Familial Hypertrophic Cardiomyopathy–Linked Mutant Troponin T Causes Stress-Induced Ventricular Tachycardia and Ca\(^{2+}\)-Dependent Action Potential Remodeling

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Abstract—The cardiac troponin T (TnT) I79N mutation has been linked to familial hypertrophic cardiomyopathy and high incidence of sudden death, despite causing little or no cardiac hypertrophy in patients. Transgenic mice expressing mutant human TnT (I79N-Tg) have increased cardiac contractility, but no ventricular hypertrophy or fibrosis. Enhanced cardiac function has been associated with myofilament Ca\(^{2+}\) sensitization, suggesting altered cellular Ca\(^{2+}\) handling. In the present study, we compare cellular Ca\(^{2+}\) transients and electrophysiological parameters of 64 I79N-Tg and 106 control mice in isolated myocytes, isolated perfused hearts, and whole animals. Ventricular action potentials (APs) measured in isolated I79N-Tg hearts and myocytes were significantly shortened only at 70% repolarization. No significant differences were found either in L-type Ca\(^{2+}\) or transient outward K\(^{+}\) currents, but inward rectifier K\(^{+}\) current (IK1) was significantly decreased. More critically, Ca\(^{2+}\) transients of field-stimulated ventricular I79N-Tg myocytes were reduced and had slow decay kinetics, consistent with increased Ca\(^{2+}\) sensitivity of I79N mutant fibers. AP differences were abolished when myocytes were diazylized with Ca\(^{2+}\) buffers or after the Na\(^{+}\)-Ca\(^{2+}\) exchanger was blocked by Li\(^{+}\). At higher pacing rates or in presence of isoproterenol, diastolic Ca\(^{2+}\) became significantly elevated in I79N-Tg compared with control myocytes. Ventricular ectopy could be induced by isoproterenol-challenge in isolated I79N-Tg hearts and anesthetized I79N-Tg mice. Freely moving I79N-Tg mice had a higher incidence of nonsustained ventricular tachycardia (VT) during mental stress (warm air jets). We conclude that the TnT-I79N mutation causes stress-induced VT even in absence of hypertrophy and/or fibrosis, arising possibly from the combination of AP remodeling related to altered Ca\(^{2+}\) transients and suppression of IK1. (Circ Res. 2003;92:428-436.)

Key Words: familial hypertrophic cardiomyopathy • troponin T • ventricular tachycardia • action potential remodeling • Ca\(^{2+}\) transient

Familial hypertrophic cardiomyopathy (FHC) is an autosomal-dominant disease resulting from mutations in genes encoding cardiac contractile proteins and is an important cause of sudden cardiac death.1 Genotype/phenotype correlation studies suggest that the prognostic significance of most mutations is related to the degree of cardiac hypertrophy and fibrosis.2,3 An exception to this are patients with cardiac troponin T (TnT) mutations such as TnT-I79N,4 who often almost universally increase myofilament Ca\(^{2+}\) sensitivity.8 Because Ca\(^{2+}\) binding to the troponin complex represents the largest component of dynamic Ca\(^{2+}\) buffering during the cardiac cycle,9 our modeling studies predict that the increased myofilament Ca\(^{2+}\) sensitivity would significantly alter intracellular Ca\(^{2+}\) transients.10 Thus, TnT mutations that change intracellular Ca\(^{2+}\) handling may lead to action potential remodeling and possibly ventricular arrhythmias responsible for sudden cardiac death. To test this hypothesis, we have generated transgenic mice with cardiac-targeted expression of mutant human cardiac TnT-I79N (I79N-Tg) that show increased myofilament Ca\(^{2+}\) sensitivity,11 enhanced cardiac contractility, and impaired relaxation,10 but no ventricular hypertrophy or fibrosis.10 In the present study, we examine the electrophysiological and Ca\(^{2+}\) signaling consequences of

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the TnT-I79N mutation in transgenic mice. Our data suggests that the TnT-I79N mutation can cause stress-induced ventricular tachycardia even in absence of hypertrophy and/or fibrosis, arising possibly from the combination of action potential remodeling, altered cytosolic Ca\textsuperscript{2+} transients, and decreased inward rectifier K\textsuperscript{+} current.

**Materials and Methods**

Groups of 4- to 6-month-old mice expressing human wild-type cardiac TnT (WT-Tg), mutant cardiac TnT (I79N-Tg), and nontransgenic littermates (Non-Tg) were used for all studies, which were performed according to NIH guidelines and approved by the institutional animal care and use committee.

