Abnormal Contractile Function in Transgenic Mice Expressing a Familial Hypertrophic Cardiomyopathy-linked Troponin T (I79N) Mutation*

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This study characterizes a transgenic animal model for the troponin T (TnT) mutation (I79N) associated with familial hypertrophic cardiomyopathy. To study the functional consequences of this mutation, we examined a wild type and two I79N-transgenic mouse lines of human cardiac TnT driven by a murine α-myosin heavy chain promoter. Extensive characterization of the transgenic I79N lines compared with wild type and/or non-transgenic mice demonstrated: 1) normal survival and no cardiac hypertrophy even with chronic exercise; 2) large increases in Ca\(^{2+}\) sensitivity of ATPase activity and force in skinned fibers; 3) a substantial increase in the rate of force activation and an increase in the rate of force relaxation; 4) lower maximal force/cross-sectional area and ATPase activity; 5) loss of sensitivity to pH-induced shifts in the Ca\(^{2+}\) dependence of force; and 6) computer simulations that reproduced experimental observations and suggested that the I79N mutation decreases the apparent off rate of Ca\(^{2+}\) from troponin C and increases cross-bridge detachment rate g. Simulations for intact living fibers predict a higher basal contractility, a faster rate of force development, slower relaxation, and increased resting tension in transgenic I79N myocardium compared with transgenic wild type. These mechanisms may contribute to mortality in humans, especially in stimulated contractile states.

Contraction of vertebrate striated (skeletal and cardiac) muscle is activated by the binding of Ca\(^{2+}\) to the Ca\(^{2+}\)-binding subunit (TnC) of the troponin complex, which together with TnI, TnT, tropomyosin, and actin form the regulatory system of the contractile apparatus (1–4). The exact function of TnT is not known, but a number of studies have shown that TnT is essential for the regulation of myofibrillar ATPase activity, the level of ATPase activation, and/or force development (5–8). Recent studies have revealed that TnT is one of the sarcomeric proteins identified in familial hypertrophic cardiomyopathy (FHC) (9, 10). FHC is an autosomal dominant disease, characterized by left ventricular hypertrophy, myofibril disarray, and sudden death. Numerous studies have shown that FHC is caused by missense mutations in various genes that encode for β-myosin heavy chain (11–14), ventricular myosin light chains 1 and 2 (15–17), myosin binding protein C (12), titin (18), actin (19), α-tropomyosin (9), troponin T (9, 10, 20), and troponin I (21, 22). Whereas individuals with β-myosin heavy chain mutations, in general, have a higher level of cardiac hypertrophy, those with TnT mutations have less hypertrophy, but a higher incidence of sudden cardiac death in young adults (10). To date, 15 human cardiac TnT mutations have been associated with FHC: I79N, R92Q/W/L, R94L, A104V, F110I, R130C, E160, E163K, E163KR, E244D, R278C, and a mutation that arises from abnormal splicing of Intron 16 (G\(_1\) → A) (23). Among these mutations, the I79N mutation is of special interest because it has been found to cause the highest risk of sudden cardiac death in young adults (10). At present, there is no clear understanding of why this TnT-I79N mutation is associated with increased sudden cardiac death. Several investigators have demonstrated an in vitro effect of the TnT-I79N mutation on the contractile properties of cardiac and skeletal muscle with conflicting results. Lin et al. (24), using rat cardiac TnT containing a mutation in an equivalent position to the TnT-I79N mutation in humans, showed that this mutant TnT had a normal affinity for actin-tropomyosin and conferred normal Ca\(^{2+}\) sensitivity to acto-S1 ATPase activity. The regulated thin filaments, however, moved 50% faster over heavy meromyosin than control filaments in an in vitro motility assay. Additional measurements utilizing the same system carried out by Homsher et al. (25) revealed that heavy meromyosin exerted reduced isometric force on single thin filaments reconstituted with the TnT-I79N mutant. Sweeney et al. (26) reported that TnT-I79N-transfected quail skeletal muscle myotubes had decreased Ca\(^{2+}\) sensitivity of force production, whereas the unloaded shortening velocity was increased about 2-fold. An embryonic isoform of rat TnT-I79N expressed in adult rat cardiac myocytes causes a decreased Ca\(^{2+}\) sensitivity of isometric force (27). Our results on TnT-I79N-reconstituted porcine fibers (28) are in accord with those of Morimoto et al. (29), who demonstrated that TnT-I79N reconstituted skinned rabbit trabeculae increased the Ca\(^{2+}\) sensitivity of contraction. A very recent study of Yanaga et al. (30) confirmed increased Ca\(^{2+}\) sensitivity of myofibrillar ATPase.
activity of TnT-I79N- reconstituted rabbit cardiac myofibrils. Part of the disparity is likely due to the different in vitro assays used by these investigators, which illustrates the need to study the effect of the mutations in an in vivo system.

Until now, a transgenic model for the TnT-I79N has not been reported, although other TnT transgenic mice have been described. A truncated CtnT in transgenic mice studied by Tar-diff et al. (31) revealed sarcomeric disarray and significant diastolic dysfunction in animals expressing protein at a very low level (<5%). Animals with higher levels of transgene expression died within 24 h of birth. Another TnT transgenic animal model reported by Marian’s lab was generated for the human cardiac TnT-R92Q mutation using a murine CtnT promoter (32). The level of expression in transgenic lines (wild type and R92Q) varied from 1 to 10% of the total CtnT pool. The authors observed diastolic dysfunction and myocyte disarray in the mutant mice as compared with wild type mice (32). The same TnT-R92Q mutant was expressed in transgenic mice in Leinwand’s laboratory where the level of R92Q expression varied from 30 to 92% (33). A murine CtnT cDNA and a rat α-myosin heavy chain promoter were used in their study. The R92Q hearts demonstrated significant induction of atrial natriuretic factor and β-myosin heavy chain transcripts, interstitial fibrosis, and mitochondrial pathology. Moreover, a basal sarcomeric activation and impaired relaxation were observed in the mutant mouse (33). In a very recent paper of Lim et al. (34), a murine α-myosin heavy chain promoter was used to produce a transgenic mouse expressing human cardiac TnT-R92Q (34). The level of protein expression was relatively low, and the mutant mice demonstrated myocyte disarray and excess interstitial collagen. Interestingly, none of these transgenic mice demonstrated significant cardiac hypertrophy.

To have an in vitro model of the TnT-I79N mutation and to possibly clarify some of the conflicting in vitro results, we have developed a transgenic model of this mutation. We examined a wild type (Tg-WT) and two I79N-transgenic mouse lines (Tg-I79N) of HCTnT driven by a murine α-myosin heavy chain promoter. The levels of expression of either Tg-WT or Tg-I79N, relative to mouse CtnT, were 71% (WT) or 35 and 52% in the two I79N lines. Extensive characterization of the Tg-I79N lines compared with Tg-WT and/or non-Tg mice demonstrated: 1) normal survival and no cardiac hypertrophy even with chronic exercise in all groups; 2) large increases in the Ca2+ sensitivity of the ATPase activity and force development in skinned fibers; 3) a substantial increase in the rate of force activation and an increase in the rate of force relaxation in flash photolysis experiments; 4) significantly lower maximal force/cross-sectional area and ATPase activity; 5) loss of sensitivity to pH-induced shifts in the Ca2+ dependence of force correlated with HCTnT-I79N expression levels; and 6) computer simulations of force-pCa relations and of flash photolysis experiments that reproduced the experimental observations and suggested that the HCTnT-I79N mutation decreases the apparent off rate of Ca2+ from the Ca2+ specific site of TnC and increases the cross-bridge apparent detachment rate g. Simulations for intact living fibers predict a higher basal contractility, a faster rate of force development, a slower isometric relaxation, and increased resting tension in Tg-I79N myocardium compared with Tg-WT. A higher basal contractility and residual resting tension limit the contractile reserve and make the ventricle vulnerable to further diastolic dysfunction. It is likely that these mechanisms contribute to the mortality observed in patients with a TnT-I79N-induced HFC especially in stimulated states of contractility such as seen during vigorous exercise or during inotropic therapy.

MATERIALS AND METHODS
Clone Construction
The cDNA for wild type human cardiac troponin T (adult isoform) was cloned by reverse transcription-PCR using primers based on the published cDNA sequence (35) and standard methods (36): HCTnT, 5'-GACCATGCTGACATAGAAGAGGT; HCTnT, 3'-AGGATTCTTCTTTCAGGCCCCTAGTT. The I79N mutant was made using overlapping sequential PCR (36). Wild type and mutant cDNAs were constructed to have an NcoI site at 5′mutantelemental ATG, and BamHI site following the stop codon to facilitate cloning into pET-3d (Novagen), which was used for bacterial expression of the proteins.

Transgene Construct
The wild type and mutant cDNAs were cloned into the unique SacI site of the plasmid, α-myosin heavy chain clone 26 (a generous gift from Dr. Jeffrey Robbins), by filling in this SacI site along with the NcoI and BamHI sites of the cDNAs and ligating the blunt fragments. The resulting construct contains about 5.5 kilobases of the mouse α-myosin heavy chain promoter, including the first 2 exons and part of the third, followed by the HCTnT cDNA (876 base pairs), and a 630-base pair 3′-untranslated region from the human growth hormone transcript.

Generation of Tg Mice
The transgene vector described above was purified on a cesium chloride gradient and restricted with Ncol to release a 7-kilobase fragment that was used for microinjection. This fragment was purified by agarose gel electrophoresis, followed by electroelution onto DEAE paper (37) and resuspended in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA at a final concentration of 5 μg/ml. Pronuclei were isolated, and the surviving embryos were implanted using standard methods (38). Founder mice were identified by preparing tail clip DNA and analyzing its hybridization to a probe corresponding to the human growth hormone 3′-untranslated region (a 630-base pair HindIII/EcoRI fragment from the transgenic construct). The PCR was also used to identify Tg mice. A forward/sense primer (5′-TTGGACCTCGAGGAGTCTT-3′) was designed. A radiolabeled oligonucleotide was used to prime reverse transcription of the cDNA sequence, and a reverse/antisense primer (5′-AGCAATCTAAATGTCCACCC-3′) was derived from human growth hormone sequence; these produced a 713-base pair fragment in mice harboring the transgene. Stable transgenic lines were generated by breeding founder Tg mice to non-Tg B6/SJL mice.

DNA and RNA Analysis of Tg Mice
Genomic Southern Blots
Large molecular weight genomic DNA was prepared from liver according to Sambrook et al. (37), and the concentration was determined by measuring the fluorescence of the Hoechst 33258 (bisbenzimide) dye (Amersham Pharmacia Biotech). Copy number was estimated by quantitative densitometry (Molecular Dynamics Densitometer) of Southern blots that had known amounts of the transgene as standards.

Northern Blots
Northern blots were performed on RNA extracted from tissues from non-Tg and Tg mice. All Tg mice hearts had an appropriately sized transcript that hybridized to the human growth hormone 3′-untranslated probe, suggesting that the transgene was being transcribed correctly. This probe was not predicted to hybridize with RNA from non-transgenic hearts or transgenic liver.

Primer Extension
To quantify the relative levels of RNA from the transgene and the endogenous mouse cardiac TnT gene, a primer extension experiment was designed (39). A radiolabeled oligonucleotide was used to prime reverse transcriptase toward the 5′ end of the RNA transcripts, resulting in radioactive products whose size reflects the length of the primer from the 5′ terminus of the RNA. The oligonucleotide primer (5′-TCCTCTGATCTCTGTGACCCACCCC-3′) was complementary to a conserved region of both transcripts that extended from nucleotide +17 to +41 relative to the ATG (i.e. the region coding for amino acids 6–14 of both human and mouse CtnT). The template for the primer extension experiment was total RNA isolated from 8–10-week-old Tg and non-Tg animals.

Reverse Transcription-PCR
RNA was prepared from hearts of transgenic and nontransgenic mice using the method of Chomczynski and Sacchi (40). Total cDNA was synthesized using Moloney murine leukemia virus reverse tran-
scriptase (Life Technologies, Inc.) according to the manufacturer’s recommendations.

Protein Analysis of Tg (Western Blotting)

Mouse hearts and pieces of human hearts were homogenized in a solution of 20% SDS and 10% β-mercaptoethanol. Small amounts of each homogenate were diluted, and their respective protein content was determined by the Coomassie Plus Bio-Rad protein assay. Homogenates were boiled in an equivalent volume of Laemmli loading buffer, mixed together in defined ratios based on protein content, electrophoresed on SDS-12.5% polyacrylamide (61:1 ratio) gels, and transferred to nitrocellulose membranes (Idena Scientific, Minneapolis, MN). Human cardiac TnT was detected using a human CtnT-specific monoclonal antibody (clone 7G7, Research Diagnostics Inc., Flanders, NJ) at a 1:2000 dilution in BLOTTO (5% nonfat dry milk, 10 mM Tris-HCl, pH 7.4, 140 mM NaCl). Total CtnT (i.e. mouse and human CtnT) was detected using a polyclonal CtnT antibody (at 1:3000 dilution) produced in our lab. The relative reactivity of the polyclonal antibody to the human and mouse CtnTs were the same based on Western blot analysis using the same protein amount of human and mouse heart tissue. HCTnT in Tg-WT and two Tg-I79N lines were determined by comparison of the immunoreactive products of the electrophoresed samples with the standard curve. The absolute amount of total CtnT was calculated from the signal intensity obtained from different ratios of human and mouse proteins (by protein content) reacted with both the monoclonal (clone 7G7) and polyclonal CtnT antibodies. Nearly identical results were obtained for the relative levels of HCTnT protein in the non-Tg and Tg mice from either a standard curve of the ratio of polyclonal antibody band intensity to monoclonal band intensity versus the percentage of HCTnT or the monoclonal band intensity versus the percentage of HCTnT (see Fig. 2). Immunoreactivity was detected using goat anti-mouse IgG labeled with horseradish peroxidase or rabbit anti-goat IgG labeled with horseradish peroxidase (both used at 1:3000 dilution; Sigma). Color was developed using diaminobenzidine/H2O2 (Sigma). Quantitative densitometry of Western blots were done using a Molecular Dynamics Densitometer. Two hearts from each transgenic line were boiled in an equivalent volume of Laemmli loading buffer, mixed together in defined ratios based on protein content, electrophoresed on SDS-12.5% polyacrylamide (61:1 ratio) gels, and transferred to nitrocellulose membranes (Idena Scientific, Minneapolis, MN). Human cardiac TnT was detected using a human CtnT-specific monoclonal antibody (clone 7G7, Research Diagnostics Inc., Flanders, NJ) at a 1:2000 dilution in BLOTTO (5% nonfat dry milk, 10 mM Tris-HCl, pH 7.4, 140 mM NaCl). Total CtnT (i.e. mouse and human CtnT) was detected using a polyclonal CtnT antibody (at 1:3000 dilution) produced in our lab. The relative reactivity of the polyclonal antibody to the human and mouse CtnTs were the same based on Western blot analysis using the same protein amount of human and mouse heart tissue. HCTnT in Tg-WT and two Tg-I79N lines were determined by comparison of the immunoreactive products of the electrophoresed samples with the standard curve. The absolute amount of total CtnT was calculated from the signal intensity obtained from different ratios of human and mouse proteins (by protein content) reacted with both the monoclonal (clone 7G7) and polyclonal CtnT antibodies. Nearly identical results were obtained for the relative levels of HCTnT protein in the non-Tg and Tg mice from either a standard curve of the ratio of polyclonal antibody band intensity to monoclonal band intensity versus the percentage of HCTnT or the monoclonal band intensity versus the percentage of HCTnT (see Fig. 2). Immunoreactivity was detected using goat anti-mouse IgG labeled with horseradish peroxidase or rabbit anti-goat IgG labeled with horseradish peroxidase (both used at 1:3000 dilution; Sigma). Color was developed using diaminobenzidine/H2O2 (Sigma). Quantitative densitometry of Western blots were done using a Molecular Dynamics Densitometer. Two hearts from each transgenic line were independently analyzed on three different blots to assess the relative levels of HCTnT protein in non-Tg, Tg-WT, and Tg-I79N mutant lines. Human heart samples, obtained from a transplant patient at Jackson Memorial Hospital adjacent to the University of Miami School of Medicine, were rapidly frozen in liquid nitrogen and stored at −150°C until use. These heart samples showed little or no sign of degradation as based on Western blotting analysis.

Mice Exercise Protocol

A swimming protocol mainly described by Geisterfer-Lowrance et al. (41) was utilized. Groups of four 2-month-old animals representing the different lines were used. Mice were acclimated to the swimming program by beginning with 10-min sessions two times a day separated by 4 h. These were incremented by 10 min/day until reaching 90 min/session. The program was completed in 4 weeks. Animals were weighed weekly. During each session, they were monitored for inability to sustain the exercise and/or sudden death. During weekly intervals the pCa 4 solution (composition is the same as pCa 8 buffer except the pCa 8 buffer was replaced by 1.3–1.7 mm and 150–250 μM [MgATP2] and relaxed in the pCa 8 solution. Steady state forces developed by non-Tg, the wild type Tg-WT and its mutant, Tg-I79N fibers were compared.

Ca2+ Dependence of Force Development—After measurements of the initial steady state force of the fibers, they were relaxed in the pCa 8 buffer and then exposed to the solutions of increasing Ca2+ concentrations (from pCa 8 to pCa 4). The maximal force was measured in each "pCa" solution followed by the short relaxation of the fibers in the pCa 8 solution. Data were analyzed using the following equations: % Force Restored = 100 × (Force Restored – Residual Force)/Initial Force; % Change in Force = 100 × [Ca2+]p/(Ca2+]p + [Ca2+]e), where [Ca2+]e is the free Ca2+ concentration that produces 50% force and n is the Hill coefficient (nH).

Rates of Force Activation—For the kinetic measurements the bundle of 3–5 fibers was attached by tweezer clips to a force transducer, placed in a 1-ml cuvette, and bathed in the pCa 8 solution. The fibers were tested for steady state force development in the pCa 4 solution and relaxed in the pCa 8 solution. Then they were exposed to 2.5 mM DM-nitrophen, 1.002 mM CaCl2, 100 μM, 1.2 mM MgCl2, 1.4 mM ATP, 10 μM glutathione, 29.4 mM (1,6-hexamethylene diamine-N,N,N’,N’-tetraacetic acid), and 20 mM creatine phosphate, pH 7.1. Subsequent to irradiation by a 1-ms UV light pulse from Xenon lamp (model XFL-250- 30717), the Ca2+ chelator was cleaved, releasing free Ca2+. Its high affinity for Ca2+ before photolysis, Kp, decreased from 5.0 × 105 to 3.0 × 103 M, following the UV flash. As a result of the rapid Ca2+ release, the fibers developed isometric tension, characterized by a two exponential time course. The rate constants of activation, was calculated according to the equation: y = A(1 − e−k1t) + B(1 − e−k2t) + C, where k1 and k2 are the rate constants and A and B are the amplitudes of the force transient. We believe that the major fast component is due to the rapid activation of contraction and that the minor slow component is due to a diffusion process related to re-equilibration of the fiber with the bulk solution after the flash.

Rates of Force Relaxation—The initial step of the fiber preparation was the same as for the measurement of the activation rates. To monitor the relaxation rates, a photolabile derivative of O,O-bis(2-amino phenyl)ethyleneglycol-N,N,N’,N’-tetraacetic acid tetrapotassium, Diazio-2, was used. Diazio-2 is able to rapidly chelate Ca2+ upon photolysis converting from a low affinity (Kp = 2.2 μM) to high affinity (Kp = 0.073 μM) for Ca2+. After testing steady state force, the fibers were immersed in the solution of 2 mM Diazio-2, 0.5 mM CaCl2, 60 mM TES, pH 7.0, 5 mM MgATP, 1 mM [Mg2+], and 10 mM creatine phosphate along with 15 units/ml creatine phosphokinase (ionic strength = 200 mM) adjusted with potassium propionate. At the ratio of total added Ca2+ to Diazio-2 give above, the resulting average initial force will be around 80% of the maximal force measured in the pCa 4 solution. The ratio provides the greatest extent of relaxation after photolysis of the Diazio-2. When force reached equilibrium, the fibers were exposed to a UV flash from Xenon lamp. The photolysis-induced relaxation was measured several times during the fiber treatment. The rate constants of relaxation was calculated according to the equation: y = Ae−k1t + Be−k2t + C, where k1 and k2 are the rate constants and A and B are the amplitudes of the force transient.

Simultaneous Force and ATPase Measurements in Fresh (Not Glycerinated) Skinned Fibers

Small preparations (~1 mm long and 50–70 μm in diameter) of mouse papillary muscle were dissected free in relaxing solution and then treated with 1% Triton X-100 for 30 min. Subsequently they were mounted in the Guth Muscle Research System, which allows for simultaneous force and ATPase measurements (42, 43). The quartz cuvette surrounding the preparation had a square cross-section of 1.0 mm2. The sarcomere length was set to 2.2 μm using a laser diffraction pattern. The solution in the cuvette was changed every 20 s using a peristaltic pump triggered by a computer. The hydrolysis of ATP was measured by the NAD+/NADH fluorescent method, in which ATP was regenerated from ADP and phosphoenolpyruvate by the enzyme pyruvate kinase (Reaction 1) (44). The reaction scheme is as follows.

PK

| ADP + PEP | ATP + pyruvate |
|---|---|
| Reaction 1 |

| pyruvate + NADH | LDH | Lactate + NAD (fluorescent) |
|---|---|---|
| Reaction 2 |

This reaction is coupled to the oxidation of NADH (fluorescent) to NAD (nonfluorescent), and the reduction of pyruvate to lactate by l-lactate dehydrogenase (Reaction 2) (45, 46). In this reaction 1 mol of phosphoenolpyruvate and NADH are used to produce 1 mol of ATP and NAD. The solution surrounding the fiber in the quartz cuvette was illuminated at 340 nm, and the decrease in NADH concentration was...
detected by a decrease in the fluorescence signal at wavelengths greater than 470 nm. The fluorescence change taking place between each solution change was converted to rate of ATP hydrolysis by comparison with NADH standards.

**Ca**²⁺ Concentration Measurements**—The **Ca**²⁺ concentration in the solution perfusing the skinned preparation was varied by use of a gradient maker (Scientific Instruments GmbH, Heidelberg, Germany) to mix two solutions of known [**Ca**²⁺] and ionic composition together (42, 43). The resulting [**Ca**²⁺] was calibrated using the fluorescent **Ca**²⁺ indicator, Calcium Green-2 (Molecular Probes). The **Kₐ** of Calcium Green-2 used to calculate p**Ca** is 4.4 µM. The concentration of Calcium Green-2 in the gradient solution was 1.0 µM. Calcium Green-2 changes its fluorescence over the range of **Ca**²⁺ required for activation of contraction and ATPase activity. The Calcium Green-2 fluorescence was excited at 480 nm, and the fluorescence was measured with a cut-off filter at 515 nm.

**Solutions**—All fresh (not glycerinated) skinned fiber solutions contained 85 mM K⁺ plus Na⁺ added with Na⁺-ATP, 2 mM MgATP²⁻, 1 mM MgCl₂, 7 mM EGTA, 10⁻⁴-10⁻⁴ M Ca²⁺, 5 mM phosphoenolpyruvate, 100 units/ml pyruvate kinase, and propionate as the major anion. Solutions for ATPase measurements also contained 0.4 mM NADH, 0.2 mM AP₅A (to inhibit myokinase), and 140 units/ml L-lactate dehydrogenase. Ionic strength was adjusted to 0.15 M, and the pH was maintained at 7.00 ± 0.02 with imidazole propionate. Relaxing solutions contained no added Ca²⁺ (10⁻⁴ M Ca²⁺). The concentrations of the various ionic species were determined by solving ionic equilibrium equations using published binding constants (48).

**Computer Modeling of Experimental Data**—Computer simulations were based on modified model of Robertson et al. (49) and a two-state cross-bridge model (50) utilizing an exponential dependence of the TnC off rate for **Ca**²⁺ (**Ca**²⁺-specific site II) on force (51, 52). A detailed description of the model is provided in the "Appendix."

**RESULTS**

**Generation and Identification of HCTnT-WT and HCTnT-I79N Mutant Tg Mice**

A total of two wild type HCTnT (Tg-WT, lines 2 and 3) and six HCTnT-I79N founder mice (Tg-I79N, lines 4–9) were identified by PCR and Southern blot analysis (data not shown). Lines 3, 8, and 9 consistently produced expected Mendelian ratios of transgenic offspring and were selected for further studies. The copy number of the transgenes were 40 for line 3 (Tg-WT) and line 8 (Tg-I79N), and 48 for line 9 (Tg-I79N) (data not shown). On Northern blot analysis, all Tg mouse hearts had an appropriately sized transcript that hybridized to the human growth hormone 3'-untranslated probe, suggesting that the transgene was being transcribed correctly (data not shown). A primer extension experiment was designed to quantify the relative levels of RNA from the transgene and the endogenous mouse CTnT gene (Fig. 1). The predicted size of products from the endogenous and transgenic transcripts was different, 113 nucleotides versus 144, respectively, mainly because the transgene contained 5' exons coding for α-myosin heavy chain 5'-untranslated RNA. This assay detected a consistent level of endogenous CTnT transcript in Tg and non-Tg mice. Transgenic RNA expression varied between lines, with considerably higher expression levels in lines 3 (WT) and 8 (I79N) and lower levels in line 9 (I79N).

**Protein Expression Levels**

To determine the expression level of HCTnT in the non-Tg, Tg-WT, and Tg-I79N lines, an HCTnT specific monoclonal antibody was utilized as described under "Materials and Methods." In Fig. 2, the average data of two mice, each run on three independent blots, are presented. The non-Tg and Tg samples were compared with the standard curve (see "Materials and Methods"), and the levels of HCTnT expression quantified. Tg-WT (line 3) contained 70.9% of the total TnT present in the mouse heart, whereas Tg-I79N contained 52% (line 8) and 34.6% (line 9).

**Heart Weight/Body Weight**

Tg mice expressing HCTnT-I79N had normal survival and no cardiac hypertrophy. A significantly decreased heart weight to body weight ratio was observed for the Tg-I79N mice compared with Tg-WT (Fig. 3A). Chronic swimming exercise (4 weeks), which has previously been used to induce cardiac hypertrophy in another murine FHC model (41), did not affect survival in all groups of Tg lines. Surprisingly, heart to body weight ratio increased only in the non-Tg and not in the Tg mice and remained significantly lower in the Tg-I79N mice versus the Tg-WT mice (Fig. 3B).

**Skinned Fibers Studies**

Our previous results on TnT-I79N-reconstituted porcine fibers (28) demonstrated a significant increase in the **Ca**²⁺ sensitivity of force development but no change in the maximal force. The effect of the TnT-I79N mutation in vivo has been examined in two sets of skinned fiber experiments. The first set was performed on glycerinated bundles of mouse papillary muscles whose diameter was between 150 and 200 µm. The second set of experiments was performed on fresh (not glycerinated) thin papillary muscle, with a diameter of ~50–70 µm.

**Glycerinated Fibers**

Steady State Force Development—After measurements of the initial steady state force (p**Ca**), non-Tg, Tg-WT, and Tg-I79N
fibers were exposed to solutions of increasing Ca\(^{2+}\) concentration, and the force-pCa relationship for the different mice was established. Fig. 4A shows a typical force-pCa curves, and Fig. 4B summarizes the pCa\(_{50}\) values for the sedentary and exercised mice. As can be seen, the Tg-I79N mice had an increased Ca\(^{2+}\) sensitivity of steady state force development in both the sedentary and exercised groups compared with non-Tg or Tg-WT. The increase was pCa\(_{50}\) ~ 0.2. No significant difference was observed between the two mutant Tg-I79N lines (line 8 and line 9). Interestingly, force per cross-sectional area was much lower for the Tg-I79N fibers versus the Tg-WT in both sedentary and exercised mice (Fig. 5).

Kinetics of Force Development—To study the rates of force activation and relaxation of the glycogenated fibers, we utilized flash photolysis of either caged calcium (DM-nitrophen) or caged chelator (Diazo-2), respectively. The Tg-I79N fibers demonstrated a significantly increased rate of force activation and moderately increased rate of relaxation compared with Tg-WT fibers. Fig. 6 illustrates representative experimental traces and the double exponential fit curves of activation (panel A) and relaxation (panel B) of force development for Tg-WT and Tg-I79N fibers. The rates of activation and relaxation were acquired from different experiments utilizing several Tg lines and averaged (only faster components with larger amplitudes). Activation of the Tg-I79N fibers was 29.8 ± 1.4 s\(^{-1}\) (n = 22), i.e. about 1.7-fold higher than Tg-WT fibers, 18.0 ± 0.7 s\(^{-1}\) (n = 12). The rate of relaxation for Tg-I79N fibers was 33.5 ± 1.8 s\(^{-1}\) (n = 17) and 28.8 ± 2.3 s\(^{-1}\) (n = 9) for Tg-WT fibers. Data are the averages of n experiments ± S.E.

Effect of pH on the Ca\(^{2+}\) Sensitivity of Force Development—Lowering pH in the physiological range (~ 7.0–6.5) is known to shift the Ca\(^{2+}\) sensitivity of force development from lower to higher calcium concentrations, especially in cardiac muscle (53–55). The Tg-WT and non-Tg mice showed this clearly (Fig. 7). However, the Tg-I79N mice had a much lower change in Ca\(^{2+}\) sensitivity to this change in pH. Changing the pH from 7.0 to pH 6.5 decreased the Ca\(^{2+}\) sensitivity of force by ΔpCa\(_{50}\) ~ −0.5 for the non-Tg or Tg-WT fibers. Both lines (8 and 9) of Tg-I79N became less sensitive to acidic pH, and a much smaller decrease in Ca\(^{2+}\) sensitivity of force development was observed. For TnT-I79N line 9, the change was ΔpCa\(_{50}\) ~ −0.39 and for line 8, ΔpCa\(_{50}\) ~ −0.2. Interestingly, line 8 had the highest level of protein expression among the Tg-I79N lines (52%), and the Ca\(^{2+}\) sensitivity change was only half of that observed for line 9 (Fig. 7A) expressing ~35% of HCTnT-I79N (Fig. 2). The effect of chronic exercise on the Ca\(^{2+}\) sensitivity of force in the two-pH conditions was examined for Tg-I79N line 9 compared with Tg-WT (Fig. 7B). As shown, exercise did not affect the level of change in Ca\(^{2+}\) sensitivity seen in the sedentary mice.

Intact Fibers

The second set of experiments was performed on fresh (not glycogenated) fibers whose diameter was between 50 and 70 μm. These freshly isolated fibers, skinned with Triton X-100, had a lower Ca\(^{2+}\) sensitivity of force development than the glycogenated ones and had an even greater difference in Ca\(^{2+}\) sensitivity (ΔpCa\(_{50}\) ~ 0.44) when comparing the Tg-WT fibers with fibers from the Tg-I79N mice (Fig. 8). Note that the force was also lower in the mutant mice compared with the wild type (Fig. 8B). The same was true for the ATPase measurements (Fig. 8A). The difference in Ca\(^{2+}\) sensitivity of the ATPase activity between Tg-WT and the Tg-I79N mice was ΔpCa\(_{50}\) ~ 0.38. When the ATPase activity was measured simultaneously with force, it was also shifted leftward. Results of the ATPase and force measurements on intact fibers were very reproduc-
Fig. 4. Effect of the HCTnT-I79N mutation on the Ca\textsuperscript{2+} sensitivity of force development in sedentary and exercised mice. A bundle of glycinated fibers (~1.3–1.7 mm long and 150–250 µm in diameter) were attached to a force transducer, bathed in the pCa 8 solution (1 ml), and tested for steady state force development in the pCa 4 solution. To determine the force-pCa dependence in different transgenic lines, the fibers were then exposed to solutions of increasing Ca\textsuperscript{2+} concentrations (from pCa 8 to pCa 4). Experimental points were fit to the Hill equation giving the pCa\textsubscript{50} and n\textsubscript{H} (Hill coefficient) values. The data represent the average of n experiments ± S.E. A and B: sedentary: non-Tg, pCa\textsubscript{50} = 5.52 ± 0.03, n\textsubscript{H} = 2.73 ± 0.17 (n = 9); Tg-WT, pCa\textsubscript{50} = 5.57 ± 0.01, n\textsubscript{H} = 3.50 ± 0.19 (n = 18); Tg-I79N L9, pCa\textsubscript{50} = 5.74 ± 0.01, n\textsubscript{H} = 2.76 ± 0.17 (n = 6); Tg-I79N L8, pCa\textsubscript{50} = 5.74 ± 0.01, n\textsubscript{H} = 2.15 ± 0.09 (n = 21). B: exercised: non-Tg, pCa\textsubscript{50} = 5.49 ± 0.03, n\textsubscript{H} = 2.31 ± 0.17 (n = 6); Tg-WT, pCa\textsubscript{50} = 5.48 ± 0.003, n\textsubscript{H} = 2.91 ± 0.12 (n = 21); Tg-I79N L9, pCa\textsubscript{50} = 5.75 ± 0.03, n\textsubscript{H} = 2.10 ± 0.06 (n = 9); Tg-I79N L8, pCa\textsubscript{50} = 5.75 ± 0.03, n\textsubscript{H} = 1.89 ± 0.06 (n = 6).

Fig. 5. Effect of the HCTnT-I79N mutation on maximal force. Maximal steady state force developed by non-Tg, Tg-WT, and two lines (L8 and L9) of Tg-I79N fibers were measured in the pCa 4 solution. Force/cross-sectional area (kN/m\textsuperscript{2}) was determined. Sedentary: non-Tg, 27.4 ± 2.60 (n = 5); Tg-WT, 31.15 ± 1.97 (n = 18); Tg-I79N L8, 22.24 ± 1.54 (n = 24); Tg-I79N L9, 18.84 ± 2.39 (n = 9). Exercised: non-Tg, 26.5 ± 4.0 (n = 3); Tg-WT, 33.52 ± 2.69 (n = 15); Tg-I79N L8, 18.80 ± 1.49 (n = 4); Tg-I79N L9, 22.50 ± 3.06 (n = 5). The data represent the average of n experiments ± S.E. (from 0.611 to 0.471) and increased k to values seen experimentally. Further fine tuning of k\textsubscript{off(TnC-Ca)} gave values of k, ∆pCa\textsubscript{50}, and peak force that very closely fit our experimental observations (see activation rates and Fig. 9). To determine whether this combination of values is unique or not, we attempted to produce the same results by altering only f and g with no changes in k\textsubscript{off(TnC-Ca)} (lower half of Table I). An increase in f causes a leftward shift in pCa\textsubscript{50}, an increase in k, and an increased peak force. Increasing g to compensate for this balanced out the effects of an increased f on ∆pCa\textsubscript{50} and peak force. A further increase in g caused a rightward shift of the pCa\textsubscript{50} and increased k way beyond values seen experimentally. To cause a leftward shift in pCa\textsubscript{50}, k\textsubscript{off(TnC-Ca)} was decreased (from 300 to 88 s\textsuperscript{-1}), and this combination resulted in an excellent fit for ∆pCa\textsubscript{50} and peak force, yet k was almost double the value seen experimentally. This is not surprising because in skinned and intact fibers increases of f and/or g increased k, the rate of force redevelopment after a quick release-stretch cycle, k\textsubscript{tr} = f + g (58). It therefore seems necessary to limit the combined values of f and g not to exceed certain values to limit the value of k. Furthermore, because f and g effects on ∆pCa\textsubscript{50} and peak force cancel each other, it appeared unnecessary to change both f and g. A change of g from 10 to 20 s\textsuperscript{-1} in addition to the aforementioned decrease in k\textsubscript{off(TnC-Ca)} was sufficient to reproduce the experimental results. In summary, a decrease in k\textsubscript{off(TnC-Ca)} from 300 to 88 s\textsuperscript{-1} combined with an increase in g from 10 to 20 s\textsuperscript{-1} provided for an excellent fit to the experimentally observed data and reproduced the DM-nitrophen flash photolysis force transient and force-pCa relationship for both wild type and TnT-I79N. Fig. 9A shows the results of the simulation of the DM-nitrophen flash photolysis-induced force transient for both Tg-WT and Tg-I79N. The rate constants of force development are very close to those observed experimentally (WT 18.39 s\textsuperscript{-1} simulated versus 18.0 s\textsuperscript{-1} observed; I79N 29.68 s\textsuperscript{-1} simulated versus 29.8 s\textsuperscript{-1} observed). Fig. 9B shows the simulation of a flash photolysis experiment with Diazo-2. 179N myocardium relaxes faster than WT with rate constants of 10.7 s\textsuperscript{-1} in the Tg-WT and 21.2 s\textsuperscript{-1} in the Tg-I79N myocardium. These values are of the same order of magnitude as those seen experimentally (28.8 s\textsuperscript{-1} WT and 33.5 s\textsuperscript{-1} I79N). Although the absolute values of the rate constant of relaxation of force were not exactly reproduced, relaxation of I79N myocardium
after a Diazo-2 flash was faster both experimentally and with the current simulation settings. Fig. 9C shows the force-pCa relation for both WT and TnT-I79N mutant. By comparing these figures with the experimental observations (Fig. 8B), it is obvious that the variables used in the simulation reproduce the data of the skinned fibers quite well. We also attempted to predict the behavior of intact ventricular myocardium afflicted with the HCTnT-I79N mutation by using the same variables of $k_{\text{effTnT-Ca}}$, $f$ and $g$ and simulated the intracellular Ca$^{2+}$ transient and force in a continuous series of twitch contractions in steady state conditions. Fig. 9D shows that the changes in $k_{\text{effTnT-Ca}}$, $f$, and $g$ derived earlier from skinned fibers data cause a smaller peak and slower decline of the intracellular free Ca$^{2+}$ transient (top panel), an increased peak force, a delayed relaxation of force and an increased rate of contraction (middle panel). To validly compare the time course of isometric relaxation in WT and I79N, we normalized the force traces to eliminate the well known effects of twitch amplitude on relaxation time course. The bottom panel of Fig. 9D clearly demonstrates that isometric relaxation in I79N myocardium is slower than in WT. If heart rate is not allowed to change, the residual force at the end of one twitch is present at the beginning of the next, and an increased end-diastolic force ensues.

**DISCUSSION**

This study describes the first transgenic animal model for the TnT-I79N mutation. We have examined three transgenic lines with high levels of cardiac muscle specific expression of human cardiac TnT under the control of the murine $\alpha$-myosin heavy chain promoter. There was no obvious correlation between transgene copy number, mRNA expression level, or endogenous TnT replacement with Tg-WT and both lines of HCTnT-I79N. However, a correlation between the mRNA expression level and endogenous TnT replacement was observed. The overall stoichiometric ratio of all sarcomeric proteins in the total cardiac extract was well preserved in all transgenic lines.

**FIG. 6.** Effect of the HCTnT-I79N mutation on the activation (A) and the relaxation (B) rates of force transient. Flash photolysis of DM-nitrophen (A) or Diaz-2 (B) was utilized as described under "Materials and Methods." A, representative experimental traces of activation of force development. Data points were fitted to $y = A(1 - e^{-kt}) + B(1 - e^{-kt}) + C$, where $A = 3.42, B = -0.96, C = 0.58, k_1 = 18.6 \text{ s}^{-1}$, and $k_2 = 0.03 \text{ s}^{-1}$ for Tg-WT, and $A = 2.33, B = -7, C = 0.48, k_1 = 38.8 \text{ s}^{-1}$, and $k_2 = 0.03 \text{ s}^{-1}$ for Tg-I79N. B, representative experimental traces of force relaxation. Data points were fitted to $y = Ae^{-kt} + Be^{-kt} + C$, where $A = 1.53, B = 0.6, C = 0.62, k_1 = 29.36 \text{ s}^{-1}$, and $k_2 = 0.01 \text{ s}^{-1}$ for Tg-WT, and $A = 1.4, B = 0.50, C = 0.67, k_1 = 33.21 \text{ s}^{-1}$, and $k_2 = 0.01 \text{ s}^{-1}$ for Tg-I79N.

**FIG. 7.** Effect of pH on the Ca$^{2+}$ sensitivity of force development in sedentary (A) and exercised (B) mice. Maximal force dependence and the force-pCa dependence were determined for non-Tg, Tg-WT, and two lines (L8 and L9) of Tg-I79N mouse fibers (sedentary and exercised) in two sets of pCa solutions, pH 7 and pH 6.5. The experimental points were fitted to the Hill equation as in Fig. 4. Sedentary pH 7.0: non-Tg, $p_{Ca_{50}} = 5.53 (n = 3)$; Tg-WT, $p_{Ca_{50}} = 5.54 \pm 0.01 (n = 15)$; Tg-I79N L8, $p_{Ca_{50}} = 5.74 \pm 0.014 (n = 18)$; Tg-I79N L9, $p_{Ca_{50}} = 5.72 \pm 0.013 (n = 9)$. Sedentary pH 6.5: non-Tg, $p_{Ca_{50}} = 5.04 (n = 3)$; Tg-WT, $p_{Ca_{50}} = 4.98 \pm 0.02 (n = 15)$; Tg-I79N L8, $p_{Ca_{50}} = 5.54 \pm 0.03 (n = 18)$; Tg-I79N L9, $p_{Ca_{50}} = 5.33 \pm 0.01 (n = 9)$. Exercised pH 7.0: Tg-WT, $p_{Ca_{50}} = 5.48 \pm 0.03 (n = 9)$; Tg-I79N L9, $p_{Ca_{50}} = 5.70 \pm 0.02 (n = 6)$. Exercised pH 6.5: Tg-WT, $p_{Ca_{50}} = 4.92 \pm 0.02 (n = 9)$; Tg-I79N L9, $p_{Ca_{50}} = 5.29 \pm 0.03 (n = 6)$. The data represent the average values from $n$ experiments $\pm$ S.E.
The Ca²⁺ preparation was changed every 20 s using a peristaltic pump triggered by the computer. The hydrolysis of ATP was measured by the NADH fluorescence method (see "Materials and Methods").

The major finding of this report is that expression of mutant HCTnT-I79N was incorporated into the thin filaments of cardiac myofilaments. They were both shifted toward lower Ca²⁺ concentrations in the Tg-I79N mice. It should be mentioned that the pCa50 values of force development in the glycerinated thicker fibers were somewhat higher than those in the smaller diameter freshly skinned fibers. This could be due to various factors determining force-pCa dependence in these different preparations, such as the fiber size and/or the glycerinating process. Moreover, the force-pCa dependence in the glycerinated fibers was determined in the pCa solutions whose free Ca²⁺ concentration was calculated using a computer program (59), whereas the simultaneous force and ATPase measurements in fresh (not glycerinated) skinned fibers utilized direct free Ca²⁺ determination with a fluorescent calcium indicator (42, 43). In any event, both methods demonstrated increased Ca²⁺ sensitivity in the Tg-I79N mice.

Interestingly, our previous experiments with HCTnT-I79N-reconstituted porcine muscle fibers demonstrated a similar increase in Ca²⁺ sensitivity of force (28). Yet, the effect seen in transgenic skinned HCTnT-I79N mouse fibers was larger than that observed in the reconstituted porcine filaments. Moreover, force/cross-sectional area was much lower for the Tg-I79N fibers versus the Tg-WT in both sedentary and exercised mice. Kinetics of force activation were also altered in the Tg-I79N mice. The rate of activation was about 1.7-fold higher for the Tg-I79N fibers compared with Tg-WT fibers, whereas the relaxation rates were only slightly different. The higher rates of activation in the Tg-I79N mice are in agreement with the results of Sweeney et al. (26), who showed that unloaded shortening velocity in TnT-I79N-transfected quail skeletal muscle myotubes was increased about 2-fold.

Transgenic studies are generally strengthened by inclusion of as many independent lines as possible. Although only two Tg-I79N lines and one Tg-WT line were extensively studied in this report, recent preliminary data from our laboratory suggest the calcium effects are specific to transgene expression, rather than insertional artifacts or epigenetic effects. Specifically, administration of propyl-thiouracil, which induces hypothyroidism and down-regulates the α-myosin heavy chain promoter (60, 61), causes the mutant phenotype to return to normal (unpublished data). Furthermore, the α-myosin heavy chain promoter has proven to be very reliable in driving cardiac-specific, developmentally regulated gene expression in other transgenic systems (33, 34, 60).

These changes in the Ca²⁺ regulation of the ATPase and force development as well as changes in maximal force and rate of force activation could be critical in understanding abnormalities observed in humans that ultimately lead to catastrophic results and sudden death of individuals carrying the TnT-I79N mutation. One could speculate that this mutation in HCTnT leads to changes in the interactions between TnT and other troponin subunits, TnI and TnC, and/or to changes in their interactions with actin-tropomyosin. This could lead to changes in contractility and possibly affect the Ca²⁺ affinity of TnC. Because TnC is a major Ca²⁺ buffer within the muscle cell, changing its Ca²⁺ affinity would alter overall [Ca²⁺] homeostasis as we observed in our computer simulation. This in turn might trigger numerous Ca²⁺-dependent cellular processes.

Abnormalities seen in the level of force development for the Tg-I79N fibers suggest possible changes in inotropic responses in the working human heart. The increased rate of force activation in the Tg-I79N mice, with more force being produced, could indeed decrease the inotropic reserve, an effect that was observed in vivo in these transgenic HCTnT-I79N mice.²

²B. C. Knollmann, S. A. Blatt, K. Horton, P de Freitas, T. Miller, M. Bell, P. R. Housmans, N. J. Weissman, M. Morad, and J. D. Potter (2001) J. Biol. Chem., in press.

FIG. 8. Simultaneous ATPase (A) and force (B) measurements in fresh (not glycerinated) skinned cardiac muscle fibers. Representative experimental curves of ATPase and force for non-Tg, Tg-WT, and Tg-I79N. The Ca²⁺ dependence of ATPase and force were very reproducible among the Tg lines, with little variation between different fibers within the line. The experiments were performed on fresh (not glycerinated) fibers whose diameter was between ~50 and 70 μm. The solution in a quartz cuvette (cross-section of 1.0 mm²) surrounding the preparation was changed every 20 s using a peristaltic pump triggered by a computer. The hydrolysis of ATP was measured by the NADH fluorescence method (see "Materials and Methods"). The Ca²⁺ concentration was varied by use of a gradient maker mixing two solutions of known [Ca²⁺] and ionic composition together. The Ca²⁺ concentration produced by the gradient maker was calibrated using the fluorescent Ca²⁺ indicator Calcium Green-2, which changes its fluorescence over the range of [Ca²⁺] required for the activation of contraction and ATPase activity. The Calcium Green-2 fluorescence was excited at 480 nm, and the fluorescence was measured with a cut-off filter at 515 nm. Note that the force was also lower in the mutant mice compared with the wild type. The same was true for the ATPase measurements. The ATPase activity was also shifted leftward.

The skinned fiber experiments demonstrated that once mutant HCTnT-I79N was incorporated into the thin filaments of murine hearts, it caused several functional abnormalities compared with transgenic HCTnT-WT or nontransgenic muscle. The major finding of this report is that expression of mutant human TnT-I79N in mice increased Ca²⁺ sensitivity of the ATPase activity and force development in cardiac myofilaments. They were both shifted toward lower Ca²⁺ concentrations in the Tg-I79N mice. It should be mentioned that the

studied. Interestingly, the animals tolerated exercise quite well, and there were no deaths or any visible exercise-induced hypertrophy in the mice. The heart weight to body weight ratio was slightly decreased in the Tg-I79N animals compared with Tg-WT mice but not significantly different from the non-Tg animals. These results are consistent with the clinical data where no overall cardiac hypertrophy, increase in maximal left ventricular wall thickness or collagen deposits were associated with the TnT-I79N mutation in humans (10).

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*Intracellular pH drops rapidly after the onset of ischemia in cardiac muscle and may play some role in the rapid drop in

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force that ensues (63–65). A decrease in pH results in the rightward shift of the Ca\(^{2+}\) dependence of force development toward higher Ca\(^{2+}\) concentrations. This effect is thought to be an adaptive as well as protective mechanism of cardiac muscle to changes in the acidic environment. Cardiac TnT, the inhibitory subunit of the troponin complex, has been implicated as the Tn subunit responsible for the effect of pH on the Ca\(^{2+}\) sensitivity of contraction (54, 66, 67). Our experiments suggest that TnT as well as TnI plays a role in this process. Lowering the pH in the physiological range (~7.0–6.5) shifted the Ca\(^{2+}\) sensitivity of force development from lower to higher calcium concentrations for non-Tg and Tg-WT mice by ΔpCa\(_{50}\) ~ 0.5. However, the Tg-I79N fibers had a much smaller change in Ca\(^{2+}\) sensitivity over this range of pH. Moreover, for the I79N line 8, which had the highest protein expression among the Tg-I79N lines (52%), the Ca\(^{2+}\) sensitivity was essentially unaffected by this change in pH. Exercising did not alter these properties. It has been postulated that the rightward shift of the Ca\(^{2+}\) dependence of force development toward higher Ca\(^{2+}\) concentrations at higher H\(^+\) concentration (lower pH) may result from a decrease in the affinity of TnC for Ca\(^{2+}\) (55). This lack of Ca\(^{2+}\) response in the Tg-I79N mice suggests that the interaction between TnT-I79N and TnC prevents TnC from lowering its affinity for Ca\(^{2+}\) and therefore interferes with the adaptive and protective mechanism of the muscle cell to function in the acidic environment that ensues during myocardial ischemia.

To determine the possible impact of the HCTnT-I79N mutation on contraction and relaxation of intact ventricular myocardium, we used a simple mathematical model of intracellular Ca\(^{2+}\) buffering and of force generation based on Huxley’s two-state cross-bridge model. The I79N mutation confers an increased Ca\(^{2+}\) sensitivity of force, a decreased peak force, and a faster development of force after a step in activation as achieved during a flash photolysis experiment with DM-nitrophen in skinned cardiac fibers. Relaxation of force during flash photolysis experiments with Diazo-2 was slightly faster in the Tg-I79N than in Tg-WT skinned fibers. Step by step simulation of these changes (Table I and Fig. 9) suggests that the dissociation of Ca\(^{2+}\) from TnC is slowed by the I79N mutation and that cross-bridge detachment is accelerated. These two opposing changes in a unique way modify peak force, pCa\(_{50}\), and the rate constant k of force development to exactly reproduce the experimental observations in skinned cardiac fibers. Therefore, in addition to increased Ca\(^{2+}\) affinity of TnC, it seems likely, based on theoretical considerations and deductive analysis of skinned fiber data, that cross-bridge kinetics are changed by HCTnT-I79N. The observed drop in force/cross-sectional area and the change in cross-bridge kinetics support this analysis. An increased rate of cross-bridge detachment was also inferred by others from their in vitro motility assays (24, 25) or TnT-I79N transfected myotubes (26), and a consistent picture regarding this mutation is beginning to emerge based on results from many different approaches. It also appears that the cross-bridge effects brought about by this mutation are distinct from the calcium effects, and it is possible that the former arise from an alteration in the interactions between TnT, tropomyosin, and F-actin, whereas the latter arise from altered TnT and TnC interactions.

Analysis of numerous simulations show that in intact cardiac fibers, a decrease in \(k_{\text{off(TnT-Ca)}}\) is invariably accompanied by a change in the time course of the intracellular Ca\(^{2+}\) transient, whereas changes in the cross-bridge kinetics do not perceptibly change the Ca\(^{2+}\) transient. Relaxation of force is slowed the most by a decreased \(k_{\text{eff(TnC-Ca)}}\) whereas this effect is somewhat attenuated by an increase in cross-bridge detachment rate g. The twitch contraction operates from a pCa range of ~7.7 at rest to a peak systolic value of 5.7. Using these pCa values, simulated peak twitch force in steady state conditions in fibers containing Tg-I79N was higher than in fibers that contained Tg-WT (Fig. 9). The model further predicts an increased rate of force development, a slower isometric relaxation, and an increased residual force or resting tension at the onset of the next contraction. If twitch amplitudes in both Tg-WT and Tg-I79N were the same or are normalized, isometric relaxation of Tg-I79N myocardium is slower than in the WT as was observed in isovolumetrically contracting isolated heart.\(^2\) The predicted slower isometric relaxation in intact fibers may appear to contradict the relaxation flash photolysis results and simulations, which show a faster relaxation in Tg-I79N than in Tg-WT. However, intact fibers operate in the pCa 7.2–5.5 range, whereas skinned fiber results were obtained at lower pCa values, and intact fibers are driven by a time-varying Ca\(^{2+}\) transient, whereas in flash photolysis pCa changed in steps from one value to another fixed value. Because the pCa step in flash photolysis experiments is virtually instantaneous, the rate-limiting step for relaxation is the detachment of cross-bridges. Yet, in intact cardiac fibers, the slower decline of the intracellular Ca\(^{2+}\) transient (Fig. 9D, upper panel) may be the rate-limiting factor for relaxation and may account for the slower isometric relaxation in I79N myocardium compared with WT.
The higher basal contractile state, the increased rate of contraction, and slower relaxation in HCTnT-I79N myocardium carries with it several implications: 1) the contractile reserve would be diminished; 2) an increase in heart rate and/or of contractility, such as after isoproterenol administration, would jeopardize relaxation and lead to further diastolic dysfunction; 3) an increased contractility and heart rate would further increase diastolic \([\mathrm{Ca}^{2+}]_\text{i}\) and cause intracellular \([\mathrm{Ca}^{2+}]_\text{i}\) overload and dysrhythmias. These predictions seem to hold true for Tg-I79N mice challenged with isoproterenol, which demonstrate an impaired inotropic response, relaxation impairment, and fatal dysrhythmias.

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In summary, this study demonstrates that transgenic expression of mutant human troponin T (I79N) in mouse hearts significantly alters contractile function and pH regulation at the myofilament level. Computer simulation predicts for the Tg-I79N myocardium compared with Tg-WT: 1) an increase in the apparent \([\mathrm{Ca}^{2+}]_\text{pl}\) affinity of TnC and an increase in the apparent cross-bridge detachment rate \(g\) and 2) a higher basal contractility, impaired relaxation, residual resting tension, and vulnerability to inotropic stimulation in intact ventricular myocardium. It is likely that these mechanisms contribute to the mortality observed in patients with a TnT-I79N-based FHC.

**APPENDIX: SIMULATION OF FLASH PHOTOLYSIS, FORCE-PCA EXPERIMENTS, AND TWITCH FORCE IN INTACT FIBERS**

Mathematical Model of Intracellular \([\mathrm{Ca}^{2+}]_\text{i}\) Handling and Force Generation

The method consists of the following steps, each of which describe a time-varying function.

**Step 1: Approximate a Free Intracellular \([\mathrm{Ca}^{2+}]_\text{i}\) Transient**

The free \([\mathrm{Ca}^{2+}]_\text{i}\) transient is assumed to have the following time course (49).

**Fig. 9. Simulation Studies.**

A. simulation of force transients of skinned cardiac fibers induced by flash photolysis of DM-nitrophen from an instantaneous change in \(\mathrm{pCa}\) from 6.2 to 4.5. The solid line represents the force transient of the Tg-WT, and the dashed line is the Tg-I79N fibers. The data on the Tg-I79N were obtained by decreasing \(k_{\text{off}(\text{TnC)}_\text{Ca}}\) from 300 to 88 s\(^{-1}\) and increasing \(g\) from 10 to 20 s\(^{-1}\). B, simulation of force transient of skinned cardiac fibers induced by flash photolysis of Diao-2 for an instantaneous change in \(\mathrm{pCa}\) from 4.9 to 6.2. The solid line is the Tg-WT, and the dashed line is the Tg-I79N mutation. Relaxation proceeds faster in the Tg-I79N mutant. C, simulation of a steady state force-\(\mathrm{pCa}\) relation for skinned cardiac fibers containing Tg-WT (solid line) or the Tg-I79N (dashed line). The \(\mathrm{pCa}_{50}\) and \(n\) values are 5.466 and 1.009 (WT) and 5.686 and 1.020 (I79N) respectively. The shape of the force-\(\mathrm{pCa}\) curve and the difference in \(\mathrm{pCa}_{50}\) values (0.4) are very close to the experimental observations (Fig. 8B). The data on the Tg-I79N mutant were obtained by decreasing \(k_{\text{aff}(\text{TnC)}_\text{Ca}}\) from 300 to 88 s\(^{-1}\) and increasing \(g\) from 10 to 20 s\(^{-1}\). D, simulation of \([\mathrm{Ca}^{2+}]_\text{i}\) transients and force curves during twitches. The figure shows the time course of the intracellular \([\mathrm{Ca}^{2+}]_\text{i}\) transient (top panel), and of corresponding force (middle panel) in an isometric twitch during repetitive stimulation at 400-ms intervals in steady state control conditions for the Tg-WT (solid lines) and for the Tg-I79N mutation (dashed lines). The bottom panel shows the normalized force traces of the same twitch and demonstrate a slower isometric relaxation in I79N myocardium than in WT. The data on the Tg-I79N mutant were obtained by decreasing \(k_{\text{off}(\text{TnC)}_\text{Ca}}\) from 300 to 88 s\(^{-1}\) and increasing \(g\) from 10 to 20 s\(^{-1}\).
\[
pCa(t) = 8 - A \times (e^{-rt} - e^{-ft})
\]  
(Eq. 1)

where \( pCa = -\log_{10}[Ca^{2+}] \), \( A \) is an amplitude factor, \( r \) and \( f \) are rising and falling time constants respectively, and \( pCa = 8 \) at time 0. We used the values for the slower cardiac pCa transient proposed by Robertson et al. (49: 2.56, 0.003, and 0.170 s, respectively, for \( A, r, \) and \( f \).

Step 2: Calculate Ca\(^{2+}\) Bound to TnC (Ca(T)) and to Calmodulin (Ca(C))—From the law of mass action one obtains the following equations.

\[
d(CaT)/dt = k_{off(TnC-Ca)} \times [T - CaT] \times [Ca^{2+}] - k_{off(TnC-Ca)} \times [CaT] 
\]  
(Eq. 2)

\[
d(CaC)/dt = k_{off(Ca-Ca)} \times [Ca - CaC] \times [Ca^{2+}] - k_{off(Ca-Ca)} \times [CaC] 
\]  
(Eq. 3)

where \( T = \) total troponin (70 \( \mu \)mol kg\(^{-1}\)), \( CaT = \) troponin occupied with \( Ca^{2+} \), \( C = \) total calmodulin (24 \( \mu \)mol kg\(^{-1}\)), and \( CaC = \) calmodulin occupied with \( Ca^{2+} \). The on and off rates for binding of \( Ca^{2+} \) to TnC and to calmodulin are, respectively, \( k_{on(TnC-Ca)} = 3.9 \times 10^7 \text{M}^{-1}\text{s}^{-1} \), \( k_{off(TnC-Ca)} = \) a function of force (300 s\(^{-1}\) at zero force; see below), \( k_{on(Ca-Ca)} = 10^8 \text{M}^{-1}\text{s}^{-1} \), and \( k_{off(Ca-Ca)} = 238 \text{s}^{-1} \). These calculations are carried out initially for equilibrium conditions at rest (\( pCa = 8 \)) to determine initial \([CaT] \) and \([CaC] \), values of which are then inserted at time 0 in Equations 2 and 3. These calculations consider only the binding of \( Ca^{2+} \) to the single \( Ca^{2+}\)-specific site II of cardiac TnC.

Step 3: Cross-bridge Model Values—For the cross-bridge on and off kinetics, we modified Huxley’s 1957 model (68): \( d(CB)/dt = f(x) \times (1 - n) - g \times n \), where \( n \) is the number of attached cross-bridges.

\[
d(CB)/dt = f(x) \times [CaT] \times ([\text{total CB}] - [CB]) - g \times [CB] 
\]  
(Eq. 4)

\([CB] \) is the instantaneous concentration of attached cross-bridges, \([\text{total CB}] \) is the total number of cross-bridges (150 \( \mu \)mol kg\(^{-1}\)) (42), \( f(x) \) is the attachment (on) rate constant of detached cross-bridges, and \( g \) is the detachment (off) rate of attached cross-bridges. The first term on the right side of Equation 4 drives the force generation based on the \( Ca^{2+} \) occupancy of the low affinity site of cardiac TnC, and the second term governs cross-bridge detachment as a first order reaction. We assumed constant values for \( f = 150,000 \text{mol}^{-1}\text{kg}^{-1}\text{s}^{-1} \) and \( g = 10 \text{s}^{-1} \).

Finally, force is displayed as normalized to maximal force that could theoretically be obtained, i.e., as the ratio \([CB]/([\text{total CB}] \). This represents almost entirely \( Ca^{2+} \) released from the SR.

Step 4: Force Dependence of Affinity of TnC for \( Ca^{2+} \)—The rate of release of \( Ca^{2+} \) from TnC is slowed by the presence of cross-bridges. \( k_{off(TnC-Ca)} \) therefore becomes smaller as cross-bridges form and force develops (47, 51, 69). The off rate of \( Ca^{2+} \) from TnC was made to depend on force as follows.

\[
k_{off(TnC-Ca)} = k_{off(TnC-Ca)} \text{rest} \times B \times (1 - e^{-c\times(CB)/(\text{total CB})})
\]  
(Eq. 5)

where \( k_{off(TnC-Ca)} \) is a force-varying function, \( k_{off(TnC-Ca)} \text{rest} \) is the TnC-\( Ca^{2+} \) off rate at rest at zero force. This value was set at 300 s\(^{-1}\) based on the \( pCa \text{rest} = 5.47 \) and the on rate of \( Ca^{2+} \) to TnC, \( k_{off(TnC-Ca)} = 3.9 \times 10^7 \text{M}^{-1}\text{s}^{-1} \). \( B \) is an amplitude factor (set at 20), and \( C \) is a gain factor (set at 1).

The exponential dependence of \( k_{off(TnC-Ca)} \) on force is based on experimental observations in cardiac and skeletal muscle (49–51).

Step 5: Derivation of Myoplasmic \( Ca^{2+} \) Delivery—To simulate \([Ca^{2+}] \), transients and force for a variety of conditions in isolation or in combination, such as changes in the apparent affinity of TnC for \( Ca^{2+} \), of cross-bridge rates \( f \) and \( g \), and/or other variables, we need to obtain the time course and amplitude of \( Ca^{2+} \) delivery into the cytoplasm, mostly derived from release of \( Ca^{2+} \) from the SR. This approach would be more valid than to assume a fixed pCa transient, which in turn will be affected by changes in buffer variables that one wishes to simulate. We used the deductive procedure of Baylor et al. (62) to derive SR \( Ca^{2+} \) release as follows: 1) The total amount of cytoplasmic (free and bound) \( Ca^{2+} \) is given by the following equation.

\[
[Ca^{2+}] = [Ca^{2+}]_1 + [CaT] + [CaC] 
\]  
(Eq. 6)

2) For the net rate of change of total cytoplasmic \( Ca^{2+} \), if the first derivative of the total \([Ca^{2+}] \) represents the algebraic sum of \( Ca^{2+} \) delivery (\( Ca^{2+} \) release, \( Ca^{2+} \) entry) and of \( Ca^{2+} \) export out of the cytoplasm (SR \( Ca^{2+} \) uptake, other mechanisms).

\[
d(Ca^{2+})/dt = d(Ca^{2+})/dt_\text{delivery} - d(Ca^{2+})/dt_\text{export} 
\]  
(Eq. 7)

3) SR \( Ca^{2+} \) uptake is a saturable first order reaction (Michaelis-Menten kinetics).

\[
d(Ca^{2+})/dt_\text{export} = -V_{\text{max}} \times [Ca^{2+}]/(K_m + [Ca^{2+}]) 
\]  
(Eq. 8)

4) From Equation 7 it follows that cytoplasmic \( Ca^{2+} \) delivery equals

\[
d(Ca^{2+})/dt_\text{delivery} = d(Ca^{2+})/dt + d(Ca^{2+})/dt_\text{export} 
\]  
(Eq. 9)

which represents almost entirely \( Ca^{2+} \) released from the SR.

Step 6: Simulation of \([Ca^{2+}] \), Transient and Force—At this stage, all components of the multi-compartment model are characterized including the time course and amplitude of myoplasmic \( Ca^{2+} \) delivery (mainly SR \( Ca^{2+} \) release). One can now change one or more components and assess the resultant changes in the intracellular \( Ca^{2+} \) transient and force generation. Intracellular calcium transients and force signals were simulated in control conditions and after changing one or more rate constants or variables by solving Equations 2–5 and 10 using Runge-Kutta fourth order numerical integration with a 50-\( \mu \)s step size.

\[
d([Ca^{2+}])/dt = (d([Ca^{2+}])/dt)_\text{delivery} - (d([Ca^{2+}])/dt) - V_{\text{max}} \times ([Ca^{2+}] - [Ca^{2+}]_\text{lim})/([Ca^{2+}]_\text{lim} + K_m) 
\]  
(Eq. 10)

Equation 10 states that the change in free \([Ca^{2+}] \), is the resultant of total \( Ca^{2+} \) delivery minus the rate of \( Ca^{2+} \) bound to TnC (Ca(T) and to calmodulin (Ca(C)), minus the rate of \( Ca^{2+} \) removed by SR uptake. The SR uptake term in Equation 10 introduces a small \( Ca^{2+} \) “leak” to maintain diastolic \([Ca^{2+}]_1 \), at a nearly constant level. In the initial control conditions, once SR \( Ca^{2+} \) release was obtained, steady state control conditions were obtained by repeating the numerical integration of Equations 2–5 and 10 over several cycles by setting the initial values of \([Ca^{2+}]_1 \), \([CaT] \), \([CaC] \), and \([CB] \) of a given cycle to their values obtained at the end of the previous cycle. Simulations carried out for a change in one or more rate constants or variables were also allowed to reach steady state conditions over several cycles. In most instances, steady state conditions were reached in three to five contractions at frequencies corresponding to heart rates of 150 min\(^{-1}\).

Derivation of Initial Variables and of Changes Incurred by TnT-179N Mutation

We used the results from skinned cardiac fiber studies, the flash photolysis force transients (DM-nitrophen flash from an estimated pCa 6.2 to 4.5, and Diaz-2 flash for a pCa step from pCa 4.9 to 6.2) and force-pCa relations to estimate the changes in the off rate of \( Ca^{2+} \) from the \( Ca^{2+}\)-specific site of TnC (\( k_{off(TnC-Ca)} \)) and the attachment and detachment rate constants of actomyosin cross-bridges, \( f \) and \( g \). To this effect, we simulated the force-pCa relationship that would be obtained for several combinations of changes of \( k_{off(TnC-Ca)} \), \( f \), and \( g \), until a simulated force-pCa relationship was obtained that reproduced the experimental results. Second, we also simulated the force transient that would be observed during a step change in pCa.
from 6.2 to 4.5; at pCa 6.2, already 15–20% of maximal force is developed in skinned cardiac fibers. The mathematical approach to these two additional types of simulation is identical to that outlined above for intact cardiac fibers, with the following modifications: 1) For simulation of force-pCa relations, all factors related to the sarcoplasmic reticulum are removed. The intracellular Ca$^{2+}$ transient becomes a fixed value and only steady state conditions are taken into account. The simulations are repeated for each pCa value from 8 to 4 in steps of 0.1 pCa unit, and steady state force was recorded and plotted as a function of pCa. 2) For simulation of flash photolysis, all factors related to the SR are removed. Force and Ca$^{2+}$ buffers are allowed to reach steady state at one pCa value (typically 6.2 in a DM-nitrophen experiment), and pCa is suddenly changed to another pCa (in this example 4.5). The transient change in force was recorded and fitted to the equation $F = F_0 + a \times (1 - e^{-kt})$ by nonlinear regression (Graphpad 5.03, SPSS Inc., Chicago, IL); $k$ is the rate constant of force decline, $F_0$ is force at the initial pCa, and $a$ is an amplitude factor. The transient change in force in the simulated Diazo-2 experiment (pCa step from pCa 4.9 to 6.2) was fitted to the equation $F = F_0 + a \times e^{-kt}$, whereby $a$ is an amplitude factor and $b$ is the rate constant of force decline.

Flash photolysis and force-pCa relations were simulated for a range of values for $k_{\text{HCT}}$ and $F_0$, and $a$ to reproduce results found in experimental observations in skinned cardiac muscle. We found that a single unique set of values of $k_{\text{HCT}}$ and $F_0$, and $a$ could be reproduced in experimental results, and these values were then subsequently used in simulations to predict what the time course and amplitude of the twitch would be in intact cardiac muscle, both Tg-WT and the Tg-I79N mutation.

**Prediction of Intracellular Ca$^{2+}$ Transient and Force in Intact Cardiac Fibers**

We simulated the intracellular Ca$^{2+}$ transient and force during a twitch that would occur in wild type and with the HCT-I79N mutation by using the values of $k_{\text{HCT}}$, $F_0$, and $a$ found with the simulation of skinned fiber studies described above. This theoretical analysis predicts muscle contraction and relaxation as will be encountered in vivo and provides a basis for the hypothesis that will be tested in the heart of animals transgenic for HCT-I79N.

All calculations were programmed in Microsoft Quickbasic 4.0 and processed on a PC, and the results (saved as ASCII files) were replotted with SigmaPlot 5.03 (SPSS, Inc., Chicago, IL). Further development of this model and the analysis of [Ca$^{2+}$], Ca$^{2+}$ buffers, and force generation using this approach are the subject of a separate report.

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