* for CTnC loaded trabeculae in Fig. 6). The shifted pK value (4.3) is similar to that found from the typical skeletal fiber *p*Sr-force response (Fig. 2). From the fact that the skeletal fiber loaded with purified STnC gave normal response (*filled triangles* in Fig. 6), it is very unlikely that the shift in the STnC-loaded trabeculae was due to a modification of STnC on purification.

Studies with Skeletal Fibers Reconstituted with Cardiac Troponin C—This was done next to test the efficacy of CTnC in a converse situation from the STnC myocardium at 180 mM salt. To our initial surprise the skeletal fibers, extracted so that the tension in *p*Ca4 was between 0–10% (10–30-min extraction period; residual TnC 20–30%, Table I), showed only marginal recovery of Ca^{2+} and Sr^{2+}-activated tensions on loading with CTnC. These results are summarized in Fig. 7 (*top left panel*). The same fibers could recover nearly full tension (mean value, 0.8 P_0) with STnC (compare *bars 3* and *4* in the *top left panel* of Fig. 7) indicating that the extraction procedure was not deleterious. Also, the same solution of CTnC used on skeletal fibers was fully effective in restoring the tension responses in the trabeculae, indicating that the limitation of CTnC-loaded skeletal fiber was not due to inactivity of CTnC on purification.

A number of additional experiments were performed to understand the limitation of CTnC in skeletal fibers. The possibility was considered that CTnC did not enter the fiber, but this was ruled out both with gels and with physiological studies. The gel runs in Fig. 8 compare an unextracted (native) psoas fiber segment (1st lane) with STnC-reconstituted fiber (3rd lane) and CTnC-reconstituted fiber (2nd lane). The 2nd lane indicates the loading with CTnC (physiological response of this segment is shown by the force traces in the *lower left* end of Fig. 9). The quantitative data from gel scans on a number of CTnC loaded fibers are summarized in Table IA and show that CTnC was accumulated to the same level as the original level of native STnC. In another experiment (Fig. 10), cardiac TnC-loaded skeletal fiber (*p*Ca4 tension after extraction, ~0.1 P_0; after loading, 0.29 P_0) was incubated with STnC for additional loading (for 90 min), but we found that there was no more effect on the tension level (0.29 P_0) with the second loading. This is additional evidence that CTnC loads into the denuded TnC sites in the fiber. The same doubly loaded fiber was next reextracted and now reloaded directly with STnC, and the tension response was then closer

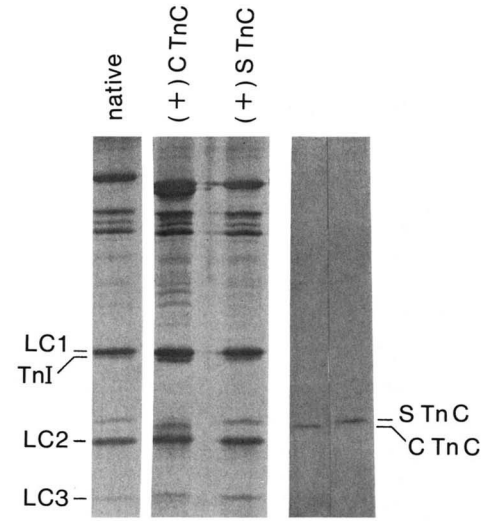

FIG. 8. **15% Silver-stained gel runs on experimental hamster psoas fibers.** The 2nd lane (+*CTnC*) was more heavily loaded than the rest to help distinguish CTnC from residual STnC, but all the data were normalized to the intensity in LC1 band (Table I).

to the original level (*f* in Fig. 10). These results suggest that partial recovery with CTnC at 180 mM ionic strength was due directly to the reduced effectiveness of CTnC in skeletal fibers.

