Cardiac Troponin T Isoforms Affect the Ca\textsuperscript{2+} Sensitivity of Force Development in the Presence of Slow Skeletal Troponin I

INSIGHTS INTO THE ROLE OF TROPONIN T ISOFORMS IN THE FETAL HEART*

Received for publication, June 30, 2004, and in revised form, August 31, 2004
Published, JBC Papers in Press, September 9, 2004, DOI 10.1074/jbc.M407340200

Aldrin V. Gomes, Gayathri Venkatraman, Jonathan P. Davis, Svetlana B. Tikunova, Patti Engel, R. John Solaro, and James D. Potter

From the \textsuperscript{1}Department of Molecular and Cellular Pharmacology and the \textsuperscript{2}Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33136, the \textsuperscript{3}Department of Physiology and Cell Biology, Ohio State University, Columbus, Ohio 43210, and the \textsuperscript{4}Department of Physiology and Biophysics, University of Illinois College of Medicine, Chicago, Illinois 60612

* This work was supported in part by National Institutes of Health Grants HL42225, HL67415 (to J. D. P.), AR20792 (to J. A. Ralli), HL073600 (to S. B. T.), and HL62426 (to R. J. S.) and by an award from the Muscular Dystrophy Association. Supported by an American Heart Association, Midwest Affiliate, \textsuperscript{4} supported by an award from the Muscular Dystrophy Association. \textsuperscript{4} To whom correspondence should be addressed: Dept. of Molecular and Cellular Pharmacology, University of Miami School of Medicine, 1600 N.W. 10th Ave., Miami, FL 33136. Tel.: 305-243-5874; Fax: 305-324-6024; E-mail: jdpotter@miami.edu.

In this study we investigated the physiological role of the cardiac troponin T (cTnT) isoforms in the presence of human slow skeletal troponin I (ssTnI). ssTnI is the main troponin I isoform in the fetal human heart. In reconstituted fibers containing the cTnT isoforms in the presence of ssTnI, cTnT1-containing fibers showed increased Ca\textsuperscript{2+} sensitivity of force development compared with cTnT3- and cTnT4-containing fibers. The maximal force in reconstituted skinned fibers was significantly greater for the cTnT1 (predominant fetal cTnT isoform) when compared with cTnT3 (adult TnT isoform) in the presence of ssTnI. Troponin (Tn) complexes containing ssTnI and reconstituted with cTnT isoforms all yielded different maximal actomyosin ATPase activities. Tn complexes containing cTnT1 and cTnT4 (both fetal isoforms) had a reduced ability to inhibit actomyosin ATPase activity when compared with cTnT3 (adult isoform) in the presence of ssTnI. The rate at which Ca\textsuperscript{2+} was released from site II of cTnC in the cTnI-cTnC complex (122/s) was 12.5-fold faster than for the ssTnI-cTnC complex (9.8/s). Addition of cTnT3 to the cTnI-cTnC complex resulted in a 3.6-fold decrease in the Ca\textsuperscript{2+} dissociation rate from site II of cTnC. Addition of cTnT3 to the ssTnI-cTnC complex resulted in a 1.9-fold increase in the Ca\textsuperscript{2+} dissociation rate from site II of cTnC. The rate at which Ca\textsuperscript{2+} dissociated from site II of cTnC in Tn complexes also depended on the cTnI isoform present. However, the TnI isoforms had greater effects on the Ca\textsuperscript{2+} dissociation rate of site II than the cTnI isoforms. These results suggest that the different N-terminal TnT isoforms would produce distinct functional properties in the presence of ssTnI when compared with cTnI and that each isoform would have a specific physiological role in cardiac muscle.

During human heart development at least four isoforms of cardiac TnT (cTnT\textsuperscript{1}) are expressed in a developmentally regulated manner (1, 2). The expression level of the main fetal isoform, TnT isoform 1 (cTnT1), decreases and the expression of TnT isoform 3 (cTnT3) increases until cTnT3 becomes the only TnT isoform in the normal adult heart. Because TnT is one of the major regulatory proteins in the thin filament (3), isoform switching of TnT during heart development may be important in the Ca\textsuperscript{2+} regulation of myocardial contraction.

Recently, we have shown that the human cTnT isoforms affect the Ca\textsuperscript{2+} sensitivity of force development and their ability to inhibit actomyosin ATPase activity in the presence of human cardiac troponin I (cTnI) (4). Other groups have also shown that TnT isoforms affect the Ca\textsuperscript{2+} sensitivity of force development and ATPase activity (5–9). These results all suggest that the N terminus of TnT affects the physiological function of troponin (Tn). However, TnI isoforms also switch during the period of TnT isoform changes. Slow skeletal TnI (ssTnI) is the predominant TnI isoform throughout fetal life and gradually decreases during the first few months of postnatal development (10). Developmental down-regulation of ssTnI occurs even in the absence of cTnI. Mice lacking cTnT are born healthy, with normal heart and body weight, because ssTnI (the fetal troponin I isoform) compensates for the absence of cTnT until 15 days after birth when ssTnI expression declines steadily, resulting in TnI deficiency and death on day 18 (11). The physiological relevance of cTnT isoforms in the human heart is still not well understood, and the physiological relevance of cTnT isoforms in fetal human cardiac Tn has not been investigated previously.

Two recent reports (12, 13) suggest that the developmental changes in the Ca\textsuperscript{2+} sensitivity of force development originate primarily from the switch of ssTnI to cTnI. Incorporation of embryonic/fetal isoforms of Tm, TnT, and TnI into the adult sarcomere by using gene transfer suggested that TnI, but not Tm or TnT embryonic isoforms, influenced calcium regulation of contraction (13). Another report (12) correlated the switching of TnT and TnI isoforms with the Ca\textsuperscript{2+} sensitivity of force generation in mice and, based on these results, suggested that the change in Ca\textsuperscript{2+} sensitivity is mainly due to TnI isoform switching. The main goal of the present study was to gain insight into the effect of the N-terminal hypervariable region in cTnT in the presence of ssTnI (fetal TnI) on cardiac muscle.