**Telemetric ECG Recordings**

Telemetric ECGs were continuously recorded from 11 mice (2 WT-Tg, 4 Non-Tg, 5 I79N-Tg) during 48 hours normal activity and during defined stress tests (see the expanded Materials and Methods section, available in the online data supplement at http://www.circresaha.org), namely: (1) 4 minutes of continuously swimming in cages filled with water (37°C); and (2) repetitive warm air stress. This “mental stress” procedure with warm air jets repetitively increased heart rate (online Figure 1) and has been reported to increase systemic blood pressure and sympathetic nerve activity in rats\textsuperscript{12} and mice.\textsuperscript{13} All stress test protocols were performed by a single operator and were performed simultaneously on matched pairs of mice.

**ECG Recordings in Anesthetized Mice**

Thirty-nine mice (12 WT-Tg, 13 Non-Tg, 14 I79N-Tg) were anesthetized with 20 mL/kg of 2% tribromoethanol via IP injection. Sixty-five mice (18 WT-Tg, 23 Non-Tg, 24 I79N-Tg) were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) IP, and bipolar limb lead ECGs recorded. After 5 minutes of continuous baseline ECG recordings, mice received either 0.1 mg/kg or 1.5 mg/kg of isoproterenol IP, followed by 15 minutes of additional ECG monitoring.

**Isolated Perfused Heart Preparation**

Sixty-five mice (18 WT-Tg, 23 Non-Tg, 24 I79N-Tg) were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) IP, and bipolar limb lead ECGs recorded. After 5 minutes of continuous baseline ECG recordings, mice received either 0.1 mg/kg or 1.5 mg/kg of isoproterenol IP, followed by 15 minutes of additional ECG monitoring.

**Volume-Conducted ECG, Transmembrane, and Monophasic Action Potential Recordings**

Volume-conducted ECG, transmembrane action potential (TAP) and monophasic action potential (MAP) recordings were obtained from isolated perfused mouse heart as previously described.\textsuperscript{15}

**Myocyte Isolation, Action Potential, and Voltage Clamp Measurements**

Ventricular myocytes were enzymatically isolated from 20 control (12 Non-Tg and 8 WT-Tg) and 13 I79N-Tg hearts (see online data supplement).\textsuperscript{14} Ca\textsuperscript{2+}-tolerant cells were used for voltage-clamp measurements of L-type Ca\textsuperscript{2+} and K\textsuperscript{+} currents and current-clamp action potential measurement using the ruptured patch method (see online data supplement), and for measurements of [Ca\textsuperscript{2+}]\textsubscript{i} during field stimulation. A subset of cells was used for simultaneous membrane potential measurements using the perforated patch method (see online data supplement). Action potentials were measured after 5 minutes of steady-state pacing at 1 Hz and 5 Hz.

**Measurement of Intracellular [Ca\textsuperscript{2+}]**

Cells were loaded with membrane-permeable fura-2 acetoxymethyl ester (fura-2AM, Molecular Probes, Inc), and intracellular Ca\textsuperscript{2+} transients were measured (see online data supplement). A subset of cells was exposed to 5 mmol/L caffeine for 5 seconds using a rapid concentration clamp system. Amplitudes of caffeine-induced Ca\textsuperscript{2+} transients were used as estimates of sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} content.

**ECG and Action Potential Data Analysis**

All data were analyzed in blinded fashion without knowledge of the genotype. All ECG recordings were manually analyzed for heart rate and PR interval during each experimental period. The entire recording periods were scrutinized for ventricular arrhythmias (see online data supplement). Microelectrode, MAP, and single-cell AP data were analyzed using a custom-built software program written in LabVIEW (National Instruments) as previously described (see online data supplement).\textsuperscript{15}

**Statistical Evaluation**

Mean±SEM values are given, unless otherwise indicated. Mean values were compared with single factor analysis of variance (ANOVA). Post hoc Student’s t test analysis was performed whenever significant differences were detected by ANOVA. Fischer’s exact test (one-tailed) was used to compare incidence of arrhythmias between groups.

**Results**

To test the hypothesis that the FHC-linked TnT-I79N mutation causes ventricular arrhythmias, we compared ECG and action potential recordings from transgenic mice with cardiac-targeted expression of human troponin T containing the single amino acid I79N mutation (I79N-Tg) with two control groups: mice expressing human wild-type troponin T (WT-Tg) or no transgene (Non-Tg). In this study, we pooled the data from the two control groups, because our previous characterization did not show significant differences between ECG parameters or incidence of ventricular arrhythmias of WT-Tg and Non-Tg mice.\textsuperscript{11}

**Telemetric ECG Recordings in Freely Moving Mice**

I79N-Tg mice (n=5) demonstrated a nonsignificant trend toward slower average heart rate and longer PR interval values compared with control mice (2 WT-Tg and 4 Non-Tg) during normal activity (heart rate, 556±21 versus 600±18 bpm; P=0.06; PR interval, 37±1.1 ms versus 33±1.1 ms; P=0.06). The average heart rate of I79N-Tg mice was significantly slower during swimming exercise (674±8 versus 740±9 bpm; P<0.05), but not during “mental stress” in form of warm air jets\textsuperscript{13} (652±14 versus 651±7 bpm; P=NS).

