Altered Regulation of Cardiac Muscle Contraction by Troponin T Mutations That Cause Familial Hypertrophic Cardiomyopathy*

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To study the effect of troponin (Tn) T mutations that cause familial hypertrophic cardiomyopathy (FHC) on cardiac muscle contraction, wild-type, and the following recombinant human cardiac TnT mutants were cloned and expressed: I79N, R92Q, F110I, E163K, R278C, and intron 16(G1 → A) (In16). These TnT FHC mutants were reconstituted into skinned cardiac muscle preparations and characterized for their effect on maximal steady state force activation, inhibition, and the Ca2⁺ sensitivity of force development. Troponin complexes containing these mutants were tested for their ability to regulate actin-tropomyosin(Tm)-activated myosin-ATPase activity. TnT(R278C) and TnT(In16) reconstituted preparations demonstrated dramatically increased Ca2⁺ sensitivity of force development, while those with TnT(R92Q) and TnT(I79N) showed a moderate increase. The deletion mutant, TnT(In16), significantly decreased both the activation and the inhibition of force, and substantially decreased the activation and the inhibition of actin-Tm-activated myosin-ATPase activity. ATPase activation was also impaired by TnT(F110I), while its inhibition was reduced by TnT(R278C). The TnT(E163K) mutation had the smallest effect on the Ca2⁺ sensitivity of force; however, it produced an elevated activation of the ATPase activity in reconstituted thin filaments. These observed changes in the Ca2⁺ regulation of force development caused by these mutations would likely cause altered contractility and contribute to the development of FHC.

Vertebrate striated (skeletal and cardiac) muscle contraction is activated by the binding of Ca2⁺ to the low affinity Ca2⁺-specific (regulatory) sites of troponin (Tn) ³ C, the Ca2⁺-binding subunit of Tn, which together with TnI, TnT, and tropomyosin (Tm) form the regulatory system of the contractile apparatus (1–4). Studies of the regulation of skeletal and cardiac muscle contraction have suggested that TnT not only anchors the TnTnC complex to the thin filaments through its interaction with TnI and Tm, but also confers the Ca2⁺ sensitivity to the actomyosin-ATPase activity and/or force (5–8).

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant disease which has been shown to be caused by mutations in almost every major sarcomeric protein, including myosin heavy chain (9–11), α-tropomyosin (12–14), myosin-binding protein C (15–17), ventricular myosin light chains 1 and 2 (18–20), human cardiac TnT (HCTnT) (12, 13, 21, 27), and TnI (HCTnI) (28). Whereas individuals with myosin heavy chain mutations, in general, have a higher level of cardiac hypertrophy, those with HCTnT mutations have less hypertrophy, but a higher incidence of sudden cardiac death at a young age (13, 29, 30). At present, 11 amino acid mutations in HCTnT have been identified to be associated with FHC: I79N, R92Q/W/L, A104V, F110I, E144D, R278C, and an In16 mutation. The latter arises from abnormal splicing of intron 16 (G1 → A) which deletes exon 16 and reads exon 17 in the wrong frame (12, 21). The resulting mRNA encodes a protein that lacks the COOH-terminal 28 amino acids (exon 16 and 17) and has an additional 7 residues. There is not a clear understanding of why these mutations in HCTnT lead to the development of FHC or sudden cardiac death.

In this study we have focused on the following HFC HCTnT mutations: TnT(I79N), TnT(R92Q), TnT(F110I), TnT(E163K), TnT(R278C), and TnT(In16). Several reports have appeared, which have examined the effects of these mutations on the biochemical and contractile properties of cardiac muscle (13, 25, 29, 31). Four of the HCTnT mutants (I79N, R92Q, F110I, and R278C) studied in this paper have been reported in a recent paper from Yanaga et al. (34), who investigated their effect on myofibrillar ATPase activity in reconstituted myofibrils. In an earlier study, Morimoto et al. (32) reported on the effect of two of these mutations (I79N, R92Q) when incorporated into skinned cardiac trabeculae (rabbit). We have reconstituted the HCTnT mutants into actomyosin, and skinned cardiac (porcine) muscle fiber preparations and studied their effects on inhibition, activation, and the Ca2⁺ sensitivity of ATPase and steady state force development. Our results are discussed in relation to those from the Ohtsuki lab (32, 34) and others (31, 33, 35).