The abbreviations used are: cTnT, cardiac troponin T; MOPS, 3-(N-morpholino)propanesulfonic acid; cTnC, cardiac troponin C; cTnI, cardiac troponin I; Tm, tropomyosin; Tn, troponin; ssTnI, slow skeletal troponin I; MHC, myosin heavy chain; DTT, dithiothreitol; fsTnT, fast skeletal TnT.
contraction to determine whether the cTnT isoforms would still have a functional role in the presence of ssTnI.

Three cTnT isoforms (Fig. 1) were investigated using several in vitro functional assays to determine whether any differences exist between the adult isoform (cTnT3) and the other isoforms of cTnT. In reconstituted fibers containing the cTnT isoforms in the presence of ssTnI, cTnT1 containing fibers showed increased Ca\(^{2+}\) sensitivity of force development (ΔpCa\(_{50}\) = +0.17) and maximal force compared with cTnT4 containing fibers. These results suggest that cTnT isoforms are able to modulate Ca\(^{2+}\) sensitivity even in the presence of ssTnI. The ability of Tn complexes to inhibit ATPase activity was significantly lower for all cTnT isoforms in the presence of ssTnI than in the presence of cTnI. The rate at which Ca\(^{2+}\) is released from site II of cTnC in Tn complexes was affected by both the cTnT and ssTnI isoforms present. cTnT had a greater effect on the kinetics of Ca\(^{2+}\) release from site II of cTnC in the presence of cTnT than in the presence of ssTnI. The N-terminal alternatively spliced region of cTnT also affected the solubility of cTnT. Our results suggest that this alternatively spliced N-terminal region of cTnT is an important modulator of the Ca\(^{2+}\) sensitivity of force development in both fetal and adult heart muscle and also affects the maximal force and the maximal and the minimal actomyosin ATPase activity. The results also suggest that the isoform of TnI present is important for some of the functional differences between the cTnT isoforms.

**MATERIALS AND METHODS**

**Expression and Purification of cTnT Isoforms**—Human cTnT isoforms were made by using a sequential overlapping PCR method as described previously (14). Standard methods previously used in this laboratory were utilized for expression and purification of the different cTnT isoforms (4). Briefly, bacterially expressed and extracted cTnTs were purified on a Fast Flow S-Sepharose column. cTnT was eluted from this column with a NaCl gradient (0–0.5 M). The semi-purified cTnT was then further purified on a cTnC affinity column. cTnT was eluted from this column with a double gradient of urea and EDTA (0–6 M and 0–3 mM, respectively). All steps were performed at 4 °C unless otherwise indicated. The purity of the isolated cTnT isoforms (>95%) was determined by SDS-PAGE.

**Expression and Purification of ssTnI**—ssTnI cDNA was cloned from a human library using standard techniques and verified by sequencing. Similar methods previously used in this laboratory for purification of cTnI were utilized for expression and purification of the ssTnI (4, 15). Briefly, bacterially expressed and extracted ssTnI was purified on a Fast Flow S-Sepharose column. ssTnI was eluted from this column with a NaCl gradient (0–0.5 M). The semi-purified ssTnI was then further purified on a cTnC affinity column. ssTnI was eluted from this column with a double gradient of urea and EDTA (0–6 M and 0–3 mM, respectively).

**Formation of the Tropinin Complex**—Formation of the human cardiac Tn complexes containing human cTnC, cTnT, and TnI was carried by a modified method of the one recently described by Szczesna et al. (15). Proteins were first diazylated against a solution containing 3 mM urea and 1 mM KCl and then in solution containing 0 mM urea and 1 mM KCl. Complexes were formed in the presence of 0 mM urea and 1 mM KCl and then diazylated against successively lower concentrations of KCl as described previously (15). Proper stoichiometry was verified by SDS-PAGE. Although not done routinely, gel filtration of these formed Tn complexes showed that this reconstitution method resulted in a single species.

**Actin-Tn-activated Myosin-ATPase Assay**—Porcine cardiac myosin, rabbit skeletal F-actin, porcine cardiac Tn, and recombinant human TnC were prepared as described previously (11, 12). The ATPase inhibitory assay was performed in a 1-mL reaction mixture of 100 mM KCl, 4 mM MgCl\(_2\), 10 mM EGTA, 2.5 mM ATP (Mg\(^{2+}\)-ATP), 0.1 mM DTT, 10 mM MOPS, pH 7. The ATPase activation assay was carried out in the same 1-mL reaction mixture with 1 mM EGTA replaced with 0.5 mM CaCl\(_2\), F-actin (3.5 μM), myosin (0.6 μM), and Tn (1 μM) were homogenized and added to the reaction tube after the addition of buffer and Tn to the assay tube. Different Tn complexes were utilized including the following: complexes containing adult cTnT3 and cTnI (Tn3cIC), Tn containing cTnT1 and cTnI (Tn1cIC), Tn containing cTnT4 and cTnI (Tn4cIC), Tn containing cTnT1 and ssTnI (Tn1ssIC), Tn containing cTnT3 and ssTnI (Tn3ssIC), and Tn containing cTnT4 and ssTnI (Tn4ssIC). All Tn complexes contained cTnC. The ATPase reaction was initiated with the addition of ATP and stopped after 20 min with trichloroacetic acid (4% final concentration). After sedimenting the precipitate, the inorganic phosphate concentration in the supernatant was determined according to the method of Fiske and SubbaRow (16). The ATPase rates were measured at 30 °C by single time points that were predetermined to be linear with time.

**Dependence of the Ca\(^{2+}\) Dissociation Rates at 22 °C**

**Dependence of the Ca\(^{2+}\) Dissociation Rates at 22 °C**

**Contraction of the skinned fibers**—The Ca\(^{2+}\) concentration was determined by measuring the fluorescence of Quin-2 in the fibres, which was excited using a 150-W xenon arc lamp. The Tn complexes containing cTnT and cTnI were reconstituted in a solution of 10 mM Tris, 10 mM MgCl\(_2\), 0.1 mM DTT, 10 mM MOPS, pH 7. The ATPase rates were measured at 30 °C by single time points that were predetermined to be linear with time.