Fig. 9 shows the results of a series of experiments on CTnC-loaded skeletal fibers where the extraction time was varied. The maximally extracted fibers (extraction time, 20–30 min; tension with *p*Ca4 prior to CTnC-loading, 0–0.1 P_0) showed relatively little tension recovery, as above. However, as the extraction time was reduced so that the tension level prior to loading with CTnC was >0.1 P_0, the tension recovery following CTnC loading was progressively enhanced. These results show that the effectiveness of CTnC was increased in moderately extracted fibers which suggests the possibility of some

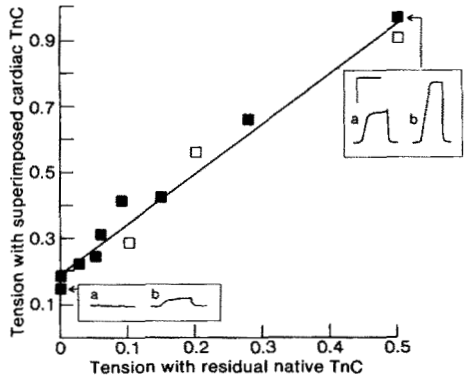

FIG. 9. **Restoring effect of cardiac TnC in skeletal muscle fiber as a function of the tension with residual TnC.** *Closed symbols* are for preparations extracted at 30 °C and *open symbols* are for extractions at 4 °C. Note that cardiac TnC is hardly effective in maximally extracted fiber, but the tension response is close to full with less drastic extraction. *Insets: lower*, maximally extracted fiber; *upper*, very light extraction. *a*, tension response to *p*Ca4 after TnC extraction; *b*, tension response after reconstitution with cardiac TnC. *Scales: horizontal bar*, 10 S; *vertical bar*, 50 kN/m^2.

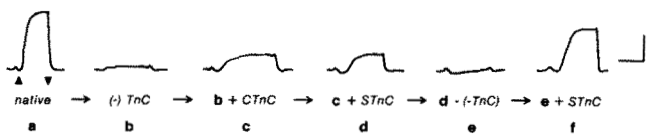

FIG. 10. **Force records (with *p*Ca4 activation) of a doubly reconstituted (STnC on top of CTnC) psoas fiber.** *Trace a* is the force of the native (skinned) fiber, *trace b* after the TnC was maximally extracted, *trace c* after reconstitution with CTnC. The same CTnC-loaded fiber was next incubated in STnC (*trace d*), but there was no further increase in force. The double loaded fiber was next extracted of TnC (*trace e*); when reconstituted with STnC, it gave a tension of 0.75 P_0 (*trace f*). The slightly lower tension in *f* than the mean in Fig. 7 (0.8 P_0 in *bar 3*) was probably the result of extended handling in the present case. Scales as in Fig. 9.

cooperative interaction between the two types of TnCs under these conditions.

DISCUSSION

The successful reconstitution of the TnC-extracted myocardium with both CTnC and STnC indicates that the intrinsic differences amongst these moieties (*e.g.* the additional regulatory site in STnC) do not interfere in activating the contractile proteins in cardiac muscle. On the other hand, our results showing that CTnC is less effective in skeletal muscle fibers at close to physiological ionic strength indicate that the modified site I (of the class of regulatory sites I and II, which trigger activation) is important in this situation. Despite that, however, the uptake of CTnC by the skeletal fiber was normal (Table I). Since the putative nonspecific Ca^{2+}-Mg^{2+} sites III and IV are similar in the two TnCs, our results thus provide additional support for the idea (see Leavis and Gergely, 1984) that these nonspecific sites help largely in maintaining the structural integrity of the troponin complexes in the fiber.

Characteristic Activation Curves: The Role of TnC Subunit—The data in Figs. 2 and 5 indicated that Sr^{2+} sensitivities for the activations of native (skinned) and CTnC reconstituted cardiac muscles are about 5-fold greater than skeletal muscle. Since on loading the trabeculae with STnC, the cardiac muscle now behaved like skeletal fibers (Fig. 6), our results indicate that TnC moiety in the regulatory complex has the key role in setting the activation characteristics and that the origin of the difference in sensitivities between cardiac and skeletal muscle is in the trigger sites I and II. The possibility is raised that site I, which is modified in CTnC, has a major role in positioning the activation curves in addition to placing the limitation on CTnC in the fast-twitch skeletal fiber for maximal activation.