We found that TnT(R278C), TnT(F110I), TnT(I79N), and TnT(R92Q)-reconstituted skinned cardiac muscle preparations had significantly increased Ca2⁺ sensitivity of force development, whereas those reconstituted with TnT(In16) had dramatically decreased maximal force and force inhibition. We also found that troponin complexes made from TnT(F110I) and TnT(In16) had substantially reduced activation of actin-Tm-activated myosin-ATPase activity while those containing TnT(R278C) and TnT(In16) demonstrated impaired inhibition of the ATPase.
Expression and Purification of Wild-type HCTnT and HCTnT Mutants

Expression and Purification of Human Cardiac TnI and TnC—The cDNAs encoding human cardiac TnI, TnC, and TnT were cloned by reverse transcriptase-polymerase chain reaction using a template of total RNA from human myocardium and oligonucleotide primers specific for the 5’ and 3’ regions of the respective coding sequences. Additionally, the six TnT FHC mutants: I79N, R92Q, F110I, E163K, R278C, and In16, a mutation in HCTnT, which arises from abnormal splicing of Intron 16 (G, → A), were made using a sequential overlapping polymerase chain reaction-based method (36). Standard methods were utilized for expression and purification of wild-type HCTnT and its FHC mutants as well as HCTnI and HCTnC (37–39). All clones were sequenced to verify the correct sequences prior to expression and purification of the respective proteins.

Actin-Tm-activated Myosin-ATPase Assays—Rabbit skeletal F-actin was prepared as described by Strzelecka et al. (40). Porcine cardiac myosin was purified according to Murakami et al. (41). Porcine cardiac Tm, TnC, and TnI were purified from pig ventricles according to Potter et al. (42). All Tn subunits (T, I, and C) were first dialyzed against 6 M KCl, 10 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 0.01% NaN₃ and then treated with the ATPase in a similar manner (43). Alternatively, the inhibition (−Ca2⁺) of the ATPase activity expressed as a percentage of the ATPase determined in the absence of troponin (basal activity). As shown in Fig. 3A, the mutants Tn(T16163K) and Tn(T278C) demonstrated elevated ATPase activity compared with the HCTnT (p < 0.05). Their ATPase activities were ~190 ± 9 and 182 ± 6%, respectively, while that of HCTnT was ~172 ± 9%. On the other hand, Tn(T6177) and Tn(In16) showed decreased ATPase activation, ~152 ± 5 and 150 ± 8%, respectively (p < 0.05), while Tn(T182) activated the ATPase in a similar manner (~177 ± 5%) to HCTnT. Alternatively, the inhibition (−Ca2⁺) of the ATPase activity was impaired with Tn(In16) (~55 ± 8%) compared with ~86 ± 6% of inhibition assessed with HCTnT (Fig. 3B). The mutants Tn(T16179N), Tn(TF110I), and Tn(T16163K) inhibited the actin-Tm-activated myosin-ATPase activity in a similar manner (~72 ± 4, 73 ± 8, and 76 ± 6%, respectively) to HCWTnT. Asterisks in Fig. 3 (A and B) indicate the statistical significance of the activation/inhibition of the ATPase activity by the troponin containing wild-type HCTnT and respective FHC TnT mutants, p < 0.05.
FHC mutations correspond to the amino acid sequence of HCTnT3. The regions thought to interact with Tm, TnC, and TnI are also illustrated.

Values of standard deviation from five to eight experiments.

Asterisk ATPase activity without troponin was 0% (the ATPase activation without troponin added. The inhibition of the level of activation of ATPase activity (pCa 8) was normalized to 100% of the actin-Tm-activated reaction. The protein concentrations were: 3.5 μM actin, 1 μM tropomyosin, 1 μM troponin, and 0.6 μM myosin. The ATPase reactions were performed in 10 mM MOPS, 50 mM KCl, 4 mM MgCl2, pH 7.0, in the presence (0.5 mM CaCl2) or absence (1 mM EGTA) of Ca2+.