**Determination of the Ca\(^{2+}\) Dissociation Rates—Ca\(^{2+}\) dissociation rates (k\(_{off}\)) were measured using an Applied Photophysics Ltd. (Leatherhead, UK) model SX.18 MV stopped-flow instrument with a dead time of 1.4 ms at 15 °C. The Tn complexes were excited using a 150-W xenon arc source. k\(_{off}\) was determined using the fluorescent Ca\(^{2+}\)-chelator Quin-2 (19, 20). Quin-2 was excited at 330 nm with its emission monitored through a 510-nm broad bandpass interference filter (Oriel Stratford, CT). Each k\(_{off}\) represents the mean of at least 21 traces fit with a single exponential or a double exponential (variance <10 × 10\(^{-4}\)) over multiple time windows leading to similar results to account for the fact that Quin-2 reports the rates of Ca\(^{2+}\) dissociation from both the N- (site II) and C-domain (sites III and IV) of cTnC in the Tn-cTnC complexes. The buffer used in all stopped-flow experiments was 10 mM MOPS, 100 mM KCl, 1 mM MgCl\(_2\), and 1 mM DTT at pH 7. Each Mg\(^{2+}\) complexes in the presence of 30 μM Quin-2 at 22 °C. Quin-2 fluorescence as it binds Ca\(^{2+}\) that dissociates from cTnC in isolation and the various complexes were converted to moles of Ca\(^{2+}\) dissociating from cTnC by mixing increasing concentrations of Ca\(^{2+}\) (0, 15, and 30 μM) with Quin-2 (21). Quin-2 fluorescence increased linearly as a function of increasing [Ca\(^{2+}\)], allowing for a conversion of the change in Quin-2 fluorescence to the number of moles of Ca\(^{2+}\) dissociating per mol of cTnC. Calibration curves were performed at the end of

**Fetal Troponin Isoforms**
an experiment using the same Quin-2 solutions and experimental conditions as used in the experiments. The amplitude of the change in Quin-2 fluorescence was extrapolated from the exponential fits of the data.

No additional Ca$^{2+}$ was added to the samples because there was enough contaminating Ca$^{2+}$ in the buffers to nearly saturate the complexes. Addition of 15 or 30 μM Ca$^{2+}$ to the various TnT-cTnC and Tn complexes prior to Quin-2 Ca$^{2+}$ dissociation led to similar cTnC N-domain Ca$^{2+}$ dissociation rates and stoichiometries. However, the addition of extra Ca$^{2+}$ to the reactions abolished the ability of Quin-2 to remove Ca$^{2+}$ from the C-terminal sites of cTnC in the various complexes.

Solubility of cTnT Isoforms—cTnT1, cTnT3, or cTnT4 (1 ml each) at various concentrations (0.4, 0.25, and 0.1 mg/ml) were dialyzed against 50 mM Tris-HCl, pH 7.0, containing 1 mM DTT and 1 M KCl. The cTnT isoforms were then successively dialyzed against the same buffer containing lower concentrations of KCl (0.7, 0.5, 0.4, 0.3, 0.2, and then 0.1 M KCl). After dialysis in buffer containing 0.1 M KCl, the cTnT isoforms were centrifuged at 15,000 rpm for 25 min at 4 °C, and the supernatant was collected. The total volume of the cTnT isoforms obtained after dialysis as well as the concentration of the cTnT isoforms in the supernatant after centrifugation were determined. The protein concentration was determined with the Coomassie Plus Bradford assay kit (Pierce) using bovine serum albumin as standard.

**Data Analysis**—Values are presented as mean ± S.D. The statistical significance of the differences between the mean values was analyzed by the Student's *t* test.

**RESULTS**

**Actomyosin ATPase Studies on Reconstituted Troponin Complexes Containing Different Troponin T and Troponin I Isoforms**—The maximal ATPase activity for Tn complexes containing the different cTnT and cTnI isoforms in the presence of Ca$^{2+}$ was similar for all the isoforms in the presence of cTnT but different in the presence of ssTnI (Fig. 2A). The myosin ATPase activity in the absence of Tn was considered to be 100% activity (0% inhibition). The ATPase activity results are presented as a bar chart so that the different cTnT and Tn combinations can be directly compared. In all experiments the amount of Tn required for maximal ATPase activation was 1 μM, which is consistent with a ratio of Tn:Tm of 1:1. The Tn complex containing cTnT3 and cTnI (cTnT3cIC) showed slightly lower (but not statistically significant) maximal ATPase activity than the other Tn complexes (cTnT1cIC or cTnT4cIC). Similar to what has been reported previously (4) (Fig. 2A). However, a Tn complex containing cTnT1 and ssTnI (cTnT1ssIC) showed a significantly higher maximal ATPase activity (175 ± 5.8%) than both cTnT3cIC (157 ± 3.9%) and cTnT4cIC (144 ± 4.5%). The maximal activity for cTnT4ssIC was also significantly lower than the maximal activity of cTnT4cIC (Fig. 2A).

The ability of the cTnT isoforms to inhibit actin-Tm-activated myosin ATPase activity in reconstituted thin filaments was also examined to determine whether the inhibitory activity of the Tn complexes containing ssTnI was affected by the deletions in the N-terminal region of cTnT. Although cTnT3cIC and cTnT4cIC were able to inhibit ATPase activity nearly fully in the presence of EGTA (-92% at 2 μM cTnT3cIC concentration), cTnT1cIC was less effective at inhibiting actomyosin ATPase activity (-80% at 2 μM Tn concentration), similar to what has been reported previously (Fig. 2B)(4). However, in the presence of ssTnI, cTnT4ssIC (47 ± 3% ATPase inhibition) was unable to inhibit the actin-Tm-activated myosin ATPase activity as well as cTnT3ssIC (66 ± 1%). Complexes containing cTnT4 were all better at inhibiting ATPase activity than complexes containing ssTnI (Fig. 2B). cTnT4 had similar inhibitory properties to cTnT1 in the presence of ssTnI.

Actomyosin ATPase assays were also carried out with equal amounts (50:50%) of different Tn complexes (Fig. 3A, A and B). All assays in the presence of Ca$^{2+}$ were carried out with a total Tn concentration of 1 μM. None of the cTnT isoforms showed a significant dominance over the other isoforms in the presence of ssTnI in affecting the maximal actomyosin ATPase activity (Fig. 3A). In the absence of Ca$^{2+}, 50:50%$ ratio of different Tn complexes (Fig. 3B) showed that cTnT1 was dominant over ssTnI for its ability to inhibit ATPase activity. 50:50% ratios of TnT1ssIC:TnT3ssIC showed a small preference for TnT1 over ssTnI (Fig. 3B). However, a Tn complex containing cTnT1ssIC showed significantly lower (but not statistically significant) ATPase activity than the other Tn complexes (cTnT3ssIC or cTnT4ssIC). Similar to what has been reported previously (4) (Fig. 3B). However, a Tn complex containing cTnT1 and ssTnI (cTnT1ssIC) showed a significantly higher maximal ATPase activity (175 ± 5.8%) than both cTnT3ssIC (157 ± 3.9%) and cTnT4ssIC (144 ± 4.5%). The maximal activity for cTnT4ssIC was also significantly lower than the maximal activity of cTnT4cIC (Fig. 2A).

The ability of the cTnT isoforms to inhibit actin-Tm-activated myosin ATPase activity in reconstituted thin filaments was also examined to determine whether the inhibitory activity of the Tn complexes containing ssTnI was affected by the deletions in the N-terminal region of cTnT. Although cTnT3cIC and cTnT4cIC were able to inhibit ATPase activity nearly fully in the presence of EGTA (-92% at 2 μM cTnT3cIC concentration), cTnT1cIC was less effective at inhibiting actomyosin ATPase activity (-80% at 2 μM Tn concentration), similar to what has been reported previously (Fig. 2B)(4). However, in the presence of ssTnI, cTnT4ssIC (47 ± 3% ATPase inhibition) was unable to inhibit the actin-Tm-activated myosin ATPase activity as well as cTnT3ssIC (66 ± 1%). Complexes containing cTnT4 were all better at inhibiting ATPase activity than complexes containing ssTnI (Fig. 2B). cTnT4 had similar inhibitory properties to cTnT1 in the presence of ssTnI.

Actomyosin ATPase assays were also carried out with equal amounts (50:50%) of different Tn complexes (Fig. 3A, A and B). All assays in the presence of Ca$^{2+}$ were carried out with a total Tn concentration of 1 μM. None of the cTnT isoforms showed a significant dominance over the other isoforms in the presence of ssTnI in affecting the maximal actomyosin ATPase activity.
FIG. 3. Effect of the cTnT isoform mixtures on the activation (+Ca²⁺) or inhibition (−Ca²⁺) of the actin-Tm-activated myosin-ATPase activity. Each data point represents the average of 4–6 separate experiments each performed in triplicate and is expressed as mean ± S.D. cTnT1; cTnT1ssIC; cTnT2; cTnT2ssIC; cTnT3; cTnT3ssIC; cTnT4; cTnT4ssIC; cTnT1; cTnT2; cTnT3; cTnT4; cTnT1ssIC; cTnT2ssIC; cTnT3ssIC; cTnT4ssIC. Assays were carried out with a total Tn concentration of 0.8 mg/ml, and the human Tn complexes reconstituted into skinned muscle fibers as reported previously (4). Table I summarizes the cTnT isoforms investigated were able to restore the Ca²⁺ dependence of force development. The Ca²⁺ dependence of force development was also measured before treating each fiber with cTnT. The level of Ca²⁺-unregulated force following the cTnT treatment was equal to ~65% of the force developed by the untreated skinned fiber preparations.

As shown in Fig. 4, a significant change in the Ca²⁺ dependence of force was observed between the fibers displaced with the different cTnT isoforms after reconstitution with the ssTnI-cTnC complex. Fig. 4A shows the increased Ca²⁺ sensitivity of force development (ΔpCa₅₀ = +0.18, p < 0.01) for cTnT3-treated fibers reconstituted with ssTnI when compared with cTnI-reconstituted fibers. Fig. 4B shows the Ca²⁺ sensitivity of force development for cTnT1- and cTnT3-treated fibers. When reconstitution of the fibers was carried out in the presence of ssTnI-cTnC, cTnT1-treated fibers had an increased Ca²⁺ sensitivity (ΔpCa₅₀ = +0.11, p < 0.05) when compared with cTnT3. cTnT3-treated fibers reconstituted with ssTnI-cTnC showed a significant decrease in Ca²⁺ sensitivity (ΔpCa₅₀ = −0.17) when compared with cTnT1 (Fig. 4C).

The maximum Ca²⁺-dependent force recovery is equivalent to the level of force developed in fibers after reconstituting the fibers with the appropriate Tn complex and treating the fiber with pCa 4 solution (relative to the original force of the fibers before displacement). The maximal force developed in skinned fibers reconstituted with the three cTnT isoforms and the ssTnI-cTnC complex was dependent on both the TnI and the cTnT isoform (Fig. 5). cTnT1-reconstituted skinned fibers showed the greatest increase in maximal force (91.2 ± 2.9%, which corresponds to a 16% increase when compared with cTnT3 reconstituted fibers). cTnT3-reconstituted fibers showed an increase in the maximal force in the presence of ssTnI (75.7 ± 2.5%) when compared with cTnT3-reconstituted fibers in the presence of cTnI (66.5 ± 3%). These results demonstrate that exons 4 and 5 of cTnT are important for modulating the maximal force in the presence of ssTnI. This increase in the maximal force observed for cTnT1 and cTnT3 in the presence of ssTnI was not seen when fibers were reconstituted with the cTnT4 isoform (Fig. 5).

Ca²⁺ Dissociation Rates from cTnC in the TnI-cTnC Complex—To ultimately determine the effect of cTnT in the kinetics of Ca²⁺ release from cTnC in the TnI-cTnC complex, cTnI-cTnC and ssTnI-cTnC complexes were investigated by using the fluorescent Ca²⁺ chelator Quin-2 in a stopped-flow apparatus (Fig. 6). Quin-2 reports the Ca²⁺ dissociation kinetics from both the N and C terminus of cTnC in the complexes. The rates from the N-terminal cTnC Ca²⁺ binding site II and C-terminal sites were separated enough in time so that there was minimal interference between them. However, the C-terminal rates were much slower and harder to measure accurately because Quin-2 is unable to remove all the Ca²⁺ from these extremely high affinity sites. In the absence of cTnI the rate of Ca²⁺ dissociation from the regulatory site of cTnC was greater than 1000/s (22), whereas Ca²⁺ dissociated from the C-terminal sites at 1.03 ± 0.08/s with a stoichiometry of 1.8 ± 0.1 mol of Ca²⁺ per mol of protein (data not shown). Thus, the rate of Ca²⁺ dissociation from the regulatory site of cTnC was slowed by cTnI or ssTnI by greater than 8.2- or 102-fold, respectively, with an average stoichiometry of 0.8 ± 0.1 mol of Ca²⁺ per mol of protein. The cTnI-cTnC complex had a 12.5-fold faster off-rate for Ca²⁺ at site II of cTnC than the ssTnI-cTnC complex (Fig. 6). The rates at which Ca²⁺ dissociated from sites III and IV of cTnC for both complexes were similar (cTnI-cTnC, 0.04 ± 0.01/s and ssTnI-cTnC, 0.036 ± 0.009/s) with an average stoichiometry of 0.8 ± 0.3 mol of Ca²⁺ per mol of protein.

Ca²⁺ Dissociation Rates from cTnC in the Tn Complex—Stopped flow fluorometric studies of Ca²⁺ removal from the Ca²⁺-Mg²⁺ and the Ca²⁺-specific regulatory site of cTnC in the
Tn complex were also conducted using Quin-2 (Fig. 7). Addition of cTnT isoforms to cTnI-cTnC resulted in a 3–4-fold decrease in Ca\(^{2+}\) dissociation rate from site II of cTnC. Addition of cTnT isoforms to ssTnI-cTnC resulted in an \(\sim 1.1–1.9\)-fold increase in Ca\(^{2+}\) dissociation rate from site II of cTnC. Most interestingly, the fetal isoform cTnT4 did not significantly affect the rate at which Ca\(^{2+}\) was released from site II of cTnC in the ssTnI-cTnC complex (Figs. 6B and 7B). The results also suggest that cTnT stabilizes the C-terminal Ca\(^{2+}\) off rates for sites III and IV of cTnC by nearly an order of magnitude above what cTnI alone does. The functional significance of this is unclear as the Ca\(^{2+}\) exchange and the structural changes involved in Ca\(^{2+}\) exchange at the Ca\(^{2+}\)/Mg\(^{2+}\) sites are too slow to be involved directly in the regulation of muscle contraction (23). The rates from the N-terminal cTnC Ca\(^{2+}\)-binding site II and the C-terminal sites were separated enough in time so that there was minimal interference between them. The average stoichiometry of Ca\(^{2+}\) dissociation from the N- and C-domains of cTnC in the Tn complexes was 0.61 ± 0.07 and 0.4 ± 0.2 mol of Ca\(^{2+}\) per mol of protein, respectively. Thus, Quin-2 is unable to remove all the Ca\(^{2+}\) from the high affinity C-domain sites making the stoichiometry for these sites less than the expected 2 mol of Ca\(^{2+}\) per mol of protein. This, however, is not surprising because the C-domain sites of cTnC in the Tn complex possess \(\sim 10\)-fold higher Ca\(^{2+}\) affinity than does Quin-2 (24) (see also Molecular Probes web site: www.probes.com). Inner filter effects preclude the possibility of utilizing a larger concentration of Quin-2. In these functional studies, Tn complexes containing ssTnI slowed the rate at which Ca\(^{2+}\) was released from site II of cTnC when compared with Tn complexes containing cTnI. These results are consistent with mice expressing ssTnI showing a decreased relaxation rate (25). Ca\(^{2+}\) is removed from the Ca\(^{2+}\)-specific sites producing an increase in Ca\(^{2+}\)-Quin-2 fluorescence at rates of 33–42/s for Tn complexes containing cTnI and rates of 11–19/s for Tn complexes containing ssTnI (Fig. 7A). Tn containing cTnI and ssTnI showed an \(\sim 1.2\)-fold increase in the Ca\(^{2+}\) dissociation rate from site II of cTnC when compared with Tn containing cTnT1 or cTnT3 (Fig. 7A). Tn containing cTnI and cTnT4 showed a similar Ca\(^{2+}\) dissociation rate from site II of cTnC to that of complexes containing cTnI and cTnT3 (Fig. 7A). In the presence of ssTnI, Tn containing cTnT1 showed an \(\sim 1.7\)-fold increase in the Ca\(^{2+}\) dissociation rate from site II of cTnC when compared with Tn containing cTnT4 (Fig. 7B). Tn containing ssTnI and cTnT1 showed a slightly slower but not significant change in the Ca\(^{2+}\) dissociation rate from site II of cTnC when compared with Tn containing cTnT3 (Fig. 7B).

As was expected (but have not yet previously shown), the presence of ssTnI in Tn complexes caused a significant decrease in the rate of Ca\(^{2+}\) removal from the Ca\(^{2+}\)-specific site II on cTnC (when compared with Tn containing cTnI), consistent with a higher affinity of the Tn complex for Ca\(^{2+}\) in the presence of ssTnI. In the presence of ssTnI the rate at which Ca\(^{2+}\) was removed from the Ca\(^{2+}\)-specific site was 2–3-fold slower than the rate for the same complexes in the presence of cTnI.

The cTnT isoforms did not affect the rates of Ca\(^{2+}\) dissociation from sites III and IV in the C terminus of cTnC (cTnT1-cTnI-cTnC, 0.0053 ± 0.0005/s; cTnT3-cTnI-cTnC, 0.005 ± 0.001/s; cTnT4-cTnI-cTnC, 0.0049 ± 0.0003/s; cTnT1-ssTnI-cTnC, 0.011 ± 0.006/s; cTnT3-ssTnI-cTnC, 0.012 ± 0.009; and cTnT4-ssTnI-cTnC, 0.007 ± 0.001). Although the average C-terminal rates for the Tn complex containing ssTnI appear to be faster than the Tn complexes containing cTnI, the large error for these values makes the results not statistically significant.

Solubility of cTnT Isoforms—To determine whether the cTnT isoforms have different solubilities, different concentrations of each cTnT isoform were dialedyzed against successively lower concentrations of KCl, and the solubility of each isoform was determined (Fig. 8). All cTnT isoforms showed low solubility under low ionic conditions. cTnT4 demonstrated the lowest solubility, whereas cTnT1 showed the greatest solubility of the three cTnT isoforms investigated.

**DISCUSSION**

cTnT1 is the predominant cTnT isoform expressed in the fetal heart, with cTnT4 being also expressed in the fetal heart at lower levels and is re-expressed in the failing adult heart, whereas cTnT3 is the dominant isoform in the adult heart (1, 2, 26). Our previous report showed that cTnT isoforms modulated the Ca\(^{2+}\) sensitivity of force development and the ability of Tn to inhibit actomyosin ATPase activity (4). Other laboratories (5, 6, 9, 27, 28) have also shown a correlation between TnT isoforms and Ca\(^{2+}\) sensitivity of force development. Significant advances in the understanding of the functional significance of the developmental transition from the ssTnI to cTnI isoform expression in cardiac myocytes have been obtained over the last 5 years. The significance of TnT isoforms in the fetal heart is not well understood but has been suggested to be minor by two recent studies (29, 30). In mice, the major switches between TnT isoforms occur before embryonic day 17, whereas the switching of ssTnI to cTnI and of β-myosin heavy chain (MHC) to α-MHC both occurs around birth. The change in Ca\(^{2+}\) sensitivity of force generation in mice starts at embryonic day 13.5. However, the main decrease in Ca\(^{2+}\) sensitivity of force generation occurs within a short time interval, between embryonic day 19.5 and 7 days after birth correlating with the change switching of ssTnI and MHC.”

**Fetal Troponin Isoforms**

**Table 1**

| cTnT Isoform utilized in the fibers | Tn Isorm | pCa\(_{50}\) | Hill coefficient | Relative force | Number of experiments |
|-----------------------------------|----------|-------------|-----------------|---------------|----------------------|
| cTnT1                             | cTnI     | 5.56 ± 0.03*| 1.71 ± 0.09    | 72.2 ± 2.6    | 4                    |
| cTnT3                             | cTnI     | 5.44 ± 0.02 | 1.90 ± 0.09    | 66.5 ± 3.0    | 4                    |
| cTnT4                             | cTnI     | 5.39 ± 0.02 | 2.00 ± 0.12*   | 67.1 ± 2.7    | 3                    |
| ssTnI                             | ssTnI    | 5.73 ± 0.03*| 1.44 ± 0.14    | 91.2 ± 2.9    | 4                    |
| cTnT3                             | ssTnI    | 5.62 ± 0.02 | 1.69 ± 0.11    | 75.7 ± 2.5    | 4                    |
| cTnT4                             | ssTnI    | 5.56 ± 0.02 | 1.56 ± 0.08    | 65.9 ± 3.0    | 3                    |

*Indicates that the pCa\(_{50}\) values for the cTnT1 isoform is significantly different from cTnT3 and cTnT4 in the presence of cTnI (\(p < 0.05\)).

**a** Indicates that the pCa\(_{50}\) values for the cTnT1 isoform is significantly different from cTnT3 and cTnT4 in the presence of ssTnI (\(p < 0.05\)).

**b** Indicates that the Hill coefficient values for the cTnT1 isoform is significantly different from cTnT3 and cTnT4 in the presence of ssTnI (\(p < 0.05\)).

**c** Indicates that the relative force values for the cTnT1 isoform is significantly different from cTnT3 and cTnT4 in the presence of ssTnI (\(p < 0.05\)).

The pCa\(_{50}\) and Hill coefficient values were the average of three to four independent fiber experiments, and the errors are the standard deviation (S.D.) values.
The developmental transition of TnI and cTnT isoforms makes it likely that cTnT isoforms, including the adult isoform, are present together with ssTnI in the absence of cTnI during some part of development in the fetal heart.

To determine the functional role of the cTnT isoforms in fetal Tn, three cTnT isoforms (cTnT1, -3, and -4) that are expressed at the protein level in human heart were investigated. This is the first report of cTnT isoforms being used in the presence of ssTnI in reconstituted systems or skinned fiber studies. Our investigations show that removal of exon 5 (10 amino acids) decreases the Ca\(^{2+}\)/H11001 sensitivity of force development in both cTnT3 (\(p_{Ca50} = 0.11\)) and cTnT4 (\(p_{Ca50} = 0.17\)) relative to cTnT1 (Fig. 3 and Table I) in the presence of ssTnI. These results suggest that cTnT isoforms are important in modulating Ca\(^{2+}\)/H11001 sensitivity in the presence of ssTnI (fetal TnI). Irrespective of the cTnT isoform utilized, ssTnI-reconstituted fibers showed a greater Ca\(^{2+}\)/H11001 sensitivity of force development (\(p_{Ca50} = 0.17–0.18\)) than cTnI-reconstituted fibers. ssTnI has been shown previously (29, 31, 32) by several laboratories to increase the Ca\(^{2+}\)/H11001 sensitivity of force development. The net result of reconstituting fibers with both fetal cTnT (cTnT1) and ssTnI is an increase in the Ca\(^{2+}\)/H11001 sensitivity of force development (\(p_{Ca50} = 0.29\)) that is equal to the sum of the effect from the cTnT1 (\(p_{Ca50} = 0.12\)) and that of ssTnI (\(p_{Ca50} = 0.17\)), when compared with adult Tn (TnT3cIC).

Removal of either exon 4 and/or exon 5 of cTnT resulted in a decrease in the maximal force recovered in skinned fibers in the presence of ssTnI (Fig. 5). These significant changes in maximal force that were observed for cTnT isoforms in the presence of ssTnI suggest that the relationship between ssTnI and cTnT isoforms is different from the relationship between the cTnT and cTnT isoforms. The increase in maximal force observed for cTnT3 (adult TnT) and ssTnI is an increase in the Ca\(^{2+}\)/H11001 sensitivity of force development (\(\Delta p_{Ca50} = +0.29\)) that is equal to the sum of the effect from the cTnT1 (\(\Delta p_{Ca50} = +0.12\)) and that of ssTnI (\(\Delta p_{Ca50} = +0.17\)), when compared with adult Tn (TnT3cIC).

Removal of either exon 4 and/or exon 5 of cTnT resulted in a decrease in the maximal force recovered in skinned fibers in the presence of ssTnI (Fig. 5). These significant changes in maximal force that were observed for cTnT isoforms in the presence of ssTnI suggest that the relationship between ssTnI and cTnT isoforms is different from the relationship between the cTnT and cTnT isoforms. The increase in maximal force observed for cTnT3 (adult TnT) in the presence of ssTnI (9%) relative to cTnT3 in the presence of cTnI was unexpected because ssTnI has been investigated previously by several groups. However, to our knowledge, this is the first investigation of the adult human cTnT isoform with ssTnI in skinned fiber studies.

Because skinned fibers containing cTnT4 gave similar maximal force in the presence of either ssTnI or cTnI, the increased maximal force is unlikely to be due to ssTnI alone but due to...
some complex interplay between the cTnT isoforms, the TnI isoforms, and the rest of the thin filament proteins. Our results also suggest that the N-terminal alternatively spliced region of cTnT affects the cooperativity of force development ($n_{1/2}$) in the presence of cTnI as shown previously (4). Removal of both exons 4 and 5 from cTnT increased the cooperativity of force development (Table I). In the presence of ssTnI, the cooperativities of force development for the cTnT isoforms were not significantly different from each other. However, the presence of ssTnI instead of cTnI resulted in a decrease in the cooperativity of force development that was statistically significant ($p < 0.05$) when cTnT4 was utilized (Table I). Although the molecular mechanism of cooperativity is still not clearly understood, the Ca$^{2+}$ dependence of muscle contraction is known to be highly cooperative with Hill coefficients ranging from 1 to 4 depending on the system used (33–35). Biochemical studies on fast skeletal TnT (fsTnT) peptides suggest that part of its N-terminal region (residues 70–159, corresponding to residues 102–189 of cTnT3) promotes cooperative interactions between functional units (36, 37). Differences in the N-terminal region may affect the binding of Tm to cTnT resulting in subtle changes in the cooperative binding seen for the different cTnT isoforms.

In the presence of cTnI, we were unable to find any significant difference between cTnT3 and cTnT4 with respect to their ability to activate (+Ca$^{2+}$) or inhibit (−Ca$^{2+}$) actomyosin ATPase activity (Table I). However, in the presence of ssTnI, cTnT3 and cTnT4 showed different abilities to activate and inhibit ATPase activity. Tn containing cTnT4 and ssTnI was unable to activate ATPase activity better than Tn complexes containing ssTnI, and this may be due to differences between the TnI isoforms in their ability to interact with actin. This may be due to a lower affinity of ssTnI binding to actin than cTnI binding to actin. Using a 50:50% ratio of Tn complexes in actomyosin ATPase assays showed that in the absence of Ca$^{2+}$, cTnI was
and represents the mean

cTnT4 was significantly less soluble than both the total amount of cTnT isoform present in the supernatant (after centrifugation) and the concentration of the cTnT isoforms in the supernatant after centrifugation were determined. Each value represents the average of 3–4 experiments and the mean ± S.D.

cTnT4 both showed similar maximal force and ATPase activity. It is possible that Tn containing ssTnI may affect the affinity of cTnT for Tm could affect the maximal rate of ATPase activity. This suggests that cTnT isoforms affect the Ca

Fig. 8. Solubility of cTnT isoforms. 1 ml of cTnT1, cTnT3, or cTnT4 at various initial concentrations (0.4, 0.25, and 0.1 mg/ml) were dialyzed against 50 mM Tris-HCl, pH 7.0, containing 1 mM DTT and 1 mM KCl. The cTnT isoforms were then successively dialyzed against the same buffer containing lower concentrations of KCl (0.7, 0.5, 0.4, 0.3, 0.2, and then 0.1 mM KCl). After dialysis in 0.1 mM KCl the cTnT isoforms were centrifuged at 15,000 rpm for 25 min at 4°C, and the supernatant was collected. The total volume of the cTnT isoforms obtained after dialysis as well as the concentration of the cTnT isoforms in the supernatant after centrifugation were determined. Each value represents the total amount of cTnT isoform present in the supernatant (after centrifugation) at 0.1 mM KCl. cTnT4 was significantly less soluble than both cTnT1 and cTnT3 irrespective of the initial concentration of cTnT utilized (p < 0.001). cTnT3 was significantly less soluble than cTnT1 when the initial concentrations of cTnT utilized were between 0.1 and 0.25 mg/ml (p < 0.01). Each value is the average of 3–4 experiments and represents the mean ± S.D.

cTnT1, cTnT3, or cTnT4 showed that cTnI plays an important role in regulating the Ca

cTnI isoform that showed an increased Ca

cTnT4 was only 1.2-fold slower for the complexes containing cTnT1 and cTnI was only 1.2-fold slower for the complexes containing cTnT1 and cTnI. This suggests that cTnT complexes containing cTnT1 and cTnI. This suggests that cTnT isoforms affect the Ca

The results also showed a clear correlation between the maximal ATPase activity and maximal force for the cTnT isoforms in the presence of either cTnI or ssTnI. In the presence of ssTnI, cTnT3 showed an increased maximal force and ATPase activity relative to cTnT4. In the presence of cTnT3, cTnT3 and cTnT4 both showed similar maximal force and ATPase activity. These results suggest that the energetic cost for muscle contraction may be similar when the different cTnT isoforms are present. The energetic cost for muscle contraction mainly depends on two components, the ATP used by the cross-bridges and the ATP used by the Ca

cTnC has not been shown previously to alter the kinetics of Ca

cTnC demonstrates in diseased human skeletal muscle (49). In rabbit skeletal muscle (49), the fsTnI may also be dominant over ssTnI irrespective of the total concentration of Tn utilized. These results suggest that the dominance of cTnI over ssTnI is not just cTnI out-competing ssTnI for sites on the thin filament.

To determine whether the cTnT isoforms have different affinities for cTnC, affinity chromatography of the cTnT isoforms on a cTnC-Sepharose column was carried out. All cTnT isoforms eluted in the same fractions suggesting that the affinities of the cTnT isoforms for cTnC are not significantly different from each other (data not shown). A previous report (38) has shown that a higher molecular weight acidic fast twitch skeletal TnT (fsTnT) bound to αβ-Tm with a greater affinity than the lower molecular weight basic fsTnT (adult isoform). TnT is involved in activating the actomyosin ATPase to levels greater than that of actomyosin-Tm alone. Changes in the affinity of cTnT for Tm could affect the maximal rate of ATPase activity. It is possible that Tn containing ssTnI may affect the localization of Tn on the actin filament relative to Tn containing cTnI. It has been suggested previously (39) that the energy barrier between thin filament states is small and that Tn serves to stabilize Tm in its inhibitory state.

The results also showed a clear correlation between the maximal ATPase activity and maximal force for the cTnT isoforms in the presence of either cTnI or ssTnI. Several groups (24, 41–44) have demonstrated that the binding of Ca

cTnC has not been examined previously. cTnT had a greater effect on the kinetics of the Ca

The cardiac muscle system is complex with many post-translational modifications (such as phosphorylation of TnI and TnT) that might regulate the effect of the cTnT isoforms on cardiac contractility. Most myofibrillar proteins exist in multiple isoforms, and many of these proteins that are present during initial myofibrillar assembly are different from the proteins that occur in adult muscle. Further complicating our understanding of muscle contraction are recent investigations that suggest that the ssTnT gene is expressed in both developing and diseased human heart (47). The ssTnI may also be transiently expressed during early cardiac muscle development in mice (48). The expression of multiple cTnT isoforms has been demonstrated in diseased human skeletal muscle (49). In rabbits, the relative TnT isoform composition was also influenced by cardiovascular stress during development, suggesting that under certain conditions the expression of TnT isoforms at the fetal level may also be varied (30).

In summary, these results suggest that myofilaments containing fetal Tn (predominantly cTnT1–ssTnI–fTnC) has greater maximal force, greater maximal ATPase activity, lower minimal ATPase activity, and greater Ca

The cardiac muscle system is complex with many post-translational modifications (such as phosphorylation of TnI and TnT) that might regulate the effect of the cTnT isoforms on cardiac contractility. Most myofibrillar proteins exist in multiple isoforms, and many of these proteins that are present during initial myofibrillar assembly are different from the proteins that occur in adult muscle. Further complicating our understanding of muscle contraction are recent investigations that suggest that the ssTnT gene is expressed in both developing and diseased human heart (47). The ssTnI may also be transiently expressed during early cardiac muscle development in mice (48). The expression of multiple cTnT isoforms has been demonstrated in diseased human skeletal muscle (49). In rabbits, the relative TnT isoform composition was also influenced by cardiovascular stress during development, suggesting that under certain conditions the expression of TnT isoforms at the fetal level may also be varied (30).

In summary, these results suggest that myofilaments containing fetal Tn (predominantly cTnT1–ssTnI–fTnC) has greater maximal force, greater maximal ATPase activity, lower minimal ATPase activity, and greater Ca

The cardiac muscle system is complex with many post-translational modifications (such as phosphorylation of TnI and TnT) that might regulate the effect of the cTnT isoforms on cardiac contractility. Most myofibrillar proteins exist in multiple isoforms, and many of these proteins that are present during initial myofibrillar assembly are different from the proteins that occur in adult muscle. Further complicating our understanding of muscle contraction are recent investigations that suggest that the ssTnT gene is expressed in both developing and diseased human heart (47). The ssTnI may also be transiently expressed during early cardiac muscle development in mice (48). The expression of multiple cTnT isoforms has been demonstrated in diseased human skeletal muscle (49). In rabbits, the relative TnT isoform composition was also influenced by cardiovascular stress during development, suggesting that under certain conditions the expression of TnT isoforms at the fetal level may also be varied (30).

In summary, these results suggest that myofilaments containing fetal Tn (predominantly cTnT1–ssTnI–fTnC) has greater maximal force, greater maximal ATPase activity, lower minimal ATPase activity, and greater Ca

The cardiac muscle system is complex with many post-translational modifications (such as phosphorylation of TnI and TnT) that might regulate the effect of the cTnT isoforms on cardiac contractility. Most myofibrillar proteins exist in multiple isoforms, and many of these proteins that are present during initial myofibrillar assembly are different from the proteins that occur in adult muscle. Further complicating our understanding of muscle contraction are recent investigations that suggest that the ssTnT gene is expressed in both developing and diseased human heart (47). The ssTnI may also be transiently expressed during early cardiac muscle development in mice (48). The expression of multiple cTnT isoforms has been demonstrated in diseased human skeletal muscle (49). In rabbits, the relative TnT isoform composition was also influenced by cardiovascular stress during development, suggesting that under certain conditions the expression of TnT isoforms at the fetal level may also be varied (30).

In summary, these results suggest that myofilaments containing fetal Tn (predominantly cTnT1–ssTnI–fTnC) has greater maximal force, greater maximal ATPase activity, lower minimal ATPase activity, and greater Ca

regulatory Ca\(^{2+}\)-binding site of cTnC. cTnT plays a much bigger role in determining the Ca\(^{2+}\) kinetics of the cTnI-cTnC complex than it does in the ssTnI-cTnC complex.

These functional characteristics of fetal Tn (cTnT1-ssTnIcTnC) may be physiologically important for the fetal heart, which appears to require greater Ca\(^{2+}\) sensitivity. The 10-residue peptide present in cTnT1 that is missing in cTnT3 is likely to be important for conformational changes in this isoform that are important for interaction with ssTnI. Overall, these results suggest that the fetal isoforms (cTnT1 and cTnT4) are functionally distinct from the adult isoform (cTnT3) in the presence of ssTnI and are likely to have a significant role in modulating cardiac muscle contraction in the fetal and adult heart.

Acknowledgment—We thank Dr. J. A. Rall for material support.