The data on mammalian slow-twitch fibers are also consistent with the present results. The amino acid sequence of TnC in slow-twitch fibers is similar to CTnC rather than STnC (Wilkinson, 1980); in tension response also the native slow fibers similarly show greater sensitivity with Sr^{2+} (and also with Ca^{2+}) than fast fibers (Sr^{2+} by about 1 *p*Sr unit and Ca^{2+} by 0.2 *p*Ca unit, which disparities are actually slightly greater than with cardiac muscle in Fig. 2; unpublished data on hamster psoas and soleus at 2.5-μm sarcomere length and 20 °C[4]; also, Stephenson and Wendt, 1984).

Increased Effectiveness of Cardiac TnC in Moderately Extracted Skeletal Fibers—Although CTnC was largely ineffective in the maximally extracted psoas fibers, the results in Fig. 9 indicated that the effectiveness of cardiac TnC, as judged by Ca^{2+}-activated tension, tended to increase in lightly extracted fibers (1–10-min extraction). One possible explanation was that prolonged extraction (10–30 min) might have caused fiber deterioration, but this appears unlikely since the homologous (skeletal) TnC restored the tension response of similarly extracted fibers close to the native level (Figs. 1 and 7). Also, this effect was found to be the same whether the fiber was extracted at 4 °C or 30 °C, except that the extraction periods were greatly prolonged at the lower temperature.

The results on CTnC loaded fibers could be explained if the changes produced by Ca^{2+} binding to a TnC moiety could be communicated to the adjacent TnC sites, possibly through the 410–423 Å-long tropomyosin molecules. Accordingly, the increased residual STnC in the moderately extracted fibers would exert a greater cumulative influence on the interspersed CTnC, and this cooperative effect might be sufficient to swtich on the entire thin filament. If 50% tension response of the lightly extracted fiber implied that the residual STnC was at a level 50–60% of the original TnC (see Fig. 8 of Moss et al. 1985), and assuming uniform distribution on the thin filament, our results would suggest that each cardiac TnC on the average must be separated by no more than one tropomyosin molecule from the native TnC for the cooperative action to be effective at 180 mM ionic strength. This explanation thus implies that while the various individual segments of the actin filament may be activated in an isolated fashion, ideally, during maximal activation, the entire thin filament is turned on in a concerted manner with communication between the adjacent segments.

If the presence of active cross-bridges affected the properties of TnC this might also explain our results. Moderately extracted fibers on Ca^{2+} activation would have a greater number of bridges as a result of the higher level of residual STnC, and these bridges could in turn exert an increased influence on CTnC improving the apparent effectiveness of CTnC. Gordon and his co-workers (Ridgway and Gordon, 1984) as well as Guth *et al.* (1986) have recently shown that cross-bridges may increase the Ca^{2+} sensitivity of TnC, but this explanation of the present results demands in addition that, in the case of CTnC in moderately extracted skeletal fibers, the more numerous bridges should help achieve a more complete conformational change in TnC during activation. As such, our results would also raise the possibility that the overall (conformational) changes produced in TnC by Ca^{2+} in native cardiac muscle are below those in native skeletal muscle.

Relation to Other Studies on TnC Exchange—Previously there was debate on the molecular origin for the marked

[4] A. Babu and J. Gulati, unpublished data.

increase in sensitivity to Sr^{2+} of the cardiac muscle over skeletal fast-twitch fibers, and the extent of TnC involvement was questioned. Ebashi and Endo (1968) were the first to find the differences in the sensitivities of these tissues. They evaluated the actomyosin ATPase activities by making superprecipitation measurements of the various composites of actomyosin, troponin, and tropomyosin. Both the skeletal and cardiac actomyosin preparations had greater sensitivity with cardiac troponin than with skeletal troponin. This is consistent with our results on skinned fibers. Indeed, since our experiments were done by replacing TnC (instead of whole troponin), the results fix TnC as the main determinant of the characteristic selectivity. In contrast, Kerrick *et al.*, (1980), on repeating the experiments of Ebashi and Endo (1968), by measuring the rates of ATP hydrolysis by skeletal actomyosin, found little difference between the regulation effects of cardiac and skeletal troponin-tropomyosins. More recently they (Kerrick *et al.*, 1985) made studies with skeletal fibers comparing the results with STnC and CTnC and found no difference here too, which is opposite to the results with trabeculae in the present study. On the other hand, Moss *et al.* (1986), also using CTnC-loaded fibers, obtained complicated results, possibly because the residual STnC was quite substantial and the salt concentration too low (Babu *et al.*, 1987), but arrived at a similar conclusion to ours that the properties of TnC determine the activation curves in fibers.[5] The tension recovery in Kerrick *et al.* (1985) was incomplete whether the loading was with STnC or CTnC (maximal tension after loading was about 55%), and this is a major caution in interpreting those results. In the present instance, then, cardiac muscle was a more convenient preparation for the TnC exchange studies.