Analysis of 48 hours of continuous telemetric ECG recordings did not reveal any significant differences in the incidence of ventricular tachycardia (VT) between I79N-Tg and control mice (Figure 1). Similarly, no VT occurred in either group during the swimming exercise protocol (5 minutes of exercise and 55 minutes of postexercise ECG monitoring). Warm air stress, however, induced short runs of nonsustained VT in 4 out of 5 I79N-Tg mice (mean 1.8±0.7 VT episodes per mouse), compared with 1 out of 6 control mice (mean 0.2±0.2 VT episodes per mouse; P=0.045). In the hour after the warm air stress, the rate of VT was higher in I79N-Tg mice compared with control mice (4.2±0.7 VT episodes per mouse in 5/5 I79N-Tg mice versus 1.2±0.2 VT episodes per mouse in 3/6 control mice; P=0.12). One I79N-Tg mouse developed several long episodes on self-terminating VT (maximal length 29 beats; Figure 1B). None of the control mice had VT episodes lasting longer than 3 consecutive premature ventricular beats (PVBs) during any recording period analyzed.
ECG Recordings in Anesthetized Mice

Under ketamine/xylazine anesthesia, heart rate and incidence of ventricular ectopy were not significantly different between 13 I79N-Tg and 25 control mice (13 WT-Tg and 12 Non-Tg). As previously reported, PR interval was significantly longer in I79N-Tg compared with control mice. On exposure to isoproterenol (ISO 0.1 mg/kg IP), heart rate increased to similar values in both groups of mice (I79N-Tg 472/1.100 vs control 473/1.100 bpm; *P<0.05 by Fisher’s exact test. Inset, Short episode of VT (4 beats) in an I79N-Tg mouse. B, Telemetric recording of nonsustained ventricular tachycardia in an I79N-Tg mouse several minutes after hot air stress challenge.

ECG Recordings in Langendorff-Perfused Hearts

Spontaneously beating, isovolumically contracting isolated hearts were perfused at a constant pressure of 75 mm Hg with Krebs-Henseleit buffer containing 1.8 mmol/L Ca²⁺. I79N-Tg hearts had significantly more ventricular ectopy (4.8+/−1.4 PVB/min, n=12) compared with control hearts (1.1+/−0.3 PVB/min, n=24 [10 WT-Tg and 14 Non-Tg]; *P<0.01). Addition of isoproterenol (100 nmol/L) to the perfusate increased heart rates to comparable levels in I79N-Tg and control mice (533+/−12 versus 539+/−11 bpm; *P=NS). As was observed in vivo, several minutes after the initial increase in heart rate, a second-degree AV conduction block developed transiently in 8 of 12 I79N-Tg hearts, but only in 7 of 24 control hearts (*P=0.04). The AV blocks resolved after

Figure 1. Mental stress caused ventricular tachycardia (VT) in freely moving I79N-Tg mice. A, Percentage of control (open columns, n=6) and I79N-Tg (filled columns, n=5) mice with episodes of nonsustained VT during normal activity, and during and 1 hour after hot air jet challenge (mental stress). *P<0.05 by Fisher’s exact test. Inset, Short episode of VT (4 beats) in an I79N-Tg mouse. B, Telemetric recording of nonsustained ventricular tachycardia in an I79N-Tg mouse several minutes after hot air stress challenge.

Figure 2. Isoproterenol challenge caused ventricular ectopy in anesthetized I79N-Tg mice. A, Rate of premature ventricular beats (PVB/min) during 15 min of continuous limb-lead ECG recordings after intraperitoneal injection of isoproterenol (ISO). Data are mean−SEM. *P<0.05, **P<0.01. Low-dose ISO (0.1 mg/kg body weight): control (8 Non-Tg+7 WT-Tg), n=15; Tg-I79N, n=9. High-dose ISO (1.5 mg/kg body weight): control (5 Non-Tg+5 WT-Tg), n=10; Tg-I79N, n=5. Inset, PVB after low-dose ISO administration. B, Representative example of recurrent VT after high-dose ISO administration in an I79N-Tg mouse.
several minutes. Complete heart block was not observed. To avoid complications secondary to AV conduction blocks, the PVB rate was quantified before the onset of AV block. Although isoproterenol increased the rate of ventricular ectopy in both groups, the rate was significantly higher in I79N-Tg (12±3.0 PVB/min) versus control hearts (5.2±1.1 PVB/min; *P<0.01).

