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Pathogenesis associated with a restrictive cardiomyopathy mutant in cardiac troponin T is due to reduced protein stability and greatly increased myofilament Ca^{2+} sensitivity☆

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ABSTRACT

Background: Dilated and hypertrophic cardiomyopathy mutations in troponin can blunt effects of protein kinase A (PKA) phosphorylation of cardiac troponin I (cTnI), decreasing myofilament Ca^{2+}-sensitivity; however this effect has never been tested for restrictive cardiomyopathy (RCM) mutants. This study explores whether an RCM cardiac troponin T mutant (cTnT-ΔE96) interferes with convergent PKA regulation and if TnT instability contributes to greatly enhanced Ca^{2+}-sensitivity in skinned fibers.
Methods: Force of contraction in skinned cardiac porcine fiber and spectroscopic studies were performed.
Results: A decrease of −0.26 and −0.25 *p*Ca units in Ca^{2+}-sensitivity of contraction after PKA incubation was observed for skinned fibers incorporated with WT or cTnT-ΔE96, respectively. To further assess whether cTnT-ΔE96 interferes solely with transmission of cTnI phosphorylation effects, skinned fibers were reconstituted with PKA pseudo-phosphorylated cTnI (cTnI-SS/DD.cTnC). Fibers displaced with cTnT-WT, reconstituted with cTnI-SS/DD.cTnC decreased Ca^{2+}-sensitivity of force (pCa_{50} = 5.61) compared to control cTnI-WT.cTnC (pCa_{50} = 5.75), similarly affecting cTnT-ΔE96 (pCa_{50} = 6.03) compared to control \cTnI-WT.cTnC (pCa_{50} = 6.14). Fluorescence studies measuring $cTnC^{IAANS}$ Ca^{2+}-affinity changes due to cTnT-ΔE96 indicated that higher complexity (thin filament) better recapitulates skinned fiber Ca^{2+} sensitive changes. Circular dichroism revealed reduced α-helicity and earlier thermal unfolding for cTnT-ΔE96 compared to WT.
Conclusions: Although ineffective in decreasing myofilament Ca^{2+}-sensitivity to normal levels, cTnT-ΔE96 does not interfere with PKA cTnI phosphorylation mediated effects; 2) cTnT-ΔE96 requires actin to increase cTnC Ca^{2+}-affinity; and 3) deletion of E96 reduces cTnT stability, likely disrupting crucial thin filament interactions.
General significance: The pathological effect of cTnT-ΔE96 is largely manifested by dramatic myofilament Ca^{2+}-sensitization which still persists even after PKA phosphorylation mediated Ca^{2+}-desensitization.
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1. Introduction

Restrictive cardiomyopathy (RCM) is an uncommon cardiomyopathic disorder that is characterized by abnormal diastolic function that results from impaired ventricular filling, increased ventricular end-diastolic pressures, and dilated atria. RCM patients generally maintain systolic function, however dysfunction may occur in late stages thus leading to heart failure. Pediatric cases of RCM typically have poor prognosis and the treatment endpoint is often transplantation [1–5].

A number of mutations have been found in the genes encoding proteins that make up the cardiac troponin complex (cTn), the resulting mutant proteins have been shown to be significant causes of genetic based cardiomyopathies [6,7]. To date, troponin-linked RCM mutations have been identified in the *TNNI3* and *TNNT2* genes. The first RCM mutation reported in the cTnT (*TNNT2* gene) was a deletion of glutamic acid found at position 96 (cTnT-ΔE96) in a pediatric patient [1]. Cardiac Tn has an important role in regulating cardiac contractility, therefore amino acid deletions or substitutions that disrupt its function can lead to dysregulation of interactions between the thin and thick filaments [8,9]. The cTn complex is constituted by three subunits: troponin C

Abbreviations: cTnT, cardiac Troponin T; RCM, restrictive cardiomyopathy; PKA, protein kinase A; cTnT-ΔE96, cardiac troponin T with glutamic acid 96 deleted; cTnI-SS/DD, cardiac troponin I with serines 23 and 24 mutated to aspartic acid; IAANS, 2-(4′-(Iodoacetomido) aniline)naphthalene-6-sulfonic acid

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(cTnC), confers the Ca^{2+} sensitive properties to striated muscle; cTnI, prevents interactions of myosin with actin at subthreshold Ca^{2+} levels; cTnT, has a key role in activation of muscle contraction and physically links the Tn complex with tropomyosin (Tm) in the thin filament [10–12]. A more refined view of cTnT function has been derived from studying cardiomyopathic mutations in cTnT which appears to have additional nuanced roles in muscle contraction including modulation of actomyosin ATPase activity and the kinetics of contraction, Ca^{2+} sensitivity of contraction as well as maximal force [6,13,14].

Previously, our group has performed *in vitro* studies that elucidated the functional defects caused by the *TNNT2* associated RCM mutation, the cTnT-ΔE96. Functional parameters of the mutant cTnT-ΔE96 were greatly altered, along with early presentation in the proband, both indicating the severity of the disease phenotype [1,15]. Skinned fibers reconstituted with the cTnT-ΔE96 mutant protein showed a large increase in Ca^{2+} sensitivity of force and an inability to fully relax; reconstituted assays also revealed that the mutant troponin complex was unable to fully inhibit myosin–actin–tropomyosin ATPase activity [15]. Our findings were further corroborated by another study that recapitulated the increased Ca^{2+} sensitivity of contraction in skinned fibers containing cTnT-ΔE96, using a different protocol for incorporation of exogenous proteins [16]. Although little is known about the pathological mechanisms underlying *TNNT2* RCM mutations, it has been previously suggested that the pathogenesis associated with *TNNI3* RCM mutations involves drastic sensitization of the myofilament to Ca^{2+} [17,18].