Comments on the Results from SDS-PAGE of Extracted Fibers—Close inspection of the data in Table I, A and B provides some additional interesting insights. For instance, distribution of the light chains in the native (skinned) fibers indicates LC1:LC2:LC3 of 1.6:2:0.4 in single fibers from hamsters, which is similar to that reported on myosin purified from rabbit muscle (1.35:2:0.65; Sarkar, 1972). Thus the majority of the myosin heads in the psoas fiber (about 75% after taking the difference in molecular weights) are in the LC1:LC2 configuration and the remaining few (less than 25%) of the heads are in the LC3:LC2. On the other hand, the trabeculae results appear to be consistent with an equimolar LC1:LC2 configuration for all heads, which is the expected result since the myocardial tissue has no alkali LC3 moiety.

Further inspection of the data shows that TnC extraction procedure caused no significant loss of LC3 or TnI in both the myocardium and skeletal muscle. In the trabeculae there was less than 10% loss of LC2, which is within the uncertainties of gel measurements.[6] There was a greater tendency for LC2 loss in the psoas muscle (15 to 35% decrease), which might also account for the somewhat lower mean value for the recovery in tension (0.8 P_0) after reconstitution (Fig. 7). Consistent with this, in tests on four fibers we found additional recovery of tension (to 0.91 P_0) with purified LC2 on top of STnC loading; LC2 also increased the tension recovery of a CTnC loaded fiber by the same amount in a separate experiment, but the final tension of this maximally extracted fiber was still far below the STnC loaded fibers.[7]

The finding that the mean values for residual TnC in extracted fibers and trabeculae were still close to 25% of the original level when the Ca^{2+}-activated tension fell nearly to zero (less than 0.1 P_0) may deserve a comment as well. Since at the present sarcomere lengths approximately 25% of the thin filament is under nonoverlap, this could have suggested that the unextracted TnC was restricted to this region. This possibility is opposed by the findings of Yates *et al.* (1986) that the extractions were enhanced at long sarcomere lengths. As another possibility, the 25% residual level of TnC for zero tension may be indicative of a threshold level for thin filament activation to initiate cross-bridge cycling, which incidentally also would be consistent with the action of the thin filament as a cooperative unit.

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[5] In part because the fibers were only lightly extracted of STnC (40–50%) in Moss *et al.* (1986b), near maximal tension level was achieved with CTnC loading. Under these conditions of a mixture of STnC and CTnC, they found that CTnC had a major effect on "n" and not on pK of the activation curve (Hill's coefficient n is known to be lower on native slow fibers than on native fast fibers; Stephenson and Wendt, 1984; also unpublished experiments of A. Babu and J. Gulati). These results may also point to interactions between STnC and CTnC when present as mixtures in fibers.

[6] A tighter binding of LC2 to cardiac myosin than to skeletal myosin is indicated. This could be the result of intrinsic differences within the heavy chains. Alternatively, recent studies indicated effects of LC2 on the exchangeability of the alkali light chains in purified skeletal (Pastra-Landis and Lowey, 1986) and scallop myosins (Ashiba and Szent-Györgyi, 1985), thereby suggesting a definite interaction between the two types of light chains (LC2 and alkali) on their binding to the heavy chains. Thus it is worth considering that LC1, LC3 heterogeneity may have a different effect on LC2 in skeletal muscle than pure LC1 in cardiac muscle.

[7] A. Babu, S. Pemrick, and J. Gulati, unpublished data.

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