**Pacing Rate and Ca²⁺ Dependence of Ventricular Ectopy**

Because the TnT-I79N mutation increases myofilament Ca²⁺ sensitivity¹¹ and impairs ventricular relaxation especially at higher extracellular [Ca²⁺],¹⁰ Ca²⁺ dependence of ventricular ectopy was examined (9 I79N-Tg and 17 control mice [8 WT-Tg and 9 Non-Tg]). Unloaded hearts were paced to avoid any complications from heart blocks and intraventricular balloon. In 1.2 mmol/L [Ca²⁺], the rate of ventricular ectopy (PVBs) was significantly different only at the highest pacing rates (Figure 3A). Episodes of nonsustained VT were not observed in either group. In 3.2 mmol/L [Ca²⁺], however, the rate of PVBs was significantly increased in I79N-Tg hearts at most pacing rates (Figure 3B). Nonsustained VT occurred in 2 of 9 I79N-Tg hearts, but in none of 16 control hearts (*P=0.12). Addition of isoproterenol in the presence of higher [Ca²⁺], further increased ventricular ectopy in both groups and increased the incidence of VT (8/9 I79N-TG versus 8/17 controls; *P=0.04). Thus, interventions that increase [Ca²⁺], (isoproterenol, fast pacing rate, higher [Ca²⁺]) appear also to increase the incidence of ventricular ectopy in I79N-Tg mice.

**Microelectrode and MAP Measurements of Action Potential in Intact Hearts**

To investigate potential cellular mechanisms responsible for the ventricular arrhythmias, KCl-filled microelectrodes were used to measure ventricular action potentials¹⁵ in isolated perfused hearts paced at 400 bpm. In 1.2 mmol/L [Ca²⁺], action potential wave shape rather than overall action potential duration was found to be significantly altered in I79N-Tg hearts (Figure 4A): the ventricular action potential had primarily a lower terminal repolarization phase, which could be quantified as a decrease in action potential duration at 70% repolarization (APD₇₀, 14±3 ms, n=8, versus 23±2.2 ms, n=10, *P<0.01, in I79N-Tg and control hearts, respectively). On the other hand, resting potential, overshoot potential, APD₀, APD₂₀, and APD₅₀ were not significantly different in the two groups of mice. Consistent with the shorter APD₇₀, there was a trend toward shorter effective refractory period in I79N-Tg (36±3 ms) compared with control mice (45±4 ms; *P=0.065).

In 1.8 mmol/L [Ca²⁺], miniaturized contact electrodes were used to record monophasic action potentials (MAPs), because it was
difficult to use microelectrodes reliably for the more vigorously contracting hearts. The reliability of MAP with respect to duration and shape of microelectrode recordings was recently shown. Figure 4 compares microelectrode (panel A) and MAP recordings (panel B) from I79N-Tg and control hearts. In a manner similar to that found when using microelectrodes, MAP wave shape rather than overall MAP duration was changed (Figure 4B): left-ventricular epicardial MAPs of I79N-Tg hearts had significantly shorter durations measured in highly Ca\(^{2+}\)-buffered myocytes than those recorded either in the isolated heart (compare Figure 4) or in myocytes using the perforated patch technique (compare Figure 6). Voltage-clamp measurements of L-type Ca\(^{2+}\) current (Figure 5B) and depolarization-activated outward K\(^+\) currents (Figure 5C) also failed to demonstrate significant differences between control and I79N-Tg myocytes. The outward component of the inward rectifier K\(^+\) current (I\(_{K1}\)) was modestly, but significantly reduced in I79N-Tg compared with control hearts (Figure 5D, inset). Because reduction of I\(_{K1}\) would tend to prolong repolarization, these data suggest that the shorter APD\(_{90}\) of I79N-Tg hearts is unlikely to result from changes in Ca\(^{2+}\) or K\(^+\) membrane currents, but may depend on the characteristics of intracellular Ca\(^{2+}\) signaling.