The level of activation of ATPase activity (A) was normalized to 100% of the ATPase activation without troponin added. The inhibition of the ATPase activity without troponin was 0% (B). The error bars represent values of standard deviation from five to eight experiments. Asterisk indicates that the ATPase values for the respective TnT mutants are significantly different from HCWTnT (p < 0.05).

**FHC TnT Mutants**—The physiological significance of the FHC TnT mutations was examined utilizing porcine skinned fibers, in which steady-state force activation, inhibition, and the Ca2+ sensitivity of force development were measured. The skinned cardiac muscle preparations were incubated with HCWTnT and its FHC mutants until a complete loss of the Ca2+ dependence of force was observed. Fig. 4 illustrates an experimental protocol for TnT(E163K)-treated preparations. The same protocol was applied for HCWTnT and the other FHC TnT mutants. As shown in Fig. 4, incubation of the fibers with TnT(E163K) for 2 h at room temperature resulted in a complete loss of the Ca2+ dependence of force following displacement of the Tn complex with TnT(E163K). The fibers became insensitive to Ca2+ and could not relax due to the absence of the TnT-TnC complex. The level of Ca2+-unregulated force following the TnT treatment did not depend on the FHC TnT mutant utilized in the displacement procedure and was equal ~60% of the force developed by the untreated skinned fiber preparations. Since the average rundown of the control fibers treated with the buffer minus proteins for the same amount of time as the experimental fiber was ~12–18%, the true level of Ca2+-unregulated force was ~67–71%. For clarity this level was set to 100% for each TnT mutant to determine the percentage of force activation and inhibition following the treatment. For two of the FHC TnT mutants, I16 and F110I, a residual Ca2+ sensitivity of force was observed following the TnT(F110I) and TnT(In16) treatment. The other FHC TnT mutants, I79N, R29Q, E163K, R278C, and wild-type HCTnT were able to replace the entire Tn complex within the time of incubation as judged by the complete loss of the Ca2+ dependence of force. When Tn-displaced fibers were incubated with a preformed human cardiac TnI-TnC complex, dissolved in the relaxing solution (pCa 8), they underwent a gradual relaxation which was monitored by the inhibition of force in the absence of Ca2+ (pCa 8). Incubation of the Tn-displaced fibers with the HCTnC-reconstituted fibers performed with the TnT(R278C) mutant. As shown, the TnT(R278C)-treated fibers had a greatly reduced endogenous TnC content (lanes 2 and 7) which was completely reconstituted with a preformed HCTnC-HCTnC complex (lanes 3 and 8). The stoichiometric ratio of other muscle proteins in the fibers: F-actin, tropomyosin, myosin light chains 1 (ELC), and 2 (RLC), was not changed following the TnT treatment/reconstitution. A Western blot of the TnT(R278C)-treated and HCTnC-reconstituted fibers performed with the TnT (A) and TnI (B) specific antibodies (Goat anti-HCTnT polyclonal and monoclonal mouse anti-TnI IE7 GAM-PO) is presented in Fig. 6. As shown, the TnT(R278C)-treated fibers lacked the endogenous TnI that was displaced (lane 1) during the treatment and was easily reconstituted with human cardiac TnI during incubation of the TnT(R278C)-treated fibers with the recombinant HCTnC-HCTnC complex (lane 2).

Steady-state Force Development—Fig. 7 summarizes the effect of the FHC TnT mutants on the relaxation (pCa 8) and force recovery (pCa 4) in skinned cardiac muscle preparations following the Tn displacement and reconstitution procedures. The dashed line indicates the level of Ca2+-unregulated force following the TnT treatment (set to 100% for each TnT mutant). The open bars represent the force developed in the presence of Ca2+, while the dashed bars indicate the level of force in the absence of Ca2+ (Fig. 7). As shown, the level of force inhibition (pCa 8) and force activation (pCa 4) following the
TnT treatment and HCTnI reconstitution depended on the FHC TnT mutant used in the Tn-displacement procedure. The fibers treated with HCWTnT and reconstituted with HCTnI inhibited (Ca²⁺⁻⁻Unregulated force) 95.5 ± 6.7% of the Ca²⁺-unregulated force (100%) and after being switched to the high Ca²⁺ solution (pCa 8), they underwent a gradual relaxation which was monitored by the inhibition of force in the absence of Ca²⁺ (pCa 8). Transferring the fibers to the solutions of increasing Ca²⁺ concentrations restored their Ca²⁺ regulation of force.

The Ca²⁺ Sensitivity of Force Development—Skinned porcine muscle fibers reconstituted with human cardiac Tn were tested for their Ca²⁺ sensitivity of force development (Fig. 8). As shown in Fig. 8A, porcine fibers reconstituted with human cardiac Tn (T, I, and C) were less sensitive to Ca²⁺ (ΔpCa50) than intact untreated fibers. However, when the porcine fibers were reconstituted with porcine Tn (T, I, and C), the Ca²⁺ sensitivity of force development was significantly increased (Fig. 8B).
HCTnI treatment the fibers were reconstituted with the recombinant diac muscle fibers treated with porcine cardiac TnT (HCWTnT) (A) and human cardiac wild-type TnT (HCWTnT) (B). After TnT treatment the fibers were reconstituted with the recombinant HCTnI-HCTnC-reconstituted complex. The midpoints ($p_{Ca_{50}}$) and the Hill coefficients ($n_H$) of Ca$^{2+}$ sensitivity curves are presented. Data points are the average of three to six independent experiments and the error bars are the S.D.

The $p_{Ca_{50}}$ and $n_H$ values are the average of three to six independent experiments, and the errors are the standard deviation (S.D.) values.

| FHC TnT mutant utilizated in the fibers | $p_{Ca_{50}}$  | Hill coefficient ($n_H$) | Number of experiments $(n)$ |
|----------------------------------------|--------------|--------------------------|----------------------------|
| HCWTnT                                 | 5.48 ± 0.01  | 1.70 ± 0.07              | 4                          |
| TnT(I79N)                               | 5.63 ± 0.02  | 1.59 ± 0.12              | 3                          |
| TnT(R92Q)                               | 5.66 ± 0.02  | 1.54 ± 0.11              | 3                          |
| TnT(F110I)                              | 5.85 ± 0.033 | 1.31 ± 0.106             | 4                          |
| TnT(E163K)                              | 5.55 ± 0.02  | 1.45 ± 0.07              | 6                          |
| TnT(R278C)                              | 5.82 ± 0.02  | 1.17 ± 0.077             | 3                          |

* Indicates that the $p_{Ca_{50}}$ and $n_H$ values for the respective TnT mutants are significantly different from HCWTnT ($p < 0.05$).

FIG. 8. Ca$^{2+}$ sensitivity of force development in skinned cardiac muscle fibers treated with porcine cardiac TnT versus human cardiac wild-type TnT (HCWTnT) (A), TnT(I79N) versus HCWTnT (B), and TnT(R278C) versus HCWTnT (C). After TnT treatment the fibers were reconstituted with the recombinant HCTnI-HCTnC complex. The midpoints ($p_{Ca_{50}}$) and the Hill coefficients ($n_H$) of Ca$^{2+}$ sensitivity curves are presented. Data points are the average of three to six experiments and the error bars are the S.D.

were compared with the Ca$^{2+}$ dependence of HCWTnT-treated, and HCTnI-HCTnC-reconstituted fibers (Fig. 8, A and C). Because of the very low level of force recovery following the TnT(In16) treatment (35 ± 3%), the Ca$^{2+}$ dependence of force development was not determined. Fig. 8B presents the Ca$^{2+}$ response of force of the TnT(I79N)-treated fibers. A slight increase in Ca$^{2+}$ sensitivity ($Δp_{Ca_{50}} \sim +0.15$) was observed compared with the HCWTnT-treated fibers. A much larger change in the Ca$^{2+}$ sensitivity of force was observed for TnT(F110I)- and TnT(R278C)-treated fibers. These two mutants caused the most dramatic increase ($Δp_{Ca_{50}} \sim +0.37$ and $\sim +0.34$, respectively) in the Ca$^{2+}$ sensitivity among all studied TnT proteins (Fig. 8C, Table I). Table I summarizes the $p_{Ca_{50}}$ values of the force-pCa relationship and the Hill coefficients for the fibers treated with wild-type HCTnI and the FHC TnT mutants. As shown, the TnT(I79N) and TnT(R92Q) increased the Ca$^{2+}$ dependence of force development by $Δp_{Ca_{50}} \sim +0.15$ and $\sim +0.18$, respectively, compared with HCWTnT and their Hill coefficients were somewhat lower than that for the HCWTnT-treated fibers. As mentioned above, the effect of TnT(R278C) was the second largest among all of the TnT mutants (Table I, Fig. 8C). However, the cooperativity parameters (Hill coefficients) of the force-pCa dependence for TnT(R278C)- and TnT(F110I)-treated fibers were significantly lower than that of the HCWTnT-treated fibers (Table I). The TnT(E163K) mutant was similar to HCWTnT when reconstituted in skinned porcine fibers, with a $Δp_{Ca_{50}} \sim +0.07$ compared with the HCWTnT-treated fibers (Table I).

DISCUSSION

Our results indicate that the FHC TnT mutations play an important role in the Ca$^{2+}$ regulation of ATPase/force development and the activation and inhibition of ATPase/force. The TnT(R278C)- and TnT(F110I)-reconstituted fibers demonstrated dramatically increased Ca$^{2+}$ sensitivity of force, while TnT(R92Q)- and TnT(I79N)-treated fibers showed a moderate increase (Table I). The deletion mutant, TnT(In16), significantly decreased both the activation and the inhibition of force, and substantially decreased the activation and the inhibition of actin-Tm-activated myosin-ATPase activity when reconstituted in thin filaments. The ATPase activation was also impaired by TnT(F110I), while the inhibition was reduced by TnT(R278C). The TnT(E163K) mutation had the smallest effect on the Ca$^{2+}$ sensitivity of force; however, it caused an elevated activation of the ATPase activity in reconstituted thin filaments. These observed changes in the Ca$^{2+}$ regulation of the ATPase and force development could be a clue to understanding the altered cardiac contractility seen in humans with these mutations (11, 13, 26). To distinguish how any particular TnT mutation could cause these alterations, a fundamental
knowledge of the role of TnT in the Ca$^{2+}$ regulation of cardiac muscle contraction is essential. The role of the regions containing these mutations is also critical.

TnT anchors the TnI-TnC complex to the thin filaments through an interaction with TnI and tropomyosin, and also confers Ca$^{2+}$ sensitivity to actomyosin-ATPase activity when complexed with TnI, TnC, and Tm (4–8). Studies with proteolytic fragments of TnT have indicated that the functionally important sites of TnT are mostly located in the COOH-terminal half of the molecule. This region of TnT interacts with Tm, TnC, TnI, and actin (7, 47–50). Two classes of interactions have been proposed to occur within the COOH-terminal domain of TnT: 1) Structural, Ca$^{2+}$-independent interactions, between the COOH-terminal domain of TnC and the NH$_2$-terminal domain of TnI; and 2) the regulatory, Ca$^{2+}$-dependent interactions between the NH$_2$-terminal, Ca$^{2+}$-specific domain of TnC, and the COOH-terminal domain of TnI (containing the inhibitory region) (7). The dependence of the Ca$^{2+}$-sensitizing function of TnT on the amino acid sequence of its NH$_2$ terminus remains an open question (8, 51). The large number of TnT isoforms, compared with other thin filament proteins suggests the importance of the NH$_2$-terminal variable region of TnT in the Ca$^{2+}$ regulation of skeletal muscle contraction.

The FHC TnT mutations studied in this paper have also been investigated by others. Several studies utilizing various techniques, including in vitro motility assays, expression of different TnT isoforms (human, rat, embryonic, and adult) in different systems such as quail myotubes, rat cardiac myocytes, feline cardiac myocytes, transgenic animal models etc., have been reported during the past few years. Some of them gave conflicting results, perhaps due to the utilization of different systems. Lin et al. (31), using recombinant rat cardiac TnT, containing a mutation in an equivalent position to the TnT(I79N) mutation, showed that troponin, containing this TnT, had normal function in terms of its affinity for Tm, Tn induced binding of Tm to actin, cooperative binding of myosin S1 to thin filaments and the Ca$^{2+}$ sensitivity of acto-S1 ATPase activity. They found, however, that regulated thin filaments, containing this Tn, moved 50% faster over HMM in an in vitro motility assay than control filaments and suggested that this could possibly lead to altered contractility in cardiac muscle. Sweeney et al. (33) reported that TnT(I79N)- and TnT(R92Q)-transfected quail myotubes demonstrated decreased Ca$^{2+}$ sensitivity of force production, whereas the unloaded shortening velocity was increased about 2-fold. In a recent paper of Yu et al. (27), an embryonic isoform of rat TnT and two FHC TnT mutations, TnT(I79N) and TnT(R92Q) made from it, were expressed in adult rat cardiac myocytes. Measurements of iso-metric force in these myocytes demonstrated significantly decreased Ca$^{2+}$ sensitivity with unaltered maximum tension. Marian et al. (23) expressed human cardiac TnT containing the R92Q mutation in feline cardiac myocytes and found that their contractility was impaired. A recent paper of Yu et al. (27) demonstrated normal myofibrillar formation and sarcomeric assembly when the R92Q mutation was expressed in adult rabbit myocardium. In a report from Leinwand’s lab (25), transgenic mouse lines containing different amounts of mouse cardiac TnT with the In16 mutation were studied. These animals exhibited sarcomeric disarray and had significant diastolic dysfunction. Animals with higher levels of transgene expression died within 24 h of birth. An earlier report from Watkins et al. (21), utilized wild-type and the same truncated mutant HCTnT to transfet quail myotubes and found that Ca$^{2+}$-activated force was significantly reduced with the mutant HCTnT compared with wild-type HCTnT.

Our results with the truncated TnT(In16) mutant are in agreement with those reported above. The level of force development in TnT(In16)-treated and HCTnT-HCTnC-reconstituted fibers was very low in comparison to HCTnT or other FHC TnT mutants. The relaxation in these fibers was also significantly impaired. Interestingly, these perturbations in force development occurred even though the mutant did not bind well to the fibers and was not efficient in displacing the Tn complex. One could speculate that this mutation could result in altered stoichiometry of the thin filament proteins and lead to dysfunctional interactions between the thick and thin filaments. It is also possible that truncation of the COOH terminus of TnT caused by this mutation may change Ca$^{2+}$-dependent interactions between TnT and the TnC-TnI complex and would ultimately result in reduced activation and relaxation during muscle contraction.

Our results are also in agreement with Morimoto et al. (32), who have reported that TnT(I79N) and TnT(R92Q) when reconstituted in skinned rabbit trabeculae, did not impair maximal force, but increased the Ca$^{2+}$ sensitivity of contraction. In a very recent study of Yanaga et al. (34), TnT(I79N), TnT(R92Q), as well as TnT(F110I), TnT(E244D), and TnT(R278C) were reconstituted in rabbit cardiac myofibrils and the Ca$^{2+}$ sensitivity of myofibrillar ATPase was measured. In concert with our results, the TnT(R278C) mutant caused the most substantial increase in Ca$^{2+}$ sensitivity of the myofibrillar ATPase, while TnT(I79N) and TnT(R92Q) produced about half of this effect. In contrast to their results with TnT(F110I), one could speculate that the observed increase in the Ca$^{2+}$ sensitivity of force development was due to the increased sensitivity of porcine cardiac muscle preparations. However, the $p_{Ca_{50}}$ of the untreated porcine fibers was only 5.67, while that of TnT(F110I)-treated was as high as 5.85. Therefore, the observed increase in Ca$^{2+}$ sensitivity of force was primarily caused by the TnT(F110I) mutation. Possibly the results of Yanaga et al. (34) were affected by the reduced efficiency of TnT(F110I) to displace Tn in myofibrils. Stoichiometric reconstitution in thin filaments omits these problems, and as we demonstrated above, the actin-Tm-activated myosin-ATPase activity was impaired (73% of HCTnT) by TnT(F110I), while in their study an elevated myofibrillar ATPase was observed.

The effect of the TnT(I79N) on the Ca$^{2+}$ sensitivity of force is quite understandable since the mutation site is located in the TnT region which is known to interact with TnI, TnC, and Tm in a Ca$^{2+}$-dependent manner (7, 30, 49, 50). The change of a positively charged Arg to a hydrophobic Cys at position 278 could alter the Ca$^{2+}$-dependent interactions between the COOH terminus of TnT and TnC, either directly or through interactions with TnI. The mutation might also alter the Ca$^{2+}$-sensitive interaction of the COOH terminus of TnT and Tm. Interestingly, an Arg at position 278 is highly conserved among other cardiac TnT isoforms, however, most of the slow and fast skeletal muscle isoforms of TnT contain an Ala residue at this position. In the other TnT(F110I) mutant, which also produced an increase in the Ca$^{2+}$ sensitivity of force, the aromatic Phe residue is replaced with an aliphatic Ile. This change could be significant since this residue is highly conserved among all TnT isoforms, across both species and tissues. The effect of TnT(F110I) on the Ca$^{2+}$ sensitivity of force development is quite surprising since this region of TnT (exon 10) is thought to interact with tropomyosin in a Ca$^{2+}$-independent manner. Perhaps an allosteric effect of this mutation on the COOH-terminal domain of TnT, which interacts with the TnI-TnC complex.
in a Ca\textsuperscript{2+}-dependent manner, occurs here. On the other hand, as determined by the displacement experiments, the F110I mutation most probably results from a decreased affinity of the TnT for Tm. This could facilitate the interaction of the COOH terminus of TnT, with TnI and TnC in a Ca\textsuperscript{2+}-dependent manner. Two other FHC mutations, TnT(I79N) and TnT(R92Q), are also located in a TnT-Tm interaction interface and are not thought to be regulated by Ca\textsuperscript{2+}. One could speculate that these mutations might affect interaction of TnT with Tm and possibly alter the movement of Tm on actin. These alterations could in turn affect the interaction of actin-Tm with the myosin heads and influence the contractility and Ca\textsuperscript{2+} sensitivity of cardiac muscle.

TnT(E163K) had the smallest effect on the Ca\textsuperscript{2+} sensitivity of force, however, when reconstituted into thin filaments, it caused the highest observed activation of actin-Tm activated myosin-ATPase activity. According to Malnic et al. (7), this residue is located in a region of cardiac TnT that contains intrinsic activation properties. The change of the negatively charged Glu to a positive Lys could possibly facilitate these properties. An elevated level of the ATPase activity observed with this mutation may produce an altered cross-bridge duty cycle and possibly alter the kinetics of actin-Tm-myosin interaction. This in turn could contribute to energetic perturbations during muscle contraction.

Dramatic increases in the Ca\textsuperscript{2+} regulation of force development observed in our skinned fiber experiments could lead to the altered cardiac contractility seen in humans with these mutations. The increased (or decreased) Ca\textsuperscript{2+} sensitivity of force that accompanies these mutations would reduce (or increase) the concentration of Ca\textsuperscript{2+} required to produce the equivalent tension response to that seen in a normal muscle fiber. Since the troponin complex is a significant buffer within the cardiac myocyte, any alteration in its Ca\textsuperscript{2+} affinity would be expected to alter overall cellular Ca\textsuperscript{2+} homeostasis. These alterations might also be expected to change the Ca\textsuperscript{2+} transient (magnitude and time course) and thereby any of the multiple Ca\textsuperscript{2+}-dependent processes within the cell, e.g. SR function, calmodulin-regulated systems, ion channels, etc. The changes in Ca\textsuperscript{2+} sensitivity with and/or without changes in the maximum level of ATPase activity or force may contribute to an impaired inotropic response. These abnormalities could lead to major alterations in critical cell signaling events and ultimately to catastrophic results, e.g. arrhythmia, sudden death, etc. Moreover, the changes in Ca\textsuperscript{2+} homeostasis could result in altered expression of a variety of genes including stress response and hypertrophy related genes that could be themselves causal for FHC. Further studies will be necessary to fully understand the mechanisms involved in the production of FHC caused by these mutations in TnT.

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