Developmentally important is the switching of TnI isoforms, from the fetal (slow skeletal TnI) to the adult isoform (cTnI) during embryogenesis and postnatal development [19–21]. Since the patient had a severe onset of disease shortly after birth, we evaluated whether the deletion of amino acid E96 in cTnT further altered regulatory mechanisms that modulate the contractile response in cTnI that contains the PKA target sites in the N-terminal extension. During β-adrenergic stimulation of the heart, cTnI is phosphorylated at serines 23 and 24 by PKA which decreases the Ca^{2+} sensitivity of contraction and enhances the relaxation rate of the heart [22–24]. Therefore, sarcomeric protein phosphorylation is a prominent mechanism for maintenance of cardiac function and homeostasis [25]. The rationale to study the effects of PKA phosphorylation in the presence of a RCM mutant is that recent reports indicate that sarcomeric mutants linked to dilated and hypertrophic cardiomyopathies interfere with the Ca^{2+} desensitizing effect of cTnI PKA phosphorylation [26–32]. However, this effect has never been tested for an RCM mutant protein incorporated into the thin filament.

This study was designed to elucidate the additional factors that could contribute to the severe disease demonstrated by the proband. The goals were twofold: 1) to test whether the RCM cTnT-ΔE96 mutant affected convergent regulation by cTnI PKA phosphorylation in skinned porcine fibers and 2) to determine whether stability of TnT underlies the mechanism causing greatly enhanced Ca^{2+} sensitivity of contraction seen in skinned fibers.

2. Experimental procedures

2.1. Cloning, expression, and purification of human cardiac troponin, cardiac troponin T isoforms WT and RCM mutant

The cDNAs for human cTnI, and cTnC were cloned as previously described [33]. The pseudo-phosphorylated human cTnI-SS/DD was produced by overlapping mutagenic primers that replaced the two adjacent Ser at position 23/24, to aspartic acid (D). Standard laboratory protocols were utilized for expression and purification of human cTnC, cTnI (WT and SS/DD), and cTnT (WT and cTnT-ΔE96) [15,33]. The porcine cardiac tropomyosin (Tm) and rabbit skeletal actin were prepared as previously described [33].

2.2. Cardiac skinned fiber studies

2.2.1. Fiber preparation

Porcine papillary muscle was isolated from porcine hearts and prepared according to the following methods [15]. The left ventricular papillary muscles were cut into strips and skinned overnight in a *p*Ca 8.0 solution containing 50% glycerol and 1% Triton X-100 at 4°C. Afterwards, the muscle strips were transferred to *p*Ca 8.0 containing 50% glycerol, without Triton X-100 and stored up to 2 months at −20°C.

2.2.2. Tn-displaced skinned cardiac fibers

The effects of the RCM cTnT-ΔE96 mutant on Ca^{2+}-dependent parameters of muscle contraction were determined upon displacement of endogenous porcine cTn with either the cTnT-WT or cTnT-ΔE96 mutant to be studied (for further details, see [15]). The fibers were then reconstituted with either binary complex: cTnI-WT.cTnC or cTnI-SS/DD.cTnC.

2.2.3. Steady state and calcium dependence of force development

Fibers were mounted on tweezer clips connected to a force transducer on one side and submerged in a 1.3-ml cuvette containing *p*Ca 8.0 solution (10^{-8} M Ca^{2+}, 1 mM Mg^{2+}, 7 mM EGTA, 2.5 mM $MgATP^{2-}$, 20 mM MOPS, pH 7.0, 20 mM creatine phosphate, and 15 units/ml creatine phosphokinase, ionic strength 150 mM). The Ca^{2+} sensitivity of contraction was measured by exposing the fibers to Ca^{2+}-containing solutions of increasing Ca^{2+} concentrations ranging from *p*Ca 8.0 to 4.0. Data were analyzed using the equation % change in force $= 100 \times [Ca]^n / ([Ca^{2+}]^n + [Ca^{2+}{}_{50}]\ n)$, where $[Ca^{2+}{}_{50}]$ is the free Ca^{2+} concentration producing 50% force, and n is the Hill coefficient. For PKA measurements the skinned fibers were incubated with 500 units/ml PKA catalytic subunit (Sigma P2645) for 30 min in *p*Ca 8.0.

2.3. Formation of ternary and binary complexes

The troponin complexes were formed as previously described [15]. The correct stoichiometry of the binary or ternary complexes was verified by SDS-PAGE before storage of complexes at −80°C.

2.4. Fluorescence labeling of cTnC

For cTn and cTn including tropomyosin, the fluorescence measurements monitoring binding of Ca^{2+} to cTnC utilized the double label configuration with 2-(4′-(iodoacetomido)aniline)naphthalene-6-sulfonic acid (IAANS) located at Cys-35 and Cys-84 (prior to complex formation). For fluorescence measurements of the thin filament, cTnC had Cys-35 mutated to Ser (C35S) and was labeled with IAANS only at Cys-84. In this case, the troponin complexes were formed with only IAANS labeled cTnC C35S. IAANS was obtained from Molecular Probes, Plano, TX. Fluorescent labeling was performed according to established methods [34,35].