Measurement of Action Potential and Membrane Currents in Isolated Ventricular Myocytes

To exclude any potential contribution of Ca\(^{2+}\) binding to mutant Troponin complex on action potential duration, myocytes were dialyzed with pipette solution containing high (14 mmol/L) concentration of EGTA that completely abolished contractions. Unlike the isolated heart, action potential durations measured in highly Ca\(^{2+}\)-buffered myocytes were not statistically different between control and I79N-Tg myocytes (Figure 5A). The duration of action potentials recorded from ventricular myocytes were on average significantly shorter than those recorded either in the isolated heart (compare Figure 4) or in myocytes using the perforated patch technique (compare Figure 6).
In I79N-Tg cells, action potential duration (APD) measured at 30%, 50%, 70%, and 90% repolarization (APD90) at 1 Hz, and shorter APD70 at 5 Hz. Control, 16 cells from 6 hearts, versus control (percent increase, 27±2% versus 19±1%; P<0.01). As a result of the slower Ca2+ transient decay kinetics, faster pacing increased diastolic [Ca2+]i significantly more in I79N-Tg myocytes compared with control myocytes (percent increase, 27±2% versus 19±1%; P<0.01). At the same time, APD90 and APD70 remained unchanged in I79N-Tg myocytes, but significantly shortened in control myocytes (compare left and right panels of Figure 6B). Together with the results from highly buffered myocytes (Figure 5), these data suggest that changes in cytosolic [Ca2+]i may contribute to the altered action potential morphology of I79N mice, possibly by causing differences in Na+-Ca2+ exchanger current.

To test this hypothesis directly, extracellular Na+ was rapidly replaced with Li+ (Figure 7A). This maneuver allows influx of Li+ through Na+ channels, but blocks Ca2+ extrusion on the exchanger.56 The block of inward exchanger current by Li+ abolished the differences in APD90 of transgenic and control mice (Figure 7B). Note that in the presence of Li+, the action potential is much shorter despite a much larger Ca2+ transient, suggesting that the inward exchanger current significantly contributes to repolarization of the action potential. In addition, Figure 7A (right) shows that enhanced Ca2+ transients produce longer action potentials after the washout of Li+. These data, together with those obtained during high Ca2+ buffering (Figure 5), suggest that changes in magnitude and kinetics of Ca2+ transients regulate the action potential of I79N transgenic mice via the Na+-Ca2+ exchanger.

**Measurement of Ca2+ Transients in Response to Isoproterenol**

Exposure to isoproterenol (500 nmol/L) increased peak Ca2+ transients more dramatically in I79N-Tg than in control myocytes (Figures 8A and 8B), such that there were no statistical differences between the two groups any longer (Fcal/Fa/o; I79N-Tg 1.15±0.13 versus control 1.21±0.19; P=0.8; Figure 8C). As expected, isoproterenol also significantly accelerated the decay rate of Ca2+ transients in both groups (Figure 8D), but the decay rate of Ca2+ transients remained significantly slower in I79N-Tg versus control myocytes (τ, 111±10 versus 65±3 ms; P<0.01). Similar to the data obtained with fast pacing, isoproterenol significantly increased diastolic [Ca2+]i in I79N-Tg versus control myocytes (Figure 8E). In addition, isoproterenol significantly increased the SR Ca2+ content of I79N-Tg versus control myocytes (Figure 8F). Thus, the data suggest that in the presence of isoproterenol, [Ca2+]i remains elevated for longer periods of time due to the slower rate of cytosolic Ca2+ decay in I79N-Tg compared with control myocytes.

**Discussion**

Our data demonstrate for the first time that expression of mutant troponin T causes ventricular arrhythmias in freely moving mice.
Results suggested that [Ca$^{2+}$] $o$ (Figure 3), action potential remodeling of I79N-Tg hearts (Figure 4) and myocytes (Figure 6) is likely the result of changes in cytosolic Ca$^{2+}$ transients (Figures 6 and 8), which cause differential activation of Ca$^{2+}$-dependent Na$^+$-Ca$^{2+}$ exchange (Figure 7). The combination of slow rate of cytosolic Ca$^{2+}$ decay, especially when Ca$^{2+}$ transients are greatly enhanced by isoproterenol (Figure 8), and reduction of I$_{Ks}$ (Figure 5D), may further contribute to the high frequency of stress-induced ventricular arrhythmias observed in I79N mice.

Mechanism of Action Potential Remodeling

In highly Ca$^{2+}$-buffered I79N-Tg and control cells, we found no significant differences in APD (Figure 5A), Ca$^{2+}$ current (Figure 5B), or transient outward K$^+$ current (Figure 5C), but I$_{Ks}$ was downregulated (Figure 5D). On the other hand, action potentials of poorly Ca$^{2+}$-buffered I79N-Tg myocytes were significantly longer (Figure 6) and had an altered morphology (shorter APD$_{50}$) similar to those measured in intact I79N-Tg hearts (Figure 4). Because the size of Ca$^{2+}$ transients, peaking between APD$_{50}$ and APD$_{90}$, is significantly reduced in I79N-Tg myocytes (Figure 6A), it is likely that the Ca$^{2+}$-extrusion mode of the exchanger would be suppressed, thereby resulting in shorter APD$_{50}$ and APD$_{90}$ in I79N-Tg myocytes. Conversely, because the decay of the Ca$^{2+}$ transient is slow in I79N-Tg myocytes, [Ca$^{2+}$] $o$ is the same or higher in the terminal phase of the action potential (Figure 6A), resulting in unchanged APD$_{90}$ at faster beating rates (Figures 4B and 6B). Supporting this idea, block of Ca$^{2+}$ influx on the Na$^+$-Ca$^{2+}$ exchanger using Li$^+$ replacement of [Na$^+$]$_o$, eliminated the differences in APD$_{90}$ (Figure 7). Together, these results suggested that [Ca$^{2+}$] $o$ strongly modulates the murine ventricular action potential via the exchanger, as also reported for rat ventricular myocytes.17

