A Troponin T Mutation That Causes Infantile Restrictive Cardiomyopathy Increases Ca\(^{2+}\) Sensitivity of Force Development and Impairs the Inhibitory Properties of Troponin*\(^{[5]}\)

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Restrictive cardiomyopathy (RCM) is a rare disorder characterized by impaired ventricular filling with decreased diastolic volume. We are reporting the functional effects of the first cardiac troponin T (CTnT) mutation linked to infantile RCM resulting from a **de novo** deletion mutation of glutamic acid 96. The mutation was introduced into adult and fetal isoforms of human cardiac TnT (HCTnT3-ΔE96 and HCTnT1-ΔE106, respectively) and studied with either cardiac troponin I (CTnI) or slow skeletal troponin I (SSTnI). Skinned cardiac fiber measurements showed a large leftward shift in the Ca\(^{2+}\) sensitivity of force development with no differences in the maximal force. HCTnT1-ΔE106 showed a significant increase in the activation of actomyosin ATPase with either CTnI or SSTnI, whereas HCTnT3-ΔE96 was only able to increase the ATPase activity with CTnI. Both mutants showed an impaired ability to inhibit the ATPase activity. The capacity of the CTnI-CTnC and SSTnI-CTnC complexes to fully relax the fibers after TnT displacement was also compromised. Experiments performed using fetal troponin isoforms showed a less severe impact compared with the adult isoforms, which is consistent with the cardioprotective role of SSTnI and the rapid onset of RCM after birth following the isoform switch. These data indicate that troponin mutations related to RCM may have specific functional phenotypes, including large leftward shifts in the Ca\(^{2+}\) sensitivity and impaired abilities to inhibit ATPase and to relax skinned fibers. All of this would account for and contribute to the severe diastolic dysfunction seen in RCM.

Cardiomyopathies are diseases that primarily affect muscle function and are associated with varying degrees of cardiac dysfunction. Morphological and pathophysiological differences of the diseased myocardium have led to the classification of three distinct types of cardiomyopathies: hypertrophic cardiomyopathy (HCM),\(^{2}\) dilated cardiomyopathy, and restrictive cardiomyopathy (RCM). HCM and dilated cardiomyopathy can be primarily classified by their morphologies, whereas RCM has a more complicated phenotype (1).

Restrictive cardiomyopathy (RCM) is a rare cardiomyopathic disorder that is characterized by abnormal diastolic function that results from impaired ventricular filling, increased ventricular end-diastolic pressure, and dilated atria. RCM is initially manifested by reduced ventricular compliance or increased stiffness (2). However, patients with RCM generally have normal to near normal systolic function until the later stages of the disease that eventually leads to heart failure. The prognosis of RCM is poor, especially in pediatric cases where patients often require heart transplantation and have average survival rates of (<50%) after diagnosis (3–5). Sudden cardiac death occurs in pediatric RCM with electrocardiographic evidence of ischemia (5). Pediatric RCM is most commonly idiopathic in origin and is noninfiltrative, and an associated interstitial fibrosis is often the sole detectable histopathologic abnormality of the myocardium (6).

Recently, the first case of infantile RCM because of a mutation in CTnT was discovered (7). Genetic analysis of the proband revealed a novel in-frame 285–287 GGA deletion in exon 9 of the cardiac TnT gene (TNNT2), resulting in a deletion of glutamic acid in position 96 (it was determined that the mutation had initially been reported in error as a glutamine).\(^{3}\) Previously, it was shown that the genetic component of RCM was mainly found in the cardiac TnT gene (TNNT3) and the cardiac desmin gene (DES) (8). The genetic evaluation revealed that the mutation arose **de novo** in the patient as both parents tested negative for this deletion in TNNT2. The patient presented with symptoms at 12 months of age and upon examination revealed a structurally normal heart with severely dilated atria. Moderate hypertrophy of the myocytes was found with significant disarray and fibrosis (7).

*In vitro* studies have indicated that TnT mutations can impart a vast array of functional defects, including reduced

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* The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

2 The abbreviations used are: HCM, hypertrophic cardiomyopathy; TnT, troponin T; TnI, troponin I; TnC, troponin C; RCM, restrictive cardiomyopathy; RLC, regulatory light chain; Tm, tropomyosin; MOPS, 4-morpholinepro-panesulfonic acid; DDT, dithiothreitol; CTnT, cardiac troponin T; CTnI, cardiac troponin I; SSTnI, slow skeletal troponin I; HCTnT, human cardiac TnT; WT, wild type.

3 B. L. Loey, personal communication.
acomyosin ATPase activity, reduced inhibitory abilities, crossbridge kinetics, myocyte contractility, and altered Ca\(^{2+}\) sensitivity (9, 10). The functional region of TnT affected by the RCM-associated deletion mutation of glutamic acid 96 is found in the main Tm-binding region located in the N-terminal half of TnT. This segment is highly conserved, and the acidic amino acids appear to be preserved across several species, indicating that they may play an essential role. Most TnT HCM mutations occur between residues 79 and 170, a region known to bind to Tm, thereby forming the link between the Tn complex and the thin filament (11). In general, mutations that occur within this region do not drastically affect the basal force or ATPase activity (12, 13). Mutations within residues 92–110 impair Tm-dependent functions of TnT and most likely affect cooperativity of the thin filament (9). Activation of the actomyosin ATPase activity is meditated by a direct interaction between amino acids 77 and 191 of sTnT with tropomyosin and actin (14).

The effects of the RCM TnT mutation were examined in both the adult and fetal human cardiac TnT isoforms (HCTnT3 and HCTnT1, respectively) to evaluate the disease progression after birth when the isoform switch occurs. HCTnT3-ΔE96 and HCTnT1-ΔE106 were incorporated into porcine cardiac skinned fibers together with either CTnI-CTnC or SSTnI-CTnC complexes using the CTn displacement method (12, 15). The Ca\(^{2+}\) sensitivity, maximal isometric force, and the ability of TnT-CTnC to inhibit unregulated force were evaluated. Both mutant isoforms showed a large increase in Ca\(^{2+}\) sensitivity when compared with their respective wild type (WT). There was no significant change in force recovery in any of these experiments. The ability of Tn to inhibit both ATPase activity and Ca\(^{2+}\)-unregulated force in the presence of the HCTnT3-ΔE96 and HCTnT1-ΔE106 mutants was impaired. Interestingly, experiments carried out with SSTnI showed smaller effects when compared with CTnI, suggesting a protective role of the slow skeletal isoforms that could explain why the affected infant did not display any RCM phenotype during early heart development.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of Human Cardiac Troponin T Isoforms and RCM Mutants**

The adult and fetal isoforms of HCTnT (HCTnT3 and HCTnT1, respectively), which had already been cloned in our laboratory (15), were used as templates for PCR using primers designed to delete 3-bp codon GAA for glutamic acid that results in the TnT-RCM mutants (HCTnT3-ΔE96 and HCTnT1-ΔE106). All subcloned DNA sequences were inserted into the pET3-d expression plasmid and sequenced to verify that they were correct prior to expression and purification. Standard methods previously used in this laboratory were utilized for expression and purification of the various HCTnTs (16). Briefly, recombinant TnT, TnI, and the TnT-RCM mutants were expressed in *Escherichia coli* BL21 (DE3) codon plus bacterial cells (Stratagene). The bacterially expressed TnTs were transformed with pET-3d constructs containing CTnI, SSTnI, or CTnC. The TnIs were first purified by column chromatography on a fast flow S-Sepharose column at 4 °C and eluted with a linear KCl gradient of 0–0.6 M. Semi-pure HCTnI and HSSTnI were dialyzed against a solution containing 50 mM Tris-HCl, pH 7.5, 1 mM NaCl, 2 mM CaCl\(_2\), and 1 mM DTT and loaded onto an HCTnC affinity column. Pure HCTnI and HSSTnI were eluted using a gradient of 0–1 mM EDTA and 0–6 M urea. The purity of the TnI proteins was determined by SDS-PAGE. CTnC was first applied onto a fast flow Q-Sepharose column and eluted with 0–0.6 M salt gradient. The clearest fractions were then dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 50 mM NaCl, and 1 mM DTT. CTnC was removed following ammonium sulfate was added to a final concentration of 0.5 M, and the protein was loaded onto a pre-equilibrated phenyl-Sepharose column. Pure CTnC was directly eluted using a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.5. Fractions of >98% purity as determined by SDS-PAGE were pooled, dialyzed against 5 mM ammonium bicarbonate, and then lyophilized.

**Formation of Troponin Complexes**

The purified individual troponin subunits were first dialyzed against 5 M urea, 1 M KCl, 10 M MOPS, 1 M DTT, and 0.1 M phenylmethanesulfonl fluoride and then twice against the same buffer excluding urea. At this point the protein concentrations of the individual subunits were determined using the Coomassie Plus kit (Pierce) and then mixed in a 1.3:1.3:1 TnT: TnI: TnC molar ratio. After 1 h, the complexes were successively dialyzed against decreasing KCl concentrations (0.7, 0.5, 0.3, 0.1, 0.05, and 0.025 M). The excess TnT and TnI that precipitated during complex formation was removed by centrifugation. Proper stoichiometry was verified by SDS-PAGE before storage of troponin complexes at −80 °C. The following troponin complexes were formed: HCTnT1 or HCTnT1-ΔE106 HCTnT1-HCTnC; HCTnT1 or HCTnT1-ΔE106-HSSTnI-HCTnC; HCTnT3 or HCTnT3-ΔE96-HCTnI-HCTnC; and HCTnT3 or HCTnT3-ΔE96-HSSTnI-HCTnC.

**Actin-Tm-activated Myosin-ATPase Assays**

Porcine cardiac myosin, rabbit skeletal F-actin, and porcine cardiac Tm were prepared as described previously (17). The protein concentrations used for actomyosin ATPase assays using a 0–0.6 M salt gradient. To obtain optimum purity, the proteins were further purified by passage over a DEAE-Sepha-

**Expression and Purification of Recombinant Human Cardiac Troponin I, Human Slow Skeletal Troponin I, and Human Cardiac Troponin C**

The cDNAs for human CTnI, SSTnI, and CTnC were previously cloned in our laboratory by reverse transcription-PCR using human heart total RNA (Stratagene), sequence-specific primers, and an Omnistrand reverse transcription kit (Qiagen) (16, 17). *E. coli* BL21 (DE3) codon plus bacterial cells were transformed with pET-3d constructs containing CTnI, SSTnI, or CTnC. The TnIs were first purified by column chromatography on a fast flow S-Sepharose column at 4 °C and eluted with a linear KCl gradient of 0–0.6 M. Semi-pure HCTnI and HSSTnI were dialyzed against a solution containing 50 mM Tris-HCl, pH 7.5, 1 mM NaCl, 2 mM CaCl\(_2\), and 1 mM DTT and loaded onto an HCTnC affinity column. Pure HCTnI and HSSTnI were eluted using a gradient of 0–1 mM EDTA and 0–6 M urea. The purity of the TnI proteins was determined by SDS-PAGE. CTnC was first applied onto a fast flow Q-Sepharose column and eluted with 0–0.6 M salt gradient. The clearest fractions were then dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 50 mM NaCl, and 1 mM DTT. CTnC was removed following ammonium sulfate was added to a final concentration of 0.5 M, and the protein was loaded onto a pre-equilibrated phenyl-Sepharose column. Pure CTnC was directly eluted using a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.5. Fractions of >98% purity as determined by SDS-PAGE were pooled, dialyzed against 5 mM ammonium bicarbonate, and then lyophilized.

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Porcine cardiac myosin, rabbit skeletal F-actin, and porcine cardiac Tm were prepared as described previously (17). The protein concentrations used for actomyosin ATPase assays
Functional Characterization of the First TnT RCM Mutation

were as follows: 0.6 μM porcine cardiac myosin, 3.5 μM rabbit skeletal F-actin, 1 μM porcine cardiac tropomyosin, and 0–2 μM of pre-formed Tn complexes as described above (15). The ATPase inhibitory assay was performed in a 0.1-mL reaction mixture of 75 mM KCl, 3.4 mM MgCl₂, 0.1 μM CaCl₂, 1.5 mM EGTA, 3.5 mM ATP, 1 mM DTT, 11.5 mM MOPS, pH 7.0, at 25 °C. The ATPase activation assay was performed using the same 0.1-mL buffer mixture with 3.3 mM MgCl₂ and 1.7 mM CaCl₂. The ATPase reaction was initiated with the addition of ATP and quenched after 20 min using trichloroacetic acid to a final concentration of 5%. The precipitated assay proteins were removed by centrifugation, and the inorganic phosphate concentration of the supernatant, which was released by ATP hydrolysis, was determined according to the method of Fiske and SubbaRow (18).

Skinned Fiber Assays

Porcine Skinned Cardiac Fiber Preparation—Skinned papillary muscles were prepared from the left ventricles of freshly slaughtered porcine hearts. Small bundles of fibers were isolated and treated overnight with a pCa 8.0 relaxing solution (10⁻⁸ M [Ca²⁺]₀, 1 mM [Mg²⁺]₀, 7 mM EGTA, 2.5 mM MgATP²⁻, 20 mM MOPS, pH 7.0, 20 mM creatine phosphate, and 15 units/ml creatine phosphokinase, I = 150 mM) containing 1% Triton X-100 and including 50% glycerol at −20 °C. Fibers were then transferred to a similar solution without Triton X-100 and kept at −20 °C.

Measurement of the Ca²⁺ Dependence of Force Development—The porcine muscle fiber bundles (a bundle of three to five fibers isolated from a batch of glycinerinized fibers) with the length and diameter varying between 1.2 and 1.5 mm and 120 and 150 μm, respectively, were attached to tweezer clips connected to a force transducer. The fibers were treated with pCa 8.0 containing 1% Triton X-100 for 30 min to ensure complete membrane removal, which allows better access to the myofilament proteins. After extensively washing the fibers with pCa 8.0 without Triton X-100, the initial maximal force was determined by transferring the fiber into pCa 4.0 solution (identical to pCa 8.0 solution except the [Ca²⁺]₀ is 10⁻⁴ M). To determine the Ca²⁺ sensitivity of force development, the fibers were gradually exposed to solutions of increasing Ca²⁺ concentration from pCa 8.0 to 4.0. Data were analyzed using the following equation: % change in force = 100 × [Ca²⁺]₀/[([Ca²⁺]₀ + [Ca²⁺]₀)²], where “[Ca²⁺]₀” is the free [Ca²⁺] that produces 50% force and “n” is the Hill coefficient. The various pCa solutions were calculated using the computer program (pCa Calculator) recently developed in our laboratory (19).

Endogenous Tn Complex Displacement—To displace the endogenous Tn complex, skinned fibers were incubated with 0.8 mg/ml HCTnT isoform 1, 3, or RCM mutants for 2.5 h at room temperature after a brief 10-min exposure to a preincubation buffer without protein (250 mM KCl, 20 mM MOPS, 5 mM MgCl₂, 5 mM EGTA, 1 mM DTT, pH 6.2). The TnT-treated fibers were then washed with preincubation buffer, and the displacement was evaluated by the level of unregulated force development measured in both pCa 8 and pCa 4 solutions. The Ca²⁺ regulation of steady-state force was restored using pre-formed human CTnI-CTnC or SSTnI-CTnC. The fibers were reconstituted using 25 μM of the binary complex in pCa 8.0 solution for ~1 h at room temperature until the force reaches a stable level. The Ca²⁺ dependence of force was determined before and after performing the displacement and reconstitution as described above.

Gel Electrophoresis and Immunoblotting Analysis

After the Ca²⁺ sensitivity assays, all skinned fibers were dissolved in 1% SDS and 10% β-mercaptoethanol and stored at −20 °C until electrophoretic analysis. Skinned fiber samples were boiled for 2 min and electrophoresed on 15% SDS-polyacrylamide gels. SDS-PAGE was used to determine protein incorporation into skinned fibers utilizing 3/4 and 1/4 of the total sample volume for Coomassie staining and Western blot analysis, respectively. Proteins were transferred to nitrocellulose membrane for Western blot (Bio-Rad) using standard protocols (20). Briefly, membranes were first immersed and incubated for 5 min in Tris-buffered saline alone and then incubated overnight in primary specific antibodies in a 50% Tris-buffered saline, 50% Rockland blocking buffer for Odyssey fluorescence detection. Specific antibodies to detect TnT (IA11; Research Diagnostic Inc, Flanders, NJ), TnI (6F9; Fitzgerald Industries International Inc., Concord, MA), and RLC (rabbit polyclonal anti-RLC serum developed in this laboratory) were used at 1:2000 dilution. After primary antibody incubation, blots were washed three times (5 min each), placed in covered boxes for 1 h, and incubated with secondary fluorescent antibody. The specific primary antibodies were detected using a 1:4000 dilution of goat anti-rabbit or goat anti-mouse IgG antibody labeled with IR800 or CY5.5 fluorescent dye. Reaction signals were measured by scanning the blot with the Odyssey Infrared Imager (LI-COR Biosciences), and the band intensities were quantified using Odyssey software.

Statistical Analysis

The experimental results were reported as x ± S.E., and analyzed for significance using Student’s t test at p < 0.05, paired or unpaired depending on the experimental design.

RESULTS

Effect of HCTnT RCM Mutant Isoforms on the Ca²⁺ Sensitivity of Force Development—To address the functional consequences of the newly discovered TnT-RCM related mutation during fetal and adult developmental stages, we inserted the mutation into two different isoforms of human cardiac TnT (HCTnT) as follows: HCTnT isoform 3 found in adult hearts with a glutamic acid deletion at position 96 (HCTnT3-ΔE96) and HCTnT isoform 1 with a glutamic acid deletion at position 106 (HCTnT1-ΔE106), the corresponding position in the fetal TnT isoform. The different number of these two isoforms is because of the fact that exon 5 is present in isoform 1 but not in isoform 3 (Fig. 1). To measure the Ca²⁺ sensitivity of force development, the endogenous porcine troponin complex was displaced using established methods by our laboratory (12). Fibers were treated with excess exogenous HCTnT and reconstituted with either human CTnI-CTnC or SSTnI-CTnC, and the Ca²⁺ sensitivity of force development was measured as a
function of increasing Ca\(^{2+}\) concentration (see Fig. 2 and "Experimental Procedures" above).

As shown in Fig. 3, drastic changes in the Ca\(^{2+}\) sensitivity of force development were observed between fibers displaced by the TnT RCM mutant and the TnT WT isoforms. Fig. 3, A and C, shows a significant and pronounced leftward shift in fibers containing HCTnT3-E96 and reconstituted with CTnI-CTnC (pCa\(_{50} = 6.16\)) or SSTnI-CTnC (pCa\(_{50} = 6.19\)) compared with their controls (pCa\(_{50} = 5.73\) and pCa\(_{50} = 5.84\), respectively). In the next series of experiments, porcine cardiac fibers were treated with HCTnT1-E106 or HCTnT1-WT and also reconstituted with either CTnI-CTnC or SSTnI-CTnC. Results depicted in Fig. 3B with CTnI-CTnC show that the Ca\(^{2+}\) sensitivity increases in fibers treated with HCTnT1-E106 (pCa\(_{50} = 6.23 \pm 0.08\)) compared with HCTnT1-WT (pCa\(_{50} = 5.81 \pm 0.02\)). However, fibers treated with HCTnT1-E106 and reconstituted with SSTnI-CTnC (Fig. 3D) do not show as large a leftward shift compared with its control (pCa\(_{50} = 0.20\)). HCTnT3-E96 in the presence of CTnI mimics the biological environment of the adult troponin complex, and under these conditions the most pronounced leftward shift was produced (ΔpCa\(_{50} = +0.43\)). Interestingly, HCTnT1-E106 in the presence of SSTnI mimics the Tn fetal heart environment and presented a smaller leftward shift in the Ca\(^{2+}\) sensitivity of force development. The mean values of pCa\(_{50}\) and the Hill coefficient (n\(_{H}\)) for each condition are summarized in the Table 1.

Fig. 3, A and C, insets, shows that porcine fibers treated with HCTnT1-WT and reconstituted with either human...
CTnI-CTnC or SSTnI-CTnC complex have an increased Ca$^{2+}$ sensitivity of force development compared with HCTnT3-WT-treated fibers. This effect of different HCTnT isoforms on the Ca$^{2+}$ sensitivity of force development has been reported several times by our laboratory (15, 16, 21). We have also observed an additive increase of Ca$^{2+}$ sensitivity in fibers reconstituted with SSTnI-CTnC compared with the CTnI-CTnC. This is because of the well known effect of SSTnI that lowers the threshold for Ca$^{2+}$ activation of force development. After the HCTnT RCM mutant isoforms were incorporated, the Hill coefficient values decreased indicating lowered cooperativity.

To address whether the TnT RCM mutants have altered affinity for the thin filament and poor incorporation in the fibers, we measured the unregulated tension at low Ca$^{2+}$ concentration ($pCa$ 8.0) after the displacement (see scheme in Fig. 2). No changes were observed in the ability of the HCTnT RCM mutants to displace the endogenous CTn complex compared with their respective HCTnT-WT. The mean values of unregulated tension are reported in Table 1. SDS-PAGE and Western blot analysis also show no evidence of poor TnT displacement (more details discussed below).

Analysis of Steady-state of Force Development—After displacement and reconstitution, the restored maximal force obtained at high Ca$^{2+}$ concentrations ($pCa$ 4.0) for each fiber was evaluated. This force was measured relative to the initial maximal force of the skinned fibers before HCTnT treatment. In Fig. 4, A and B, we observed a slight decrease in the restored maximal force developed by fibers displaced with the HCTnT RCM mutant isoforms reconstituted with either cardiac or slow skeletal TnI-CTnC complexes. However, the difference
TABLE 1
Summary of pCa force relationship curves in fibers reconstituted with either cardiac or slow skeletal TnI-TnC complex at pH 7.0

The pCa2+–unregulated force values are the average of many independent fiber experiments, and the errors are the S.E. values. The Ca2+-unregulated force was calculated by the following equation: (Fp/Ca4-atp) × 100, where the Fp/Ca4 and Fp/Ca4-atp are the force at pCa 8.0 and pCa 4.0 solutions, respectively. p values compare the RCM mutant in TnT3 and TnT1 against their respective TnT-WT with the same Tn isoform.

| TnT    | TnI isoform   | pCa2+_S.E. | Hill coefficient | ΔpCa2+_S.E. | %Ca2+-unregulated force | No. of experiments |
|--------|---------------|------------|------------------|-------------|-------------------------|------------------|
| HCTnT3 | Cardiac       | 5.73 ± 0.02| 2.34 ± 0.34      | +0.43       | 84.5 ± 5.2              | 4                |
| HCTnT3-ΔE96a | Cardiac     | 6.16 ± 0.06b | 1.30 ± 0.31c    | +0.35       | 81.2 ± 4.9              | 6                |
| HCTnT3 | Slow skeletal | 5.84 ± 0.03| 1.78 ± 0.23      | +0.35       | 86.6 ± 3.1              | 4                |
| HCTnT3-ΔE96 | Slow skeletal | 6.19 ± 0.03c | 1.38 ± 0.25c    | +0.35       | 86.6 ± 3.1              | 4                |
| HCTnT1 | Cardiac       | 5.81 ± 0.02| 1.77 ± 0.22      | +0.35       | 84.8 ± 2.7              | 6                |
| HCTnT1-ΔE106a | Cardiac     | 6.23 ± 0.08b | 1.21 ± 0.33c    | +0.42       | 90.4 ± 3.0              | 6                |
| HCTnT1 | Slow skeletal | 5.91 ± 0.02| 1.86 ± 0.27      | +0.20       | 83.9 ± 5.6              | 5                |
| HCTnT1-ΔE106 | Slow skeletal | 6.11 ± 0.07c | 1.09 ± 0.30c    | +0.20       | 91.0 ± 1.0              | 6                |

*p < 0.001.

p<sub>0.05</sub>.

*p < 0.01.

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Actomyosin ATPase Assays Using Reconstituted Troponin Complexes Containing RCM Mutant Isoforms—It is well known that actin accelerates myosin ATP hydrolysis, and that in the presence of the Tn complex, the myosin activity is regulated by Ca<sup>2+</sup>. In the next set of experiments we evaluated the capability of the Tn complexes containing the HCTnT WT or RCM mutants to activate or inhibit the actomyosin ATPase activity. Fig. 5, A and B, illustrates the activation of the ATPase activity in the presence of Ca<sup>2+</sup> by Tn complexes containing CTnI-CTnC or SSTnI-CTnC. The actomyosin ATPase activity in the absence of Tn complex was considered to be the basal activity level for both activation and inhibition and was set as 100%. Upon evaluation of the entire curve, the low Tn concentration points (0.5 and 0.8 μM) showed significant increases in ATPase activation by complexes containing the HCTnT RCM mutant isoforms compared with their respective WT and matching Tn isoform.

The activity of the HCTnT-RCM containing complexes to inhibit the actomyosin ATPase in the absence of Ca<sup>2+</sup> was also examined to determine whether the inhibitory activity of the HCTnT complex is affected by this specific TnT deletion. Fig. 5, C and D, shows an impaired ability of the Tn complex to inhibit ATPase activity. At 1 μM Tn, which reflects the proper stoichiometry of the regulatory complex (1 μM Tn, 1 μM Tn), the activation of the actomyosin ATPase with HCTnT RCM mutant isoforms was compared with their respective WT and appeared not to be significant.

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ble of generating tension when incubated in a solution without Ca$^{2+}$ ($p_{Ca}$ 8.0). Basically, this phenomenon occurs when TnI and TnC are absent from the thin filament and as the whole Tn complex is displaced by excess exogenous TnT. After that, the fibers are incubated with a pre-formed TnI-TnC complex, and the tension starts to decrease because of the TnI-TnC inhibitory effect in the absence of Ca$^{2+}$ (see schematic in Fig. 2). In the next experiment we measured the ability of the pre-formed TnI-TnC complex to fully relax the fibers after the TnT RCM mutant isoforms were used in the displacement step. This kind of experiment would indicate whether the TnT RCM-related mutant is interfering with the ability of TnI to inhibit muscle contraction in skinned fibers.

Fig. 6, A and B, shows the residual unregulated force in fibers that were TnT-displaced after reconstitution with human SSTnI-CTnC or CTnI-CTnC complexes. For clarification, the residual unregulated force indicates the amount of tension remaining in the fibers after they reach a plateau of relaxation. The level of force remaining after CTnI-CTnC reconstitution in fibers displaced with the RCM mutants HCTnT3-ΔE96 and HCTnT1-ΔE106 was 17.3 and 29.4%, respectively. However, fibers displaced with HCTnT3-WT and HCTnT1-WT had 3.5 and 17.2% force remaining, respectively. In fibers reconstituted with SSTnI-CTnC, the HCTnT3-ΔE96 did not interfere with its ability to fully relax the fibers as compared with HCTnT3-WT. However, fibers displaced with HCTnT1-ΔE106 showed ~10% more residual unregulated force than WT after reconstitution with SSTnI-CTnC.

SDS-PAGE and Western Blot Analysis of the Experimental Fibers—To address whether the impaired relaxation produced by the TnT RCM mutant isoforms in the skinned fiber experi-
HCTnT RCM mutant isoforms compared with their controls. This difference is because of an increase in band intensities for the HCTnT because it migrates together with PCTnI on SDS-PAGE. For these TnI measurements pig cardiac RLC was used as a loading control.

DISCUSSION

In this study we investigated the effects of a new TnT deletion mutation located in its N-terminal domain that is linked to infantile RCM (7). We present data that supports clinical findings of impaired contractile and diastolic dysfunction, a consequence of RCM that may be due to perturbations in the Tn complex inhibitory function and Ca\(^{2+}\) affinity. The glutamic acid 96 deletion occurs at a position highly conserved across many species and causes loss of a negative charge in the coiled-coil region that is likely to affect TnT-Tm-actin interactions (23–25). Our approach was to determine how loss of this amino acid in the fetal and adult TnT isoforms affects the function of the thin filament using skinned fibers and reconstituted actomyosin systems.

Skinned fiber experiments demonstrated that the removal of glutamic acid 96 or 106 in TnT drastically increased the Ca\(^{2+}\) sensitivity of force development in fibers reconstituted with the adult and fetal TnT isoforms; however, the results were more profound in the adult Tn environment (Fig. 3). Our lab has shown that different TnT isoforms present at spatially regulated times during cardiac muscle development show distinctly altered abilities to modulate Ca\(^{2+}\) sensitivity of the myofilament (15). The variability found between isoforms 1 and 3 lies in the absence of exon 5 from the N-terminal domain of isoform 3 (Fig. 1). Also, other studies using avian and rodent myocardium reported that as TnT switches from the fetal to the adult isoforms, the Ca\(^{2+}\) sensitivity also decreases (26, 27). In the past, TnT function remained elusive and was thought to strictly mediate interactions between Tn and the thin filament. However, these new results reveal that the function of TnT in the muscle regulatory process is significantly more complex than originally thought (28). It has been proposed that the N terminus of TnT has a fine-tuning function that alters myofilament sensitivity to Ca\(^{2+}\) and influences force production (15, 29).

We have been investigating the inhibitory properties of Tn mutations associated with cardiomyopathies. HCM and RCM Tn mutations have been shown to interfere with muscle inhibitory responses; however, RCM appears to have a more dramatic effect (12, 30). In Figs. 5 and 6, the TnT RCM mutant isoforms, HCTnT3-ΔE96 and HCTnT1-ΔE106 either in the presence of adult or fetal TnI, showed a definite inability to inhibit ATPase activity and a profound inability to decrease unregulated force after TnT displacement and TnI-Ctnc reconstitution. In addition, Fig. 7 demonstrates a consistent TnI content in all experimental fibers. Szczesna et al. (12) evaluated the basal force after displacement by six different TnT mutants related to HCM and reconstitution with Ctnc in skinned fibers. Three of them showed a reduced ability to induce relaxation in the treated fibers. Another study showed that the Tn complex containing the TnI R145G mutation related to HCM was unable to fully inhibit both ATPase activity and unregulated force in reconstituted fibers (17). Gomes et al. (30) ana-
lyzed the functional effects of five TnI mutants related to RCM. All of these mutants showed a reduced ability to fully inhibit the ATPase activity and relaxation in skinned fibers; however, the L144Q and R145W mutants showed the lowest inhibitory ability. All of these functional results considered together demonstrate specific phenotypes characteristic of each cardiomyopathy.

It is clear that Tn mutants related to RCM have the ability to impair parameters associated with muscle relaxation, and from these findings it can be concluded that glutamic acid 96 in TnT, which was found to be deleted, has a critical role in controlling muscle inhibition. However, it is still unknown how HCM mutations provoke the same effects as RCM but are manifested in vastly distinct diseases. It may be possible that clinical diagnostic techniques lack the ability to differentiate subtleties that exist between these two diseases (12, 17, 30, 31).

The pCa50 and n values obtained from two different methods of calculation from skinned fiber experiments indicated that there was a substantial increase in Ca2+ sensitivity of contraction and a decrease in the cooperativity of the thin filament because of the TnT RCM mutant isoforms; however, the effect was even more dramatic in the presence of HCTnT1-complexed with CTnI (adult Tn environment) compared with was even more dramatic in the presence of HCTnT3-complexed with CTnI (adult Tn environment) because of the TnT RCM mutant isoforms; however, the effect exists between these two diseases (12, 17, 30, 31).

Because the N-terminal domain of TnT has a major role in connecting the troponin complex to the thin filament, we would expect that this deletion could change interactions between Tn and Tm affecting cooperativity and Ca2+ sensitivity of the thin filament. It is known that residues 70–159 of sTnT are responsible for the cooperative interactions between functional units (34, 35). Interestingly, the HCM CTnT mutation R92Q, which is located very close to our CTnT mutation in this study, was also able to change the cooperativity of the thin filament (36). In addition, glutamic acid 96 is located in the CTnT region that interacts with the Tm head-to-tail overlap region (37). This Tm region promotes the cooperative interaction of myosin on the actin filament, showing that this residue is affecting essential functions of this region (38–40). Our results show that the functional alteration produced by this specific RCM mutation is too complex to be elucidated with our current level of knowledge.

In general, Tn mutations related to cardiomyopathy can lead to alterations in the cooperativity of the thin filament and Ca2+ sensitivity (9, 17, 30, 41–43). The TnR RM mutations discovered to date show decreased cooperativity of thin filament interactions, as reported previously by our laboratory (30). The effects of lowered cooperativity in the RCM mutants appear to be consistent during heart development at all stages (see Table 1). These results indicate that this residue plays an important role in the cooperativity
of the thin filament and that patients carrying this mutation would be subject to cardiac dysfunction because of the lowered cooperative interactions of the thin filament during heart development. An interesting concept is that increased Ca²⁺ sensitivity and decreased cooperativity in the myocardium from such an early stage of development might increase the tension-dependent ATP consumption leading to an earlier onset and a more severe form of disease (41). The histological findings from the patient carrying the TnT RCM mutation had unusually shaped and elongated mitochondria (7) that may indicate that there was an alteration in energy production or an unusually high cardiac energy demand because of the impaired cardiac muscle physiology.

The skinned fiber results show a specific phenotype with large increases in Ca²⁺ sensitivity and no effects on maximal force (Figs. 3 and 4). At the molecular level we could hypothesize two scenarios that might explain these phenomena; the mutation might be increasing or decreasing the ratio between strong and weak cross-bridges or may be directly affecting CTnC, thereby altering its Ca²⁺ affinity. It is known that increasing the ratio of strong cross-bridges in skinned fibers can cause an increase in Ca²⁺ binding to TnC and also affect the maximal force (44–46). However, 2,3-butanedione monoxime, which acts to increase the weak cross-bridge ratio, decreases the Ca²⁺ sensitivity and maximal force (47, 48). Our functional studies do not support the idea that these TnT RCM mutant isoforms act by changing the cross-bridge ratio. Bepridil, a known Ca²⁺ sensitizer, is able to increase the Ca²⁺ sensitivity without affecting maximal force (49, 50). More recently, some reports showed that bepridil acts directly on TnC affecting Ca²⁺ binding to its N-terminal domain (51–53). Our mutation may be directly altering Ca²⁺ binding to TnC producing a similar effect to bepridil because our skinned fiber data displayed the same functional phenotype. We also believe that the TnT RCM mutation may have dual effects as follows: where Ca²⁺ sensitivity is increased and muscle inhibitory function is impaired. More investigations are needed to understand the molecular mechanisms where a troponin mutation is able to alter biochemical and biophysical properties of muscle.

It is well known that TnI switches isoforms during heart development. The SSTnI isoform is the predominant TnI isoform throughout fetal life and gradually decreases during the first few months of postnatal development (54). Upon structural and physiological analysis, it was determined that these TnI isoforms confer distinct contractile properties on the myocardium (55). Evaluating the physiological effects of the TnI isoforms is important in understanding the development of the disease. Our results show a protective effect of the SSTnI compared with CTnI against the changes produced by the RCM mutation when compared with their appropriate controls. These findings confirm the known effects of SSTnI that assist cardiac function during extreme conditions (56–58).

In conclusion, RCM is a form of cardiomyopathy that restricts the heart from properly stretching and filling with blood. Our functional studies recapitulate the clinical findings that demonstrate a diminished capacity of the diseased heart to fully relax during diastole. As discussed above, the TnT mutation leading to RCM may have a dual effect at the level of TnC and at the level of the actomyosin interface. Oliveira et al. (14) using TnT N-terminal fragments showed that the amino acid sequence 77–191 is responsible for ATPase activation in the absence of TnI and TnC. Therefore, the TnT mutation located in this peptide could potentiate this effect by interfering with cross-bridges at resting conditions. The other explanation could be that there is a direct effect on Tn function because structural changes were observed in the RCM-TnI mutations (59). Functional analysis of the first mutation in TnT linked to RCM has strengthened our understanding of this heart dysfunction and could help distinguish RCM from other cardiomyopathies. These results indicate that much more knowledge is needed to fully elucidate the molecular mechanism involved in Tn-based cardiomyopathies.

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