Different Functional Properties of Troponin T Mutants That Cause Dilated Cardiomyopathy*

The effects of Troponin T (TnT) mutants R141W and ΔK210, the only two currently known mutations in TnT that cause dilated cardiomyopathy (DCM) independent of familial hypertrophic cardiomyopathy (FHC), and TnT-K273E, a mutation that leads to a progression from FHC to DCM, were investigated. Studies on the Ca\(^{2+}\) sensitivity of force development in porcine cardiac filaments demonstrated that TnT-ΔK210 caused a significant decrease in Ca\(^{2+}\) sensitivity, whereas the TnT-R141W did not result in any change in Ca\(^{2+}\) sensitivity when compared with human cardiac wild-type TnT (HC-WtTnT). TnT-ΔK210 also caused a decrease in maximal force when compared with HC-WtTnT and TnT-R141W. In addition, the TnT-ΔK210 mutant decreased maximal ATPase activity in the presence of Ca\(^{2+}\). However, the TnT-K273E mutation caused a significant increase in Ca\(^{2+}\) sensitivity but behaved similarly to HC-WtTnT in actomyosin activation assays. Inhibition of ATPase activity in reconstituted actin-activated myosin ATPase assays was similar for all three TnT mutants and HC-WtTnT. Additionally, circular dichroism studies suggest that the secondary structure of all three TnT mutants was similar to that of the HC-WtTnT. These results suggest that a rightward shift in Ca\(^{2+}\) sensitivity is not the only determinant for the phenotype of DCM.

Contraction of cardiac muscle is initiated by the binding of Ca\(^{2+}\) to the Ca\(^{2+}\)-specific regulatory site in Troponin C (TnC),\(^1\) the Ca\(^{2+}\) binding subunit of the troponin (Tn) complex, which also contains Troponin I (TnI) and Troponin T (TnT). The Tn complex, together with tropomyosin (Tm), forms the regulatory system of the contractile apparatus (1, 2). Contraction results from the ATP-driven interaction of the thin, actin-containing filaments and the thick, myosin-containing filaments. This interaction is regulated by Tm-Tn in a Ca\(^{2+}\)-dependent manner. Upon binding of Ca\(^{2+}\) to the single Ca\(^{2+}\)-specific site of cardiac TnC, the C terminus of TnT interacts with the amino terminus of TnC and dissociates from the actin-Tm complex, allowing myosin to bind to actin. TnT is thought to stabilize the troponin complex, but it also regulates the Ca\(^{2+}\) sensitivity of actomyosin ATPase activity and the level of ATPase activation as well as force development (2, 3).

Recently, mutations in genes for various sarcomeric proteins were shown to cause dilated cardiomyopathy (DCM) (4). DCM is a relatively common disorder that results in heart failure and premature death (5). DCM is characterized by ventricular dilation and impaired systolic function. Pathological manifestations of DCM include modest hypertrophy, myocyte degeneration, and increased interstitial fibrosis (4). Proposed causes of DCM include alcohol toxicity, ischemia, metabolic and nutritional deficiency, infectious diseases, and familial and genetic factors. Among these factors, it is estimated that heritable gene mutations account for 25–30% of cases (4). The first thin filament-associated DCM mutation was described in the cardiac actin gene (6). Subsequently, mutations were found in other sarcomeric proteins, including β-myosin heavy chain, cardiac myosin binding protein C, titin, α-Tm, and cardiac TnT (7). The presumed mechanism by which these mutations cause DCM is not clearly understood. So far there are four TnT mutations (R92W, R141W, ΔK210, and K273E) that are known to cause DCM. However, only two of these mutations (R141W and ΔK210) are known to cause DCM independent of FHC.

After genotypic analysis, Kamisago et al. (8) reported the first TnT mutation as the cause of DCM in two unrelated families. This mutation occurs in exon 13 of cardiac TnT because of a deletion of the basic amino acid lysine at position 210. None of the individuals affected with DCM exhibited ventricular hypertrophy, a hallmark feature of hypertrophic cardiomyopathy. Medical records of deceased individuals from these families revealed sudden deaths of two infants with infantile cardiomyopathy and three young adults (8). Li et al. (9) reported another novel TnT mutation, TnT-R141W, in the Tm-binding site of TnT in a large family of 72 members. Fourteen living members of the family clinically manifested DCM, predominantly in the second decade of their life. Seventeen members of this family died of heart failure before the genotypic studies were carried out. It is worth mentioning that arginine at position 141 of cardiac TnT (cTnT) is conserved in many species, including mouse, rat, chicken, quail, and nematode as well as among the slow or fast skeletal muscle isoform of TnT (9).

Fujino et al. (10) reported another novel mutation in the cardiac TnT gene at position 273, where the highly conserved amino acid lysine is switched to glutamic acid. Nine family members from two unrelated pedigrees inherited the mutation, and this mutation is associated with a high incidence of sudden cardiac death in one family. This mutation primarily caused asymmetric septal hypertrophy in seven of nine family mem-

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‡¶ The abbreviations used are: TnC, troponin C; FHC, familial hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; WT, wild type; HCTnT, human cardiac wild-type troponin T; CD, circular dichroism; cTnT, cardiac TnT; RLC, regulatory light chain; MOPS, 4-morpholinepropanesulfonic acid; Tm, tropomyosin.
bers with the mutation. Interestingly, the mutation caused a complete transition from FHC to DCM in one of the two family members whose disease progression was monitored for 12–14 years.

To date, the biochemical mechanisms regulating cardiac diastole are not clearly understood. In this study, we have investigated the functional consequences of the DCM-causing TnT mutations R141W, ΔK210, and K273E on the regulatory properties of the thin filament. Previously, Morimoto et al. (11) investigated TnT-ΔK210 and hypothesized that the Ca$^{2+}$-sensitizing effect of this mutation is the primary cause of dilated cardiomyopathy. Because Ca$^{2+}$ is the primary activator of the thin filament, we investigated how this mutation affects the Ca$^{2+}$ sensitivity of force development. Our results are in agreement with Morimoto’s group, which showed that the mutation TnT-ΔK210 caused a decrease in Ca$^{2+}$ sensitivity. However, we also observed a significant decrease in the Ca$^{2+}$-activated maximal force in TnT-ΔK210-reconstituted fibers when compared with HCWTnT-reconstituted fibers, which was not previously observed by Morimoto et al. (11). However, ATPase activation assays done in the presence of Ca$^{2+}$ showed differences for both the TnT-DCM mutants compared with HCWTnT. Both TnT-DCM mutants showed a significant decrease in the maximum ATPase activity when compared with HCWTnT, with the ΔK210 mutation having a larger impairment of maximal ATPase activity compared with the R141W mutation. So far, no one has investigated the functional effect caused by the TnT-R141W or the TnT-K273E mutation. We found that the DCM mutant TnT-R141W did not result in any change in Ca$^{2+}$ sensitivity and maximal force when compared with HCWTnT. The TnT-K273E mutation sensitized the muscle to lower Ca$^{2+}$ concentrations, consistent with most TnT-FHC-causing mutations. These results suggest that distinct DCM mutations within the same sarcomeric protein, TnT, have different effects in regulating muscle contraction.

**Materials and Methods**

**Mutation, Expression, and Purification of Cardiac TnT**—The cDNA for human cardiac TnT was previously cloned in our laboratory (12). The TnT-ΔK210, R141W, and K273E mutations were introduced in human cTnT by the method of sequential overlapping PCR (13). The purified DNA was sequenced to verify the presence of mutations prior to expression in BL21 bacterial cells. Standard methods were utilized for purification of HCWTnT and TnT-DCM mutants R141W and ΔK210. TnT-K273E, and TnI and TnC (14–16). The purity of the proteins was checked on SDS-PAGE after purification.

**Skinned Fiber Studies**—This experiment is used to measure the steady-state force and the Ca$^{2+}$ sensitivity of force development. The experiment is performed after displacement of the endogenous Tn complex and replacement with HCWTnT or TnT mutants and reconstitution with the human cardiac TnI and TnC complex. This protocol is well established in this laboratory (12, 17, 18). Porcine muscle fiber bundles were mounted on a force transducer and treated with the pCa 8.0 relaxing solution containing 1% Triton X-100 for ~1 h. The composition of pCa 8.0 solution was 10-4 M Ca$^{2+}$, 1 mM Mg$^{2+}$, 7 mM EGTA, 5 mM MgCl$_2$, 20 mM imidazole, pH 7.0 (20, 21, and 17 units/ml creatinine phosphokinase; ionic strength = 150 mM. Subsequently, the fibers were transferred to pCa 8.0 solution without Triton X-100 and then into pCa 4.0 solution for the initial force determination. The composition of the pCa 4.0 solution is the same as that of the pCa 8.0 buffer, except that the Ca$^{2+}$ concentration was ~10-5 M. To determine the Ca$^{2+}$ sensitivity of force development, the fibers were gradually exposed to the solutions of increasing Ca$^{2+}$ concentrations, from pCa 8.0 to pCa 4.0. To displace the endogenous Tn complex, the fibers were incubated in a solution containing 250 mM KC1, 20 mM MOPS, pH 6.2, 5 mM MgCl$_2$, 5 mM EGTA, 0.5 mM dithiothreitol, and ~0.8–1.0 mg/ml HCWTnT or TnT-DCM mutants, for 1 h at room temperature. After a 1-h incubation, the Ca$^{2+}$ sensitivity of force was evaluated in each experimental condition, the fibers were washed with the same solution without the protein (10 min at room temperature) and tested for the Ca$^{2+}$-unregulated force that developed due to the absence of the endogenous porcine cardiac TnI and TnC. The Ca$^{2+}$ sensitivity of force development was restored with a preformed human cardiac TnI and TnC complex. The reconstitution with the TnI and TnC complex (30 μM) was performed in the pCa 8 solution for ~1.5 h at room temperature for the force to reach a stable level. Control fibers were run in parallel and treated with the same solutions without the proteins. The final Ca$^{2+}$ sensitivity of force development was determined after human cardiac TnI and TnC reconstitution to check if by exposing the fiber to increasing Ca$^{2+}$ concentration from pCa 8.0 to pCa 4.0. The data was analyzed using the Hill equation in Sigma plot. The Hill equation is written as follows: pRelative force = 100 × [Ca$^{2+}$/$K_{50}\text{Ca}^{2+}$] + pCa$^{2+}$, where pCa$^{2+}$ denotes Ca$^{2+}$ concentration of the solution in which 50% of force is produced and nH is the Hill coefficient.

**CD Spectra of the Reconstituted Cardiac Troponin Complexes**—To determine the secondary structural content of the recombinant cardiac troponin complex, CD spectra of the HCWTnT and the TnT-reconstituted complexes were analyzed. The CD spectra were obtained using a Jasco J-720 spectropolarimeter using a cell path length of 0.1 cm at 200 nm and subtracted from solutions containing 0.01 M Tris, pH 7.0, 0.25 mM CaCl$_2$, 1 mM dithiothreitol, and 0.01% NaN$_3$. After dialysis they were mixed at a molar ratio 1:25:1 (TnT:HCWTnT:TnC) and incubated for 1 h on ice before being dialyzed against buffers with decreasing concentrations of urea. The dialysis buffer contained 1 M KCl, 10 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 0.01% NaN$_3$, and 6 M urea. Subsequently, the complexes were gradually dialyzed against buffers containing decreasing concentrations of KCl, starting from 1 M, then 0.7, 0.5, 0.3, and finally 0.1 M KCl. The excess TnT and TnC that precipitated at the low ionic strength without urea buffer was removed by centrifugation at 20,000 rpm for 30 min. The molar ratio of the reconstituted troponin complexes was verified by 1:1.5:1 (HCWTnT:TnI:TnC) at 210 nm. All ATPase assays were performed in the presence of 0.5 mM CaCl$_2$ and absence (1 mM EGTA) or presence (1 M MOPS, 50 mM KC1, 4 mM MgCl$_2$, pH 7.0) of 2.5 mM ATP. After 20 min of incubation at 37 °C, the reaction was terminated with 5% trichloroacetic acid. The inorganic phosphate that was released was measured according to the method of Fiske and Subbarow (22).

**Circular Dichroism (CD)**—Circular dichroism spectra of the HCWTnT and the TnT-reconstituted complexes were recorded on a Jasco J-720 spectropolarimeter using a cell path length of 0.1 cm at ambient temperature (22 °C) in a 10 mM sodium phosphate, pH 7.0, and 500 mM KC1 solution. The CD spectrum was recorded at 190–250 nm in the far UV region with a bandwidth of 1 nm at a speed of 50 nm/min and
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at a resolution of 0.2 nm. The concentration of HCWTnT and the TnT mutants was determined by Bradford assay using HCWTnT as a standard. The concentration of HCWTnT was accurately determined by nitrogen analysis. Analysis and processing of data was done using the Jasco system software. Ten scans were averaged, base lines were subtracted, and no numerical smoothing was applied. The mean residue ellipticity \([\theta]_{\text{MRE},222}\), in degrees cm\(^2\) dmol\(^{-1}\) for the spectra was calculated using the equation \([\theta]_{\text{MRE}} = [\theta]_{222} \times 10^2\), where \([\theta]_{222}\) is the measured ellipticity in millidegrees, \(C_r\) is the mean residue molar concentration, and I is the path length in cm.

The \(\alpha\)-helical content for each protein was calculated using the equation for \([\theta]_{222}\) at 222 nm (23) \([\theta]_{222} = -30300\, f_H - 2340\) where \(f_H\) is the fraction of \(\alpha\)-helical content \((f_H \times 100, \text{expressed in } \%\)). Spectra are presented as the mean residue ellipticity.

Data Analysis—Statistical analysis of the differences between mean values was done using the Student’s t test. Level of significance was set at \(p \leq 0.05\). Data values are presented as mean ± S.D.

RESULTS

Ca\(^{2+}\) Sensitivity of Force Development in Porcine Skinned Fibers—Fig. 1A compares the pCa-force relationship in the porcine fibers exchanged with HCWTnT and reconstituted with human cardiac TnI (HCtN) and human cardiac TnC (HCTnC) complex. As shown in Fig. 1A, porcine fibers exchanged and reconstituted with human Tn complex show a decrease in Ca\(^{2+}\) sensitivity \((\Delta pC_{50} = -0.14)\) when compared with untreated fibers. This result is consistent with what was previously observed in our laboratory (17, 18). This difference in Ca\(^{2+}\) sensitivity is due to the substitution of human Tn complex into the porcine fibers. In subsequent figures, the Ca\(^{2+}\) dependence of force development for both TnT-DCM mutants was compared with the Ca\(^{2+}\) dependence of force development of HCWTnT-treated fibers. Before TnT exchange, the Ca\(^{2+}\) dependence of force development was measured, and there was no statistically significant difference in Ca\(^{2+}\) sensitivity and cooperativity between the different groups of fibers used (data not shown). Fig. 1B shows the Ca\(^{2+}\) sensitivity of fibers exchanged with TnT-ΔK210. We observed a significant decrease in Ca\(^{2+}\) sensitivity \((\Delta pC_{50} = 5.27 ± 0.02)\) compared with HCWTnT-treated fibers \((pC_{50} = 5.39 ± 0.02)\). Fig. 1C shows the Ca\(^{2+}\) sensitivity of fibers exchanged with TnT-R141W. The TnT-R141W mutation caused a small but not statistically significant decrease in Ca\(^{2+}\) sensitivity of force development when compared with HCWTnT-treated fibers. We also determined the effect on the Ca\(^{2+}\) dependence of force development for the TnT-K273E mutation (Fig. 1D). Because a different batch of fibers was used for this set, we had to determine the effect of HCWTnT on these porcine fibers. As with the other batch of fibers, we observed a \(\Delta pC_{50}\) of −0.14 in porcine fibers treated with HCWTnT. When TnT-K273E was employed to displace the endogenous troponin from porcine fibers, a leftward shift in Ca\(^{2+}\) sensitivity where a \(\Delta pC_{50}\) of +0.17 was observed. Table I summarizes the pCa\(_{50}\) values of the pCa-force relationship.
and the Hill coefficient for the fibers treated with HCWTnT and TnT mutants. There was no significant difference in the Hill coefficient (which is a measure of cooperativity) between HCWTnT and TnT mutant-treated fiber groups.

**Ca**²⁺ *Regulation of Steady-state Force Development*—In our fiber studies, we measure the force produced under relaxing conditions (*pCa* 8.0) as the basal force and the force produced at *pCa* 4.0 as 100% in a typical untreated fiber. After *Tn* exchange and reconstitution, 70% of the force was recovered (Fig. 2) in HCWTnT-treated fibers. As described previously, we used a different set of fibers for the TnT-K273E mutation analysis, and the HCWTnT that we used as control produced the same amount of force as the other set of fibers used for the other mutations (70%). Therefore, the data for the HCWTnT-displaced fibers was pooled from both sets of fibers. The TnT-R141W and TnT-K273E-treated fibers showed no significant decrease in maximal force compared with fibers exchanged with HCWTnT (Fig. 2).

**Western Blot Analysis of Control, Troponin-displaced, and TnI/TnC-reconstituted Fibers**—We did Western blot analysis of the control fibers (fibers treated with 1% Triton X-100), the fibers displaced with WT or mutant TnT (these fibers were not reconstituted with TnI/TnC), and fibers displaced with WT or mutant TnT and reconstituted with TnI/TnC. We checked both displacement and reconstitution for each mutant. We used double antibody staining for human cardiac TnI, and regulatory light chain (RLC) served as a loading control. Our analysis indicates that the displacement of troponin was essentially complete. Fig. 3 is a Western blot of the control, displaced and reconstituted fibers immunostained for TnI and RLC. Fig. 3, lane 2 shows a control fiber treated with 1% Triton for half an hour. The amount of TnI present in the control fiber is taken as 100%. The TnI present in TnT-displaced fiber and TnI/TnC-reconstituted fiber is stated relative to the control fiber. Fig. 3, lane 3 is a TnT-WT-displaced fiber. Densitometric analysis showed that 98% ± 5% of endogenous porcine TnI is displaced. Fig. 3, lane 4 is a TnT-R141W displaced fiber. Lane 5 is a TnT-ΔK210-displaced fiber; lane 6 is the TnT-K273E-displaced fiber. We observed 95% ± 6%, 95 ± 5% and 97 ± 4% displacement of endogenous TnI for TnT-R141W, ΔK210, and K273E, respectively. Lane 7 is the TnT-WT-displaced and TnI/TnC-reconstituted fiber. Here, we can see that after TnI/TnC treatment, the displaced TnI is readily reconstituted. Our quantification indicates that the amount of TnI reconstituted is 106% ± 5% for HCWTnT, 104 ± 6% for R141W, 105 ± 4% for ΔK210, and 108 ± 4% for K273E (data not shown).

**ATPase Assays**—ATPase assays were carried out in the presence and absence of Ca²⁺ to investigate the activation and inhibition of actomyosin ATPase activity. Fig. 4 shows inhibition and Fig. 5 shows activation of ATPase activity. The myosin ATPase activity in the absence of HCTn was considered as the basal activity (0% inhibition). The inhibition of ATPase activity was found to be similar for Tn complexes containing HCWTnT or TnT mutants R141W, ΔK210, and K273E. At 2-μM Tn concentration, the Tn complex containing HCWTnT and DCM mutants was able to inhibit the ATPase activity to a similar extent (~85% inhibition for all four proteins investigated). At 1 μM, TnT-R141W inhibits slightly better than HCWTnT (61% ± 6% for HCWTnT versus 70% ± 2% for TnT-R141W). However, this difference in inhibition was not found to be statistically significant by Student’s *t* test. As shown in Fig. 5, the bar chart compares the ATPase activity in the presence of Ca²⁺ between HCWTnT and TnT mutants. The experiment was performed by

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**Table I**

| HCTnT utilized in the fiber | *pCa*₂⁰ | Hill coefficient (nH) | No. of experiments (n) |
|---------------------------|---------|-----------------------|------------------------|
| HCWTnT                    | 5.39 ± 0.02 | 1.82 ± 0.11 | 4                      |
| TnT-ΔK210                 | 5.27 ± 0.02* | 1.74 ± 0.11 | 4                      |
| TnT-R141W                 | 5.35 ± 0.03 | 1.74 ± 0.04 | 4                      |
| HCWTnTb                   | 5.52 ± 0.02 | 2.24 ± 0.09 | 3                      |
| TnT-K273E                 | 5.67 ± 0.20* | 2.11 ± 0.2  | 3                      |

* The *pCa*₂⁰ value for the TnT- ΔK210 and TnT-K273E is significantly different from HCWTnT (*p* < 0.01).

* The experiment was performed in a different batch of fibers.

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**FIG. 2.** *Effect of TnT mutation on maximal force.* The Ca²⁺ dependence of maximal force produced after reconstitution with whole troponin complex containing either HCWTnT or TnT mutants is shown. *+* indicates that the maximal force produced when TnT-ΔK210 was utilized in the whole troponin complex is significantly altered to lower values when compared with fibers treated with troponin complex containing HCWTnT. Values are relative to the initial force obtained by the skinned fiber before reconstitution.

**FIG. 3.** *Western blot of the Control, TnT (WT and mutants)-treated, and HCTnHCTnC-reconstituted fibers.* Lane 1, TnI standard; lane 2, control fiber; lane 3, HCWTnT-treated fiber; lane 4, TnT-R141W-treated fiber; lane 5, TnT-ΔK210-treated fiber; lane 6, TnT-K273E-treated fiber; lane 7, fiber displaced with HCWTnT and reconstituted with HCTnHCTnC complex; lane 8, Bio-Rad prestained SDS-PAGE standards; lane 9, RLC standard. The blots were stained with monoclonal anti-cardiac Troponin I and a polyclonal rabbit RLC antibody. Anti-TnI goat anti-mouse-polyclonal and anti-RLC goat anti-rabbit polyclonal secondary antibodies were utilized.
studies were carried out to determine whether the TnT-DCM concentration. The assay conditions were as follows. 3.5 μM actin, 1 μM Tm, 0–2 μM Tn, and 0.6 μM myosin were dissolved in 10 mM MOPS, 50 mM KCl, 4 mM MgCl₂, pH 7.0, and 1 mM EGTA. Each data point represents the average of four experiments, each performed in triplicate, and is expressed as mean ± S.D.

varying the Tn concentration such that the final concentrations were 0, 0.5, 1, 1.5 μM. At Tn concentrations above 1 μM, the maximum ATPase activity did not increase further and plateaued. The ATPase activity in the absence of Tn was considered to be the basal activity or 100% activity in this assay. As shown in Fig. 5, TnT-DCM mutants showed a decrease in ATPase activity compared with HCWTnT. The ATPase activity of HCWTnT was 134% compared with HCWTnT, whereas the TnT-R141W mutant showed 41.9% and 38.9%, respectively. This change in α-helical content is not a significant change in secondary structure when compared with HCWTnT.

Circular Dichroism of HCWTnT and TnT Mutants—CD studies were carried out to determine whether the TnT-DCM mutants affected the secondary structure of TnT (Fig. 6). The average mean residue molar ellipticity for HCWTnT at 222 nm was −13,854 (corresponding to an α-helical content of 39.6%). The α-helical content of TnT-R141W mutant was 40.5%, whereas the other mutants, TnT-ΔK210 and TnT-K273E, showed 41.9% and 38.9%, respectively. This change in α-helical content is not a significant change in secondary structure when compared with HCWTnT.

FIG. 4. Regulation of actomyosin ATPase activity in the absence of Ca²⁺. The effect of HCWTnT and TnT mutants on the inhibition of actomyosin ATPase activity was measured as a function of Tn concentration. The assay conditions are as follows. 3.5 μM actin, 1 μM Tm, 0–2 μM Tn, and 0.6 μM myosin were dissolved in 10 mM MOPS, 50 mM KCl, 4 mM MgCl₂, pH 7.0, and 1 mM EGTA. Each data point represents the average of four experiments, each performed in triplicate, and is expressed as mean ± S.D.

FIG. 5. Regulation of actomyosin ATPase activity in the presence of Ca²⁺. The effect of HCWTnT and TnT mutants on the activation of actomyosin ATPase activity is measured by varying troponin concentrations; the effect at 1 μM Tn, the concentration of Tn that gave the maximal activity, is shown in the figure. The assay conditions are as follows. 3.5 μM actin, 1 μM Tm, 0 and 1 μM Tn, and 0.6 μM myosin were dissolved in 10 mM MOPS, 50 mM KCl, 4 mM MgCl₂, pH 7.0, and 0.6 mM CaCl₂. Each data point represents the average of four-five experiments, each performed in triplicate, and is expressed as mean ± S.D. 100% corresponds to the activity in the absence of Tn. * indicates level of significance with p < 0.05. ** indicates level of significance with p < 0.001.

FIG. 6. Circular dichroism spectra of HCWTnT and TnT mutants. All the samples were used at a concentration of 0.3 mg/ml in 10 mM NaPO₄, pH 7.0, containing 0.5 M KCl. The average mean residue ellipticity [θ] for the proteins used (degrees·cm²/dmol⁻¹) is as follows: HCWTnT, −13,854; TnT-ΔK210, −15037; TnT-R141W, −14610. Each data point represents an average of three experiments; each experiment consisted of an average of 10 scans and is expressed as the mean.

Discussion

The main goal of this study was to determine whether the TnT mutants so far known to cause DCM have the same or different properties in the regulation of muscle contraction. Mutations in other cytoskeletal and sarcomeric proteins show that the pathogenesis of DCM could occur because of failure at various levels: defects in the force generating capability of the sarcomere, defects in force transmission, defects in energy production, and increased susceptibility of the sarcomere to degenerative processes such as apoptosis (7, 24).

Mutations in sarcomeric proteins cause cardiomyopathies, including FHC, restrictive cardiomyopathy, and DCM. The functional consequences of TnT mutations causing FHC have been extensively characterized (12, 17, 25–27). Previously, our laboratory and others have shown that FHC-causing mutations in TnT predominantly increase myofilament Ca²⁺ sensitivity (12, 25, 28). In a few cases, some FHC mutants show no change in Ca²⁺ sensitivity. Fiber studies in transgenic mice harboring the TnT-FHC mutations (I79N and R92Q) also showed increased Ca²⁺ sensitivity, which possibly leads to altered cardiac energetics through increased cost of force production in vivo (29, 30). In addition to changes in Ca²⁺ sensitivity, FHC TnT mutations lead to alterations in ATPase activity, troponin solubility and folding, thin filament sliding speed, and the Ca²⁺ affinity of troponin C (27, 28, 31). Recently six mutations in TnI have been shown to be associated with restrictive cardiomyopathy (32). However, the functional consequences of these TnI mutations have not been investigated.

So far, only two HCTnT mutations have been shown to cause DCM independent of FHC. To determine how the TnT-DCM mutations affect the regulatory properties of the thin filament, we analyzed the effect of the mutations in two different systems, reconstituted thin filaments and skinned cardiac muscle fiber preparations. Studies on the Ca²⁺ sensitivity of force development in porcine cardiac fibers demonstrated that TnT-
ΔK210 caused a significant decrease in Ca\textsuperscript{2+} sensitivity (Fig. 1B). This mutation has been studied by two other groups. Morimoto et al. (11) investigated this first DCM-TnT mutation and reported a rightward shift in Ca\textsuperscript{2+} sensitivity in skinned rabbit cardiac muscle fibers. We obtained similar results with respect to the Ca\textsuperscript{2+} sensitivity and, in fact, the magnitude of shift we observed was greater than the 0.1 ΔpCa shift seen by Morimoto et al. (11). In addition, we observed a decrease in the maximal force for TnT-ΔK210-treated fibers with no significant change in cooperativity when compared with HCWTnT (Fig. 2 and Table I). This is the first report of this TnT-ΔK210 mutation causing a decrease in maximal force, because no decrease in maximal force was detected by Morimoto et al. (11).

We also examined the ability of TnT-ΔK210 to inhibit and activate actin-Tn-activated myosin ATPase activity in reconstituted thin filament systems to determine whether these activities of the Tn complexes containing the TnT deletion mutant are affected. We found that the TnT-ΔK210 was able to inhibit ATPase activity nearly as fully (∼80% at 2 μM) as the HCWTnT (Fig. 4). TnT-ΔK210 is located in a region of TnT that binds to TnI as well as TnC and Tm. However, it is likely, based on data from the preliminarily reported atomic structure of Tn (33), that this specific region (residues 200–220 of TnT does not bind to the inhibitory regions of TnI and, as such, would not affect the inhibitory activity of Tn. However, we found that the maximal ATPase activity in Tn containing the deletion mutation was significantly decreased when compared with Tn containing HCWTnT (Fig. 5). Tn containing the TnT-ΔK210 showed a striking decrease in its inability to activate myosin to hydrolyze ATP in the presence of calcium. These results are in agreement with recent observations by Robinson et al. (34) who also investigated the TnT-ΔK210 mutation and found that the mutation caused significantly less activation at pCa 4.5 when compared with wild type. They also observed no difference in the ability of Tn containing this mutation to inhibit ATPase activity, consistent with our results.

It has been previously shown by our group that Ca\textsuperscript{2+} binding to TnC, in addition to relieving inhibition of TnI, activates ATPase through interaction with TnT (35). Chemical cross-linking studies by Leszyk et al. (36) suggest that the lysine residues 208–210 in TnT form a tight binary complex with TnC. The removal of one of these lysine residues may decrease the affinity of TnC for TnT, resulting in conformational changes in either or both TnT and TnC, resulting in a change in the affinity of TnC for Ca\textsuperscript{2+}. It is likely that the TnT-ΔK210 impaired the ATPase cycling rate by affecting the rate of attachment of force generating cross bridges producing reduced force under physiological Ca\textsuperscript{2+} concentrations. To compensate for this reduced force, the ventricle may undergo remodeling leading to dilation.

No studies have so far been reported on the functional consequence of the other DCM mutant, TnT-R141W. Investigation of the TnT-R141W mutation showed that this mutation behaved differently from the TnT-ΔK210 mutation with respect to the Ca\textsuperscript{2+} sensitivity of force development (Fig. 1C). Fibers containing TnT-R141W showed a slight but not significant decrease in the Ca\textsuperscript{2+} sensitivity when compared with HCWTnT-treated fibers. In addition, unlike TnT-ΔK210, the R141W mutation did not cause a change in maximal Ca\textsuperscript{2+}-activated force when compared with HCWTnT (Fig. 2). Morimoto et al. (11) postulated that a decrease in Ca\textsuperscript{2+} sensitivity was the main cause of DCM. However, the results from the TnT-R141W mutant suggest that this may not be the case for all TnT-DCM mutations. In our ATPase inhibitory assays, we found that the TnT-R141W mutation was also able to inhibit the ATPase activity −80%. This value is similar to what was seen with HCWTnT and TnT-ΔK210 (Fig. 4), indicating that the TnT-DCM mutations are not disrupting the inhibitory function of TnI. Interestingly, although we did not see a significant decrease in Ca\textsuperscript{2+} sensitivity of force development, we observed a significant decrease in maximal ATPase activity by TnT-R141W when compared with Tn containing HCWTnT. However, the decrease in maximal ATPase activity by TnT-R141W mutant was less pronounced when compared with TnT-ΔK210 (Fig. 5). One possible reason for this could be that the TnT-R141W mutation is present in the N-terminal region of TnT that binds to only Tm. Although many FHC-causing mutations occur within the N-terminal Tm binding region of TnT, no FHC mutant occurs within 10 amino acids of the TnT-R141W mutation. Palm et al. (37) introduced nine mutations in a TnT fragment containing residues 70–170 and found that five TnT-FHC mutations between residues 92 and 110 impaired Tm-dependent functions of TnT. Most FHC mutations in this region bound less strongly to a Tm affinity column and were less able to stabilize the Tm overlap complex. TnT-FHC mutations outside the region did not affect the above mentioned Tm-dependent function. Recently, it has been shown by Tobacman et al. (38) that critical interactions between Tn and Tm depend on highly conserved TnT residues between 112–136 in the bovine cardiac TnT (this region corresponds to residues 115–139 in humans). The R141W mutation is two residues away from this potential Tm-anchoring region and may cause subtle conformational changes in this region that alter the Tn-Tm interaction. The decreased ATPase activity seen for both mutants will likely result in the systolic dysfunction observed in patients with these mutations.

CD spectroscopy was carried out to determine whether the TnT-DCM mutants affected the secondary structure of TnT (Fig. 6). Although little difference was found between the α-helical content of TnT-R141W (40.5%) and TnT-ΔK210 (41.9%) when compared with wild-type TnT (39.6%), the small difference in α-helical content of the TnT-ΔK210 (41.9%) may be functionally important for the observed differences between HCTnT. The S.D. for wild-type TnT was ± 220 degree-cm\textsuperscript{2}-dmol\textsuperscript{-1} (based on three independent spectra), which corresponds to an error of less than 1% α-helical content. It is also possible that the TnT-R141W mutation causes changes in tertiary structure that are not observed at the secondary structure level. It is not known what regions(s) of the HCTnT mutants are affected by these mutations. Two other TnT mutations that cause DCM—R92W and K273E—are of interest. However, these mutations first cause FHC, which later progresses to DCM (10, 39). Investigation of these mutations will allow us to determine whether these FHC to DCM-causing mutations are similar/different to either FHC- or DCM-causing mutations. The TnT-R141W mutation, in TnT, leads to asymmetric septal hypertrophy as well as DCM in affected individuals. Moreover, in certain individuals with typical hypertrophy, the disease progresses to DCM. It is not clear what factors contribute to this transition from hypertrophy to progressive dilation. In our experiments, the TnT-R273E mutation sensitized the muscle to lower calcium concentrations. The leftward shift in calcium sensitivity is consistent with other TnT-FHC-causing mutations. The patients who inherited this mutation exhibited variable cardiac morphologies. Interestingly, Freeman et al. (40) have shown that this kind of transition from FHC to DCM is also observed in an animal model with the R403Q mutation in the cardiac α-myosin heavy chain, which suggests that FHC and DCM may be part of the same pathological spectrum. There is a high incidence of sudden cardiac death in infants as well as adults among patients harboring the TnT-ΔK210 mutation.
Properties of TnT Mutants Causing Dilated Cardiomyopathy

The decreased ATPase activity and Ca\(^{2+}\) sensitivity as well as impaired force production that we observe could provide the inciting stimulus for the sarcomere to remodel leading to DCM. The differences observed between the two DCM mutations investigated in this report may be consistent with the different phenotypes produced by the different mutations. The clinical features exhibited by patients having the TnT-R141W mutation are different from the TnT-ΔK210 mutant, although the TnT-R141W mutation also causes primary DCM in the affected individuals. None of the family members had left ventricular wall thickness >13 mm, confirming that DCM occurs in this family independent of FHC. The authors noted that this mutation did not cause sudden cardiac death (9). Moreover, only half of the fourteen patients who inherited the TnT-R141W mutation had an ejection fraction of less than 50%, resembling the hallmark features of DCM, in contrast with the TnT-ΔK210 mutation in patients is highly variable with moderate to high penetrance. The in vitro results presented here suggest that the R141W mutation would cause a less severe DCM phenotype than the ΔK210 mutation. It is likely that the location of the mutation determines the severity of the disease.

Although the detailed molecular function of TnT still remains unclear, the two DCM mutations in different functional regions of the TnT molecule appear to alter its function in a different way. Because Ca\(^{2+}\) is the primary activator of the thin filament leading to muscle contraction, alterations in the Ca\(^{2+}\) sensitivity of force development in either direction are expected to affect the regulatory properties of thin filament. These results suggest that a decrease in Ca\(^{2+}\) sensitivity is unlikely to be a common denominator in the pathogenesis of DCM caused by TnT mutations.