Mechanism of Ventricular Arrhythmias

There are several possibilities how the remodeling of repolarization wave-shape (Figure 4), induced by differences in Ca$^{2+}$ transients (Figures 5 to 8), could be arrhythmogenic. (1) Differences in repolarization wave-shape may contribute to larger spatial heterogeneity of refractoriness, which can cause reentrant ventricular tachycardia.18 (2) The slow decay of Ca$^{2+}$ transient may lead to increased calmodulin kinase 2 activation, shown to cause early afterdepolarizations in mice,19 even in the absence of APD prolongation. (3) The combined decrease of I$_{Ks}$ (Figure 5) and increase of diastolic Ca$^{2+}$, at fast pacing rates or in presence of isoproterenol (Figure 8) may trigger delayed afterdepolarizations,20 and/or spontaneous Ca$^{2+}$ oscillations.21 (4) Abnormal Ca$^{2+}$ handling may in itself contribute to the initiation of reentrant arrhythmias by mechanisms distinct from enhanced dispersion of refractoriness or triggered activity, as recently demonstrated in a murine heart failure model.22 Exactly which mechanism(s) are responsible for the ventricular tachycardia of I79N-Tg mice remains to be determined.

Mechanism of Altered Ca$^{2+}$ Transients

The altered Ca$^{2+}$ transients in I79N-Tg myocytes (Figures 6 and 8) could be a direct consequence of the increased Ca$^{2+}$ sensitivity of I79N-mutant myofilaments,11 because Ca$^{2+}$ binding to myofilaments represents a substantial portion of cytoplasmic Ca$^{2+}$ buffering.9 Experimentally increasing cytoplasmic Ca$^{2+}$ buffering also results in reduced and prolonged Ca$^{2+}$ transients.23 Likewise, transgenic mice expressing a TnI mutation with increased myofilament Ca$^{2+}$ sensitivity also show depressed and prolonged Ca$^{2+}$ transients.24 Based on experimental data from skinned fibers expressing the TnT-I79N mutation, mathematical modeling predicts smaller and slowly decaying Ca$^{2+}$ transients in I79N compared with wild-type TnT fibers, assuming unchanged SR Ca$^{2+}$ release and sarcolemmal Ca$^{2+}$ flux.11 Thus, our finding of smaller and slower Ca$^{2+}$ transients (Figures 6 and 8) with no change in L-type Ca$^{2+}$ current (Figure 5B) and SR Ca$^{2+}$ content (Figure 8F) suggests that initially more Ca$^{2+}$ is bound to TnT-I79N containing myofilaments, but later on, as the muscle starts to relax, the additional Ca$^{2+}$ that comes off the myofilaments produces the slower decay of Ca$^{2+}$ transients. It may seem somewhat surprising that peak caffeine-induced Ca$^{2+}$ transients (Figure 8F) were not depressed in I79N myocytes. However, it should be noted that, unlike the fast twitch Ca$^{2+}$ transients (Figure 8A), a sustained rise in [Ca$^{2+}$]$_i$ in the presence of caffeine would almost fully saturate TnC Ca$^{2+}$ binding sites (>90% assuming peak caffeine-induced free [Ca$^{2+}$]) of 1 μmol/L$^9$ and high cooperativity of Ca$^{2+}$ binding and myofilament activation in intact muscle$^{25}$). Under such conditions, the TnT-I79N mutation may change the total cytosolic Ca$^{2+}$ buffering capacity by less than 5% (given that TnT-I79N left-shifts pCa50 by 0.21)). Alternatively, the data could simply imply that I79N-Tg cells have decreased SR Ca$^{2+}$ release and depressed cytoplasmic Ca$^{2+}$ removal. Thus, future studies will need to independently quantify SR Ca$^{2+}$ content and Ca$^{2+}$ buffering capacity of I79N-Tg myocytes and address possible contribution of Na$^+$-Ca$^{2+}$ exchanger and SR Ca$^{2+}$-ATPase to the altered Ca$^{2+}$ homeostasis described here.
Limitations and Potential Implications