2.5. Determination of apparent Ca^{2+} affinities by fluorescence

Thin filaments were constructed using the protocol established in our laboratory [34]. Steady state fluorescence measurements were performed in a Jasco 6500 spectrofluorometer where IAANS fluorescence was excited at 330 nm and emission was detected at 450 nm. The protein concentrations used for the cTn, cTn with tropomyosin and thin filaments were 0.25 μM, 0.54 μM and 0.025 mg/ml, respectively. The concentration of free Ca^{2+} and amounts of titrated Ca^{2+} were calculated using the *p*Ca calculator program [36]. The data were fitted to a version of the Hill equation that accounted for the spectral changes that occur at a low Ca^{2+} concentration.

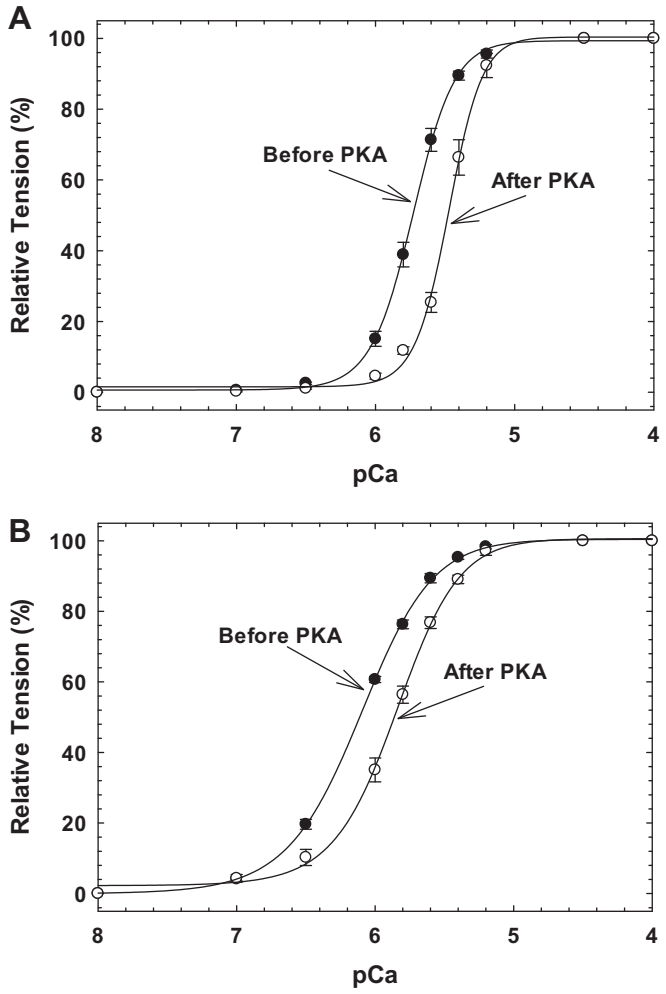

Fig. 1. Normalized *p*Ca force relationship in skinned cardiac muscle fibers before and after PKA incubation. The Ca^{2+} dependence of force development was measured before (filled symbols) and after (open symbols) PKA catalytic subunit incubation. A) Fibers displaced with cTnT-WT; B) Fibers displaced with cTnT-ΔE96. Data are expressed as mean ± S.E.

2.6. Circular dichroism measurements

Far-UV CD spectra were collected using a 1-mm-path quartz cell in a Jasco J-720 spectropolarimeter. Spectra were recorded at 195–250 nm with a bandwidth of 1 nm at a speed of 50 nm/min, and a resolution of 0.5 nm at room temperature (20°C). Ten scans were averaged, and no numerical smoothing was applied. The optical activity of the buffer was subtracted from relevant protein spectra. Mean residue ellipticities ($[\theta]_{MRE}$ in millidegree·cm^2/dmol) for the spectra were calculated using Jasco system software and the following equation: $[\theta]_{MRE} = [\theta] / (10 \times Cr \times L)$ where $[\theta]$ is the measured ellipticity in millidegrees, Cr is the mean residue molar concentration, and L is the path length in cm. Protein concentrations were determined by the biuret reaction using bovine serum albumin as a standard. The experimental protein concentration for the cTnT-WT and cTnT-ΔE96 was 0.2 mg/ml. The buffer used contained 10 mM sodium phosphate pH 7.0, 0.5 M NaF and 1 mM DTT. For the thermal denaturation studies the wavelength was set at 222 nm (which represents the α-helical content) and the temperature was successively increased from 20 to 80°C.

2.7. Statistical analysis

The experimental results are reported as mean ± S.E. and were analyzed for significance using Student's *t* test at $p < 0.05$ (paired or unpaired depending on the experimental design).

3. Results

3.1. Cardiac skinned fiber experiments

3.1.1. PKA incubation

Skinned fibers were displaced with cTnT-WT or cTnT-ΔE96, reconstituted with the binary complex cTnI.cTnC and the Ca^{2+} sensitivity of contraction was measured before and after PKA catalytic subunit incubation. Similar to what was previously published by our group [15], cTnT-ΔE96 sensitized the myofilament 0.38 *p*Ca units compared to cTnT-WT (pCa_{50} 6.11 ± 0.02 vs 5.73 ± 0.02). These same fibers, after PKA incubation, displayed a 0.26 and 0.25 *p*Ca unit rightward shift in the Ca^{2+} sensitivity of force for the cTnT-WT and cTnT-ΔE96, respectively (Fig. 1A and B and Table 1). Prior to PKA treatment, the cooperativity of thin filament activation (*n*H) was decreased in fibers displaced with the cTnT-ΔE96 mutant compared to cTnT-WT (Table 1). However, after PKA phosphorylation the *n*H only significantly increased in fibers containing cTnT-WT (Table 1). Note that even after PKA incubation, the fibers displaced with cTnT-ΔE96 still displayed increased myofilament Ca^{2+} sensitivity of 0.39 *p*Ca units compared to cTnT-WT.

3.1.2. PKA phosphorylation mimetic cTnI

Since PKA has been shown to have several targets in the myofilament including myosin binding protein C and troponin T, which could in turn affect myofilament Ca^{2+} sensitivity; we looked at the effects of the cTnT-ΔE96 mutant in the presence of a pseudo-phosphorylated cTnI (cTnI-SS/DD) where the two serine sites 23 and 24 were replaced by aspartic acid. Skinned porcine fibers displaced with recombinantly expressed cTnT-WT and replaced with phosphorylation mimetic binary complex cTnI-SS/DD.cTnC recapitulated the effects of β-adrenergic stimulation, a decrease in Ca^{2+} sensitivity of force development $\Delta pCa_{50} = -0.14$ was seen compared to fibers replaced with cTnI-WT.cTnC (See Fig. 2A and Table 2). When the cTnT-ΔE96 mutant displaced cardiac skinned fibers were replaced with the PKA phosphorylation mimetic cTnI-SS/DD.cTnC, a similar decrease in Ca^{2+} sensitivity of force development $\Delta pCa_{50} = -0.11$ was achieved (Fig. 2B and Table 2). Whereas, displacement of endogenous cTnT with the cTnT-ΔE96 mutant led to decreased cooperativity indicated by the Hill

Table 1
Summary of *p*Ca–force relationship curves before and after PKA incubation in fibers reconstituted with cardiac TnI.TnC complex at pH 7.0
The pCa_{50}, *n*H and % Ca^{2+} unregulated force values are the average of many independent fiber experiments, and the errors are reported as S.E. values. The Ca^{2+} unregulated force was calculated using the following equation: (*Fp*Ca8/*Fp*Ca4) × 100, where the *Fp*Ca8 and *Fp*Ca4 are the force at *p*Ca 8.0 and *p*Ca 4.0 solutions, respectively.

	Before PKA treatment		After PKA treatment				
cTnT	*p*Ca50	Hill coefficient, *n*H	*p*Ca50	Hill coefficient, *n*H	Δ*p*Ca50[a]	% Ca^{2+} Unregulated Force	N
cTnT-WT	5.73 ± 0.02	2.95 ± 0.08	5.47 ± 0.02[b]	3.73 ± 0.19[b]	−0.26	87.88 ± 4.95	6
cTnT-ΔE96	6.11 ± 0.02[c]	1.66 ± 0.05[c]	5.86 ± 0.03[b,d]	1.98 ± 0.17[d]	−0.25	96.19 ± 2.59	6

[a] Δ*p*Ca50 = pCa_{50} before PKA − pCa_{50} after PKA.
[b] $p < 0.05$ same cTnT before vs after PKA treatment.
[c] $p < 0.05$: cTnT-ΔE96 vs cTnT-WT before PKA treatment.
[d] $p < 0.05$: cTnT-ΔE96 vs cTnT-WT after PKA treatment.

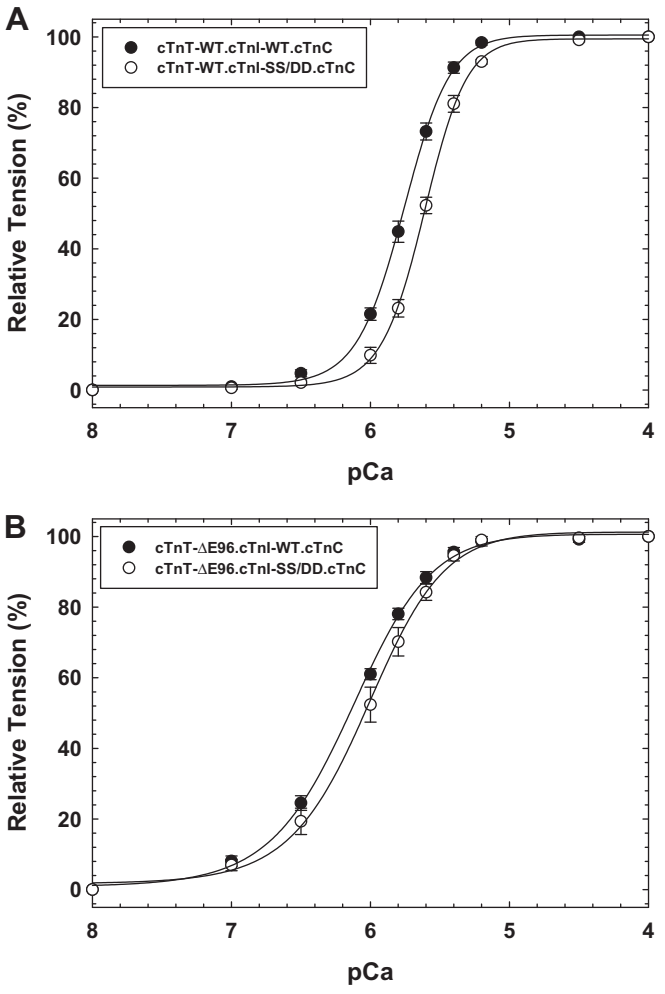

Fig. 2. Normalized *p*Ca force relationship in skinned cardiac muscle fibers in the presence of PKA pseudo-phosphorylated cTnI. The Ca^{2+} dependence of force development was measured in each preparation after cTnT displacement and binary complex reconstitution. In A) the *p*Ca force relationship of fibers displaced with cTnT-WT and reconstituted with either cTnI-WT.cTnC (filled symbols) or PKA phosphorylation mimetic cTnI-SS/DD.cTnC complex (open symbols). Where in B) the skinned fibers were displaced with cTnT-ΔE96 and reconstituted with either cTnI-WT.cTnC (filled symbols) or the cTnI-SS/DD.cTnC (open symbols) complex. Data are expressed as mean ± S.E.

coefficient (n_H) in the presence of the phosphomimetic cTnI as well (Fig. 2 and Table 2). Note that the difference in Ca^{2+} sensitivity of contraction between fibers displaced with cTnT-WT and cTnT-ΔE96 and reconstituted with cTnI-SS/DD.cTnC remained the same (+0.42 *p*Ca units). No statistical differences in maximal force recovery (%) were found for the cTnT-ΔE96 mutant compared to cTnT-WT in the presence of cTnI-WT or cTnI-SS/DD (see Supplemental Fig. 1).

3.2. IAANS fluorescence measurements

The Ca^{2+} affinity measurements for cTnT-ΔE96 mutant were compared to that of cTnT-WT at different levels of thin filament complexity and changes of the fluorescence signal could be detected due to alterations in the conformation/environment of the extrinsic IAANS probe(s) bound to cTnC. The Ca^{2+} affinity of the cTn complex containing cTnI-WT was slightly decreased but significant for the cTn-ΔE96 mutant (pCa_{50} 6.66 ± 0.01) versus cTn-WT (pCa_{50} 6.69 ± 0.01) (Fig. 3A and Table 3). When cTnT-ΔE96 was included in the cTn complex containing the pseudo-phosphorylated cTnI, there was a large decrease in Ca^{2+} affinity ($\Delta pCa_{50} = -0.17$) of the cTnC.cTnI-SS/DD.cTnT-ΔE96 mutant complex compared to cTnC.cTnI-SS/DD.cTnT-WT (Table 3). When porcine cardiac tropomyosin (Tm) was added, the Ca^{2+} affinity of cTn was similar for either the cTn-ΔE96 mutant or cTn-WT (Fig. 3B and Table 3). However, a large increase ($\Delta pCa_{50} = +0.23$) in Ca^{2+} affinity was detected for thin filaments containing the cTn-ΔE96 mutant versus cTn-WT (Fig. 3C and Table 3). When the cTnT-ΔE96 mutant was incorporated into the thin filament containing cTnI-SS/DD an even greater increase in the Ca^{2+} affinity was seen ($\Delta pCa_{50} = +0.30$), this result is consistent with the skinned fiber data that showed that the cTnT-ΔE96 mutant did not ablate the Ca^{2+} sensitivity of contraction (Tables 2 and 3).

3.3. Stability of the RCM cTnT mutant

3.3.1. Circular dichroism analysis

The secondary structural characteristics of isolated cTnT-ΔE96 mutant were compared to cTnT-WT and it was found that the mutant had lower β-sheet content [θ]MRE = −12138.93 ± 265.52 at λ = 222nm than cTnT-WT with −14494.21 ± 138.99 at 21°C (Fig. 4).

3.3.2. Thermal denaturation

Circular dichroism was used to further assess the structural stability MRE% of the cTnT-ΔE96 mutant compared to the cTnT-WT, the proteins were subjected to thermal denaturation by incrementally increasing the temperature over a range of 20–80°C. The cTnT-ΔE96 mutant was physically less stable and had a lower melting temperature, T_{50} of 39.21 ± 0.93°C compared to the cTnT-WT, T_{50} of 43.49 ± 0.63°C (Fig. 5A and B). This represents a loss of structural stability due to the deletion of glutamic acid 96 in cTnT, which could be transmitted to the rest of the cTn complex and the adjoining thin filament. The alterations in T_M or slope of a transition indicate that thermodynamic stability of the proteins is altered [37].

4. Discussion

Sarcomeric protein mutations linked to HCM and DCM have been shown to uncouple the Ca^{2+} sensitivity from β-adrenergic mediated regulation of the myofilament [26–32,38]. The lusitropic effect of PKA phosphorylation of cTnI modulates cardiac contractility by increasing the rate of Ca^{2+} dissociation from the N-domain of cTnC [23,24,39–41].

Table 2
Summary of *p*Ca–force relationship curves in fibers reconstituted with different cardiac TnI.TnC complexes at pH 7.0
The pCa_{50}, n_H and % Ca^{2+} unregulated force values are the average of many independent fiber experiments, and the errors are reported as S.E. values. The Ca^{2+} unregulated force was calculated by the following equation: $(F_{pCa8}/F_{pCa4}) \times 100$, where the F_{pCa8} and F_{pCa4} are the force at *p*Ca 8.0 and *p*Ca 4.0 solutions, respectively.

cTnT	cTnI	pCa_{50}	Hill coefficient, n_H	ΔpCa_{50}[a]	% Ca^{2+} unregulated Force	N
cTnT-WT	WT	5.76 ± 0.02	2.68 ± 0.11	–	90.3 ± 3.4	8
cTnT-WT	SS/DD	5.62 ± 0.01[b]	2.88 ± 0.22	−0.14	82.2 ± 4.7	6
cTnT-ΔE96	WT	6.14 ± 0.03[c]	1.52 ± 0.05[c]	–	95.5 ± 3.1	8
cTnT-ΔE96	SS/DD	6.03 ± 0.06[b,c]	1.63 ± 0.09[c]	−0.11	99.7 ± 0.3[c]	5

N = number of experiments.
[a] ΔpCa_{50}: cTnT + cTnI-SS/DD pCa_{50} − cTnT + cTnI-WT pCa_{50}.
[b] $p < 0.05$ cTnI-SS/DD vs cTnI-WT with the same cTnT.
[c] $p < 0.05$ cTnT-ΔE96 vs cTnT-WT with the same cTnI.

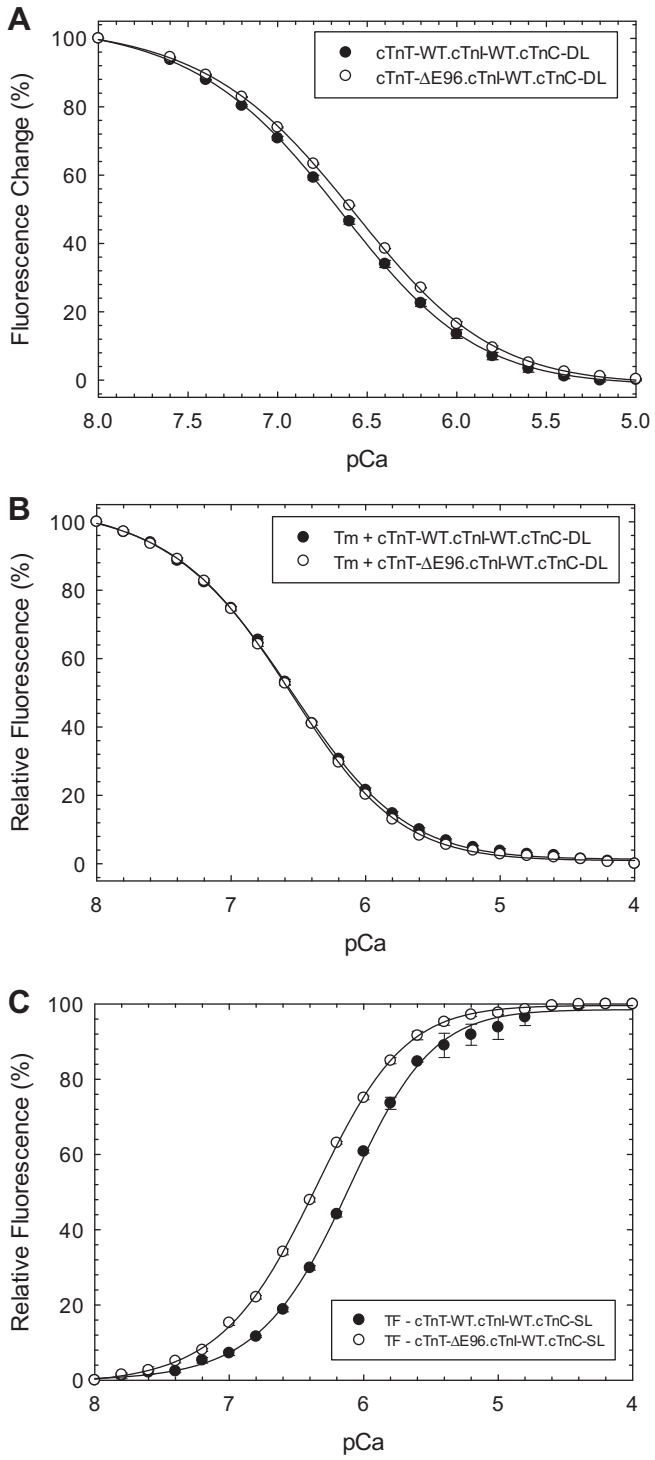

Fig. 3. Determination of the apparent Ca^{2+} affinities of troponin complexes containing cTnT-ΔE96 by fluorescence — Steady state fluorescence measurements (see Experimental procedures for details). A) Troponin complex; B) tropomyosin and troponin; and C) thin filament. Data are expressed as mean ± S.E.

Importantly, it has been shown that amino acid substitutions in other cTn subunits may cause the thin filament to become refractory to signal transduction by β-adrenergic pathways, as seen particularly with the phosphorylation of serines 23 and 24 in cTnI. Therefore, we evaluated the effects of the RCM cTnT-E96 deletion on the ability of cTnI to desensitize the myofilament to Ca^{2+} upon PKA phosphorylation. One of the questions that we addressed is whether an RCM mutant in the cTn complex has the same ability as HCM and DCM-linked mutants to impair cTnI PKA phosphorylation function at serines 23 and 24. Fibers incorporated with the RCM cTnT-ΔE96 mutant maintained the large increase in Ca^{2+} sensitivity compared to the WT-cTnT replaced fibers in every condition tested; therefore, the E96 deletion does not affect the ability of cTnI to modulate the Ca^{2+} sensitivity of contraction post PKA phosphorylation or in the presence of pseudo-phosphorylated cTnI. The phosphorylation-induced enhancement of the Ca^{2+} dissociation rate appears to be associated with global conformational changes in cTnI as shown by fluorescence anisotropy [42] and FRET measurements [43]. Additional functional changes induced by cTnI phosphorylation may be related to altered protein–protein interactions within the cTn complex [44–46].

The results obtained for the Ca^{2+} sensitivity of contraction and the cooperative activation of the myofilament, obtained using the phosphorylation mimetic cTnI-SS/DD, were consistent with that found when skinned fibers were incubated with PKA. The reduction in the pCa_{50} was less pronounced using this method compared to PKA incubation. This was not unexpected since PKA has numerous targets within the thin filament which would be phosphorylated when the entire skinned fiber was exposed to the PKA catalytic subunit. Therefore, the PKA-incubated skinned fibers more accurately portray what is happening in the myofilament during β-adrenergic stimulation since PKA phosphorylation of additional sites alters myofilament function [25,47]. Since impairment of the PKA phosphorylation effects on myofilament Ca^{2+} sensitivity has not yet been studied in the presence of an RCM associated mutant; then, how would this lead to diastolic dysfunction? In this case, the degree of desensitization imposed by PKA phosphorylation may not effectively induce lusitropy due to the substantial Ca^{2+} sensitization caused by the RCM mutant. Furthermore, RCM is characterized by restrictive ventricular filling, therefore the properties at the myofilament level including increased basal force [15,18] and enhanced contractility may result from ineffective modulation by β-adrenergic stimulation, contributing to ventricular stiffness and the severe diastolic dysfunction associated with RCM. Exploration of the thin filament hierarchy to determine the source of altered Ca^{2+} affinity of cTnC, due to the cTnT-ΔE96 mutant, revealed that only subtle changes occurred at the level of the troponin complex, with a small decrease in Ca^{2+} affinity for the mutant containing complex, with the phenotypic manifestations more pronounced in the thin filament. This finding is similar to what was shown by others [48–51]. In our study, the addition of Tm to Tn complex containing the (cTnT-WT or the cTnT-ΔE96 mutant) did not alter cTnC Ca^{2+} affinity. The Ca^{2+} affinity became increased in the more complex system (addition of actin) for the RCM-containing thin filament, indicating that the deletion of glutamic acid in cTnT, which lies at the TnT–Tm interface, alters the cTn–Tm interaction with actin. The location of the E96 deletion in the hypervariable N-terminal cTnT tail underlies its significant functional effects since this portion of cTnT is important for maintenance of diastolic function. As shown by Tobacman et al., this portion of cTnT (1–153) was able to establish the blocked state without the presence of cTnI [13]. Transition from the blocked to closed state, is a known mechanism of increasing actomyosin ATPase activity at low Ca^{2+} [9,52] and this RCM mutation has been previously shown to impair the ability of the troponin complex to inhibit actomyosin ATPase activity in the absence of Ca^{2+} [15]. Therefore, increased Ca^{2+} affinity of cTnC in the presence of the RCM deletion mutant may interfere with maintenance of the blocked state and subsequently alter interactions with actin. In addition, mutations (within the region 92–110) were shown to alter the Tm-dependent functions of the TnT fragment 70–170, such as binding to actin [53]. These data allow us to conclude that the E96 deletion in TnT indirectly affects Ca^{2+} binding to the cTnC N-terminal domain, most likely through direct effects on tropomyosin binding to actin. The fluorescence studies provided insight on the origin of altered Ca^{2+} affinity, that it was altered at the tropomyosin:actin interface.

In regard to cardiac function, a number of studies have shown that cardiomyopathy-associated mutants globally affect the troponin tail

Table 3
Summary of the fluorescence experiments.

	Troponin (cTnI-WT)		Tropomyosin + troponin (cTnI-WT)		Thin filament (cTnI-WT)	
	cTn-WT	cTn-ΔE96	cTn-WT	cTn-ΔE96	cTn-WT	cTn-ΔE96
pCa_{50}	6.69 ± 0.01	6.66 ± 0.01[a]	6.58 ± 0.01	6.59 ± 0.01	6.15 ± 0.02	6.38 ± 0.01[a]
n_{Hill}	0.99 ± 0.02	1.05 ± 0.01[a]	1.00 ± 0.01	1.04 ± 0.01[a]	1.34 ± 0.04	1.28 ± 0.02
	Troponin (cTnI-SS/DD)		Tropomyosin + troponin (cTnI-SS/DD)		Thin filament (cTnI-SS/DD)	
	cTn-WT	cTn-ΔE96	cTn-WT	cTn-ΔE96	cTn-WT	cTn-ΔE96
pCa_{50}	6.71 ± 0.01	6.54 ± 0.01[a,b]	n/a	n/a	6.03 ± 0.02[c]	6.33 ± 0.01[a,c]
n_{Hill}	1.16 ± 0.01[b]	0.96 ± 0.01[a,b]	n/a	n/a	1.39 ± 0.05	1.37 ± 0.05

n/a = not measured.
Data are presented as mean ± .S.E. n = 4–5.
[a] $p < 0.05$ RCM cTnT-ΔE96 mutant vs cTnT-WT within the same complex system.
[b] $p < 0.05$ troponin containing cTnT-WT.cTnI-WT vs troponin containing cTnT-WT.cTnI-SS/DD, or troponin containing cTnT-ΔE96.cTnI-WT vs troponin containing cTnT-ΔE96.cTnI-SS/DD.
[c] $p < 0.05$ thin filament containing cTnT-WT.cTnI-WT vs thin filament containing cTnT-WT.cTnI-SS/DD, or thin filament containing cTnT-ΔE96.cTnI-WT vs thin filament containing cTnT-ΔE96.cTnI-SS/DD.

domain, in contrast to local effects manifested at the site of the substitution. To assess whether the ΔE96 deletion in TnT, altered its function in the thin filament, we determined whether the altered physical properties of cTnT-ΔE96 protein could be due to increased flexibility/decreased thermal stability. Two different groups found that the stability and/or flexibility of the TnT1 (tail domain) are crucial for the regulatory properties of tropomyosin and actin. In reference to our current study, it was previously shown that introduction of the mutants R92W, R94L, A104V and F110I into cTnT, changed the peptide T_m between −0.6 and −5.9°C, lower than the WT protein [53]. Although, these amino acid substitutions (between 92 and 110) introduced seemingly small changes in the physical properties of cTnT, the mutants have a diminished ability to stabilize the tropomyosin head-to-tail overlap complex. In another study, Hinkle and Tobacman showed that the R92Q (−1.8°C) and A104V (−4.2°C) mutants decreased the thermal stability of the TnT1 peptide (1–156) [54]. Since the troponin tail domain makes its primary interactions with Tm, any actin associated effects are thought to be indirect [54]. This results in weaker binding of Tn to Tm for most mutants in this region [53], as well as supernormal or sub-normal folding stability of the mutant TnT [54]. From this and previous studies we can suggest that altered interactions by TnT due to increased flexibility may be a common feature of cardiomyopathy-linked mutants located at the Tn–Tm interface. These changes in the properties of TnT, thus alters its ability to precisely regulate the transition from the active to inactive state during systole and diastole, respectively. An emerging concept is that mutations in tropomyosin that increase its flexibility are correlated with increased myofilament Ca^{2+} sensitivity [55–57], while tropomyosin mutations that decrease its flexibility are associated with decreased Ca^{2+} sensitivity [58–60]. These results have also been confirmed in mouse models [61]. Our study as well as others suggest that the flexibility of the troponin T tail may control/modulate Ca^{2+} sensitization in the same manner described for tropomyosin.

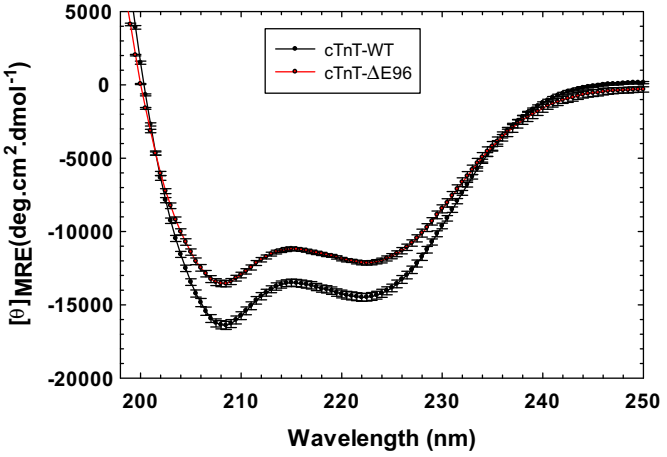

Fig. 4. Determination of the secondary structural characteristics of the cTnT-ΔE96 mutant versus cTnT-WT. Far-UV Circular dichroism spectra were recorded at 195–250 nm at room temperature (20°C). Data are expressed as mean S.E. and n = 7 performed for cTnT-WT and n = 6 for cTnT-ΔE96.

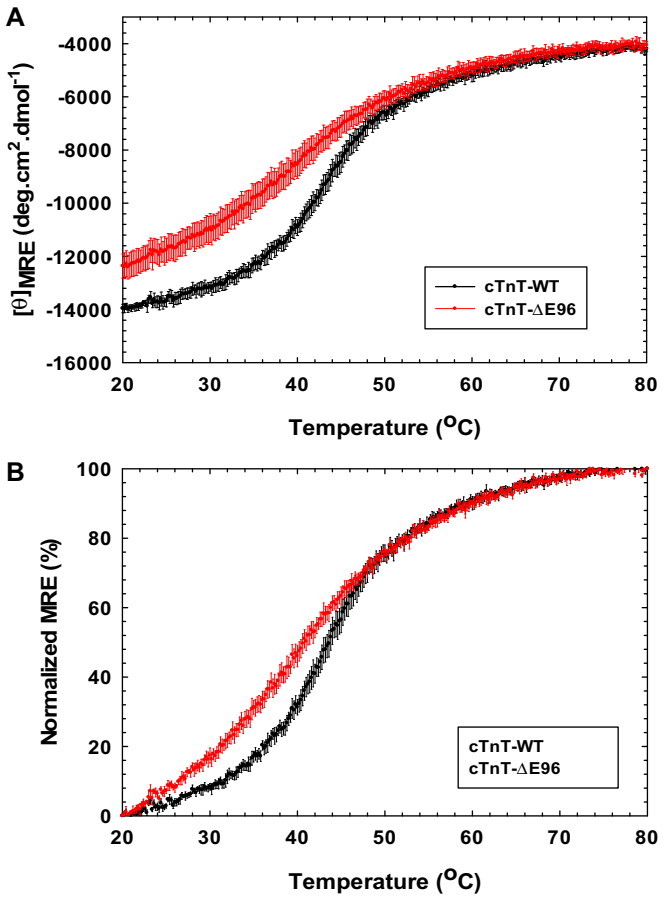

Fig. 5. Circular dichroism thermal denaturation curve monitored at a fixed wavelength. The data was collected at λ = 222 nm. A) The thermal denaturation curve ([θ]MRE) of the RCM mutant cTnT-ΔE96 mutant versus cTnT-WT. B) Normalized graph of the thermal denaturation curve for the cTnT-ΔE96 RCM mutant versus cTnT-WT. Data are expressed as mean S.E. and n = 4 performed for cTnT-WT and cTnT-ΔE96.

5. Conclusion

This study has explored the physical properties of the mutant cTnT-ΔE96 and effects of β-adrenergic regulation of the cTn complex containing the deletion. The severe phenotype manifested (early development of cardiac impairment) demonstrated by patient data including severe diastolic dysfunction and our functional data suggests that the cTnT-ΔE96 deletion mutant has substantial deleterious consequences that warranted further investigation. In summary, we have found that this deletion in cTnT contributes to disease development through altered protein stability which compromises its function, the heightened Ca^{2+} sensitivity in skinned fibers is improperly regulated by PKA mediated pathways which though not refractory, are ineffective at decreasing the Ca^{2+} sensitivity to the normal range, thus interfering with relaxation. Therefore, instability of cTnT caused by deletions of amino acid substitutions may be a major determinant leading to development of cardiomyopathy. Furthermore, it needs to be investigated whether ineffective PKA phosphorylation impairment is a trend or common phenotype for RCM mutations.

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