REFERENCES

1. Gergely, J. (1998) Adv. Exp. Med. Biol. 453, 169–176
2. Zat, A. S., and Potter, J. D. (1987) Annu. Rev. Biophys. Biophys. Chem. 16, 535–559
3. Tobacman, L. S. (1996) Annu. Rev. Physiol. 58, 447–481
4. Seidman, J. G., and Seidman, C. (2000) Annu. Rev. Physiol. 62, 945–989
5. Kamisago, M., Sharma, S. D., DePalma, S. R., Solomon, S., Sharma, P., McDougleigh, B., Smoot, L., Mullen, M. P., Woolf, P. K., Wile, E. D., Seidman, J. G., and Seidman, C. E. (2000) N. Engl. J. Med. 343, 1688–1696
6. Li, D., Czernuszewicz, G. Z., Gonzalez, O., Tappcott, T., Karibe, A., Durand, J. B., Brugada, R., Hill, R., Gregortich, J. M., Anderson, J. L., Quinones, M., Bachinski, L. L., and Roberts, R. (2001) Circulation 104, 2188–2193
7. Fujino, N., Shimizu, M., Ino, H., Yamaguchi, M., Yasuda, T., Nagata, M., Konno, T., and Mabuchi, H. (2002) Am. J. Cardiol. 89, 29–33
8. Morimoto, S., Lu, Q. W., Harada, K., Takahashi-Yanaga, F., Minakami, R., Ohta, M., Sasaguri, T., and Ohtsuki, I. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 913–918
9. Szczesna, D., Zhang, R., Zhao, J., Jones, M., Guzman, G., and Potter, J. D. (2000) J. Biol. Chem. 275, 624–630
10. Austubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) in Current Protocols in Molecular Biology (Janesen, K., ed.) pp. 8.0.1–8.5.9, John Wiley & Sons, New York
11. Pan, B. S., and Potter, J. D. (1992) J. Biol. Chem. 267, 23052–23056
12. Sheng, Z., Pan, B. S., Miller, T. E., and Potter, J. D. (1992) J. Biol. Chem. 267, 25497–25413
13. Szczesna, D., Guzman, G., Miller, T., Zhao, J., Farokhi, K., Ellemberger, H., and Potter, J. D. (1996) J. Biol. Chem. 271, 8381–8386
14. Lang, R., Gomez, A. V., Zhao, J., Housmans, P. R., Miller, T., and Potter, J. D. (2002) J. Biol. Chem. 277, 11670–11678
15. Gomez, A. V., Guzman, G., Zhao, J., and Potter, J. D. (2002) J. Biol. Chem. 277, 35341–35349
16. Strzelecka-Golaszewska, H., Jakubiak, M., and Drabikowski, W. (1975) Eur. J. Biochem. 55, 221–230
17. Murakami, U., Uchida, K., and Hiratsuka, T. (1976) J. Biochem. (Tokyo) 80, 611–619
18. Potter, J. D. (1982) Methods Enzymol. 85, 241–263
19. Fiske, C. H., and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
20. Chen, Y. H., and Yang, J. T. (1971) Biochem. Biophys. Res. Commun. 44, 1285–1291
21. Schonberger, L., and Seidman, C. E. (2001) Am. J. Hum. Genet. 69, 249–360
22. Redwood, C., Lohmann, K., Bing, W., Esposito, G. M., Elliott, K., Abdulrazzak, H., Knott, A., Purcell, I., Marston, S., and Watkins, H. (2000) Circ. Res. 86, 1146–1152
23. Takahashi-Yanaga, F., Ohtsuki, I., and Morimoto, S. (2001) J. Biochem. (Tokyo) 130, 130–131
24. Tobacman, L. S., Lin, D., Butten, C., Landis, C., Back, N., Pavlov, D., and Homsher, E. (1999) J. Biol. Chem. 274, 26363–26370
25. Hernandez, O. M., Housmans, P. R., and Potter, J. D. (2001) J. Appl. Physiol. 90, 1125–1136
26. Czernuszewicz, G. Z., Gonzalez, O., Tappcott, T., Karibe, A., Durand, J. B., Brugada, R., Hill, R., Gregoritich, J. M., Anderson, J. L., Quinones, M., Bachinski, L. L., and Roberts, R. (2001) J. Clin. Invest. 111, 209–216
27. Takeda, S., Yamaahita, A., Maeda, K., and Maeda, Y. (2002) Biophys. J. 82, 170a (abstr.)
28. Robinson, P., Mirza, M., Knott, A., Abdulrazzak, H., Willott, R. Marston, S., Watkins, H., and Redwood, C. (2002) J. Biol. Chem. 277, 40710–40716
29. Potter, J. D., Sheng, Z., Pan, B. S., and Zhao, J. (1995) J. Biol. Chem. 270, 2557–2562
30. Leszyk, J., Collins, J. H., Leavis, P. C., and Tao, T. (1998) Biochemistry 27, 6983–6987
31. Palm, T., Grubeski, S., Hitchcock-DeGregori, S. E., and Greenfield, N. J. (2001) Biophys. J. 81, 2857–2887
32. Hinkle, A., and Tobacman, L. S. (2003) J. Biol. Chem. 278, 506–513
33. Fujino, N., Shimizu, M., Ino, H., Okeke, K., Yamaguchi, M., Yasuda, T., Kokado, H., and Mabuchi, H. (2001) Clin. Cardiol. 24, 397–402
34. Freeman, K., Colon-Rivera, C., Olsson, M. C., Moore, R. L., Weinberger, H. D., Grupp, I. L., Vikstrom, K. L., Iaccarino, G., Koch, W. J., and Leinwand, L. A. (2001) Am. J. Physiol. 280, H151–H159
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