Murine and human cardiac electrophysiology differ substantially, and action potential changes in response to expression of mutant TnT may be different in humans. Furthermore, ventricular tachyarrhythmias were nonsustained in I79N-Tg mice and did not cause significant mortality. As previously reported, I79N-Tg mice died after isoproterenol injection with complete heart block captured on ECG records. Although heart block did not occur in freely moving mice or in isolated perfused hearts, and isoproterenol-induced cardiac contractile dysfunction preceded the heart block, we cannot exclude that atrioventricular conduction abnormalities may also contribute to sudden deaths related to the I79N mutation. Nevertheless, our data provide the first direct evidence that the TnT-I79N mutation could cause ventricular tachycardia in an in vivo mouse model, even in absence of significant cardiac hypertrophy or fibrosis.

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Figure 8. Characterization of cytosolic Ca^2+ transients. A, Original traces of Ca^2+ transients at 1.8 mmol/L [Ca^2+]o, (left) and in presence of 500 mmol/L isoproterenol (ISO, right) recorded in control and I79N-Tg fura-2AM-loaded ventricular myocytes field-stimulated at 1 Hz. All Ca^2+ transients were fitted by monoexponential function for data analysis. B, Representative superimposed traces of Ca^2+ transients from a control (left) and a I79N-Tg myocytes (right) at 1.8 mmol/L [Ca^2+]o, and in presence of ISO. C through F, Comparison of Ca^2+ transients amplitude (C), decay time constant (D), diastolic [Ca^2+]i (E), and SR Ca^2+ content (F). 1.8 mmol/L [Ca^2+]o, 26 cells from 4 control (Non-Tg) and 25 cells from 5 I79N-Tg hearts; 1.8 mmol/L [Ca^2+]o/ISO, 14 cells from 4 control and 12 cells from 5 I79N-Tg hearts; SR Ca^2+ content, 12 cells from 4 control and 9 cells from 4 I79N-Tg hearts. Data are mean ± SEM; *P < 0.05, **P < 0.01.


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Groups of 4- to 6-month old mice expressing human wild-type cardiac TnT (WT-Tg), mutant cardiac TnT (I79N-Tg) and non-transgenic littermates (Non-Tg), strain B6SJL, were used for all studies, which were carried out according to NIH guidelines and approved by the institutional animal care and use committee.

Telemetric ECG recordings

Eleven mice (2 WT-Tg, 4 Non-Tg, 5 I79N-Tg) were instrumented with an intraperitoneal telemetric ECG transmitter (Data Sciences International, St Paul, MN) during general anesthesia (ketamine 50mg/kg, xylazine 20mg/kg) via intraperitoneal (i.p.) injection. This transmitter continuously records and transmits a surface ECG recorded between two wire electrodes placed subcutaneously in the region of the right shoulder and left limb, mimicking lead II of the Einthoven ECG. The signals were digitized via a commercially available digitization system (Data Sciences International, Saint Paul, MN) and stored on hard disk and optical media for off line analysis using a custom-designed software package. After a postoperative recovery period of 10 days, we continuously and pair-wise simultaneously recorded ECGs during periods of normal activity (48 hours continuous recording) and during defined stress tests, namely: 1. Swimming exercise in cages filled with water (37 °C). This setup required the mice to swim continuously for 4 minutes. 2. Repetitive warm air stress. The mice were subjected to jets of warm air generated by a conventional hair dryer (15 seconds of warm air alternating with 45 seconds without air exposure for 10 minutes, figure 1 online). The experimental setup was designed to deliver warm, but not overheated air jets within the mouse cage. The setup was identical for all mice. This “repetitive mental stress” procedure significantly increases heart rate,
systemic blood pressure, and sympathetic nerve activity in rats and mice. Repetitive air jets provoked a repetitive increase in heart rate in this study (figure online). Prior to each stress test, the mice were monitored continuously during normal activity. ECG recordings were continuously obtained from one hour before until one hour after the beginning of the stress challenge. Prior to the stress test, heart rate was not different from heart rates measured at other periods of normal activity. All stress test protocols were performed by a single operator, and were performed simultaneously on matched pairs of mice.

**Limb lead ECG recordings**

Thirty-nine mice (12 WT-Tg, 13 Non-Tg, 14 I79N-Tg) were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) i.p., and bipolar limb lead ECGs were recorded as described previously. After 5 minutes of continuous baseline ECG recordings, mice received either 0.1 mg/kg or 1.5 mg/kg of isoproterenol i.p., followed by 20 minutes of additional ECG monitoring.

**Isolated Perfused Heart Preparation**

Sixty-five mice (18 WT-Tg, 23 Non-Tg, 24 I79N-Tg) were anesthetized with 20 ml/kg of 2% tribromoethanol via i.p. injection. Hearts were isolated and perfused at a constant perfusion pressure of 75 mmHg at 37°C as previously described. Krebs-Henseleit (KH) buffer containing (mmol/L): NaCl (118), KCl (4.7), MgSO₄ (1.2), Na-EDTA (0.7), NaHCO₃ (25), KH₂PO₄ (1.2), and glucose (11) was prepared at the time of the experiment and equilibrated with a mixture 95% O₂ and 5% CO₂ for one hour to achieve a pH of 7.4 and a pO₂ of at least 500 mmHg.

Experiments were carried out either in unloaded or isovolumetrically-beating hearts. Hearts were allowed to equilibrate for 15 minutes before any measurements were obtained. To abolish the fast intrinsic rate and allow for ventricular pacing over a wider range of cycle lengths,
the atrioventricular node was ablated in a subset of animals. A pair of small platinum wire hooks was inserted into the right ventricular myocardium for bipolar pacing. To examine the effects of pacing rate, hearts were paced at 2.5, 3.3, 5, 6.7 and 10 Hz (20 pacing trains of 50 beats separated by a 2 sec pause at each frequency). To examine the effects of different inotropic states, CaCl$_2$ (final free [Ca$^{2+}$] of 1.2, 1.8 and 3.2 mmol/L) and/or isoproterenol (final concentration of 100 nmol/L) were added to the equilibrated KH perfusion buffer.

**Volume-conducted ECG, transmembrane and monophasic action potential recordings**

Volume-conducted ECG, transmembrane action potential (TAP) and monophasic action potential (MAP) recordings were obtained from isolated perfused mouse heart as previously described.$^6$

**Myocyte isolation**

Ventricular myocytes were enzymatically isolated from 20 control (12 Non-Tg and 8 WT-Tg) and 13 I79N-Tg hearts as described.$^4$ Briefly, after heart removal under surgical plane of anesthesia (tribromoethanol 2%, 30 ml/kg i.p.), hearts were perfused for 5 min with Tyrode’s buffer (in mmol/L: NaCl 137, KCl 5.4, MgCl2 0.5, HEPES 10, and glucose 10, pH 7.4, 36.5 °C) and for 7 to 9 min with 50 ml Tyrode’s buffer containing 25 mg collagenase B, 15 mg collagenase D, (Boehringer Ingelheim) and 2 mg protease (type IV, Sigma). The left ventricle was removed and minced in 50 ml of isolation solution. The myocytes suspension was filtered (1.2 micron) and allowed to settle for 15 min. Up to this step, all solutions also contained albumin (1mg/ml) and 2,3-butanedione monoxime (BDM, 10 mmol/L). To make cells Ca$^{2+}$ tolerant, cells were then repeatedly washed with Tyrode solutions containing increasing Ca$^{2+}$ (0.06, 0.2, 0.6 and 1.2 mmol/L) and decreasing BDM (9.5, 8, 5 and 0 mmol/L) concentrations.
Whole-cell voltage and current clamp measurements - ruptured patch

Ca\(^{2+}\)-tolerant cells were used for voltage-clamp measurements of L-type Ca\(^{2+}\) and K\(^+\) currents and current-clamp action potential measurement using the ruptured patch method as described.\(^4\) Briefly, ionic currents were recorded at 33 °C in the whole–cell, voltage–clamp configuration of patch-clamp technique using 1.5/1.12 mm OD/ID borosilicate glass electrodes (World Precision Instruments), which had tip resistances of 1-2 MΩ when filled with internal solution. The electrodes were connected to an Axopatch 200 B amplifier (Axon Instruments) controlled by pClamp 8.1, which was used for data acquisition and analysis. After formation of a high-resistance seal >1 GΩ between recording electrode and myocyte membrane, electrode capacitance was fully compensated before breaking the membrane patch. After the membrane rupture, series resistance was compensated (70% - 80%). Only cells with “leak currents” of less then 100 pA were used. Cells were superfused with Tyrode’s solution (33±0.5 °C) containing (in mmol/L): NaCl 137, KCl 5.4, CaCl\(_2\) 2, HEPES 10, MgCl\(_2\) 1, Glucose 10, pH=7.4 with NaOH. To record K\(^+\) currents, 5 µmol/L nifedipine was added to the superfusate to block I\(_{Ca}\). To measure action potentials and K\(^+\) currents, pipette solution contained (mmol/L): NaCl 10, KCl 100, EGTA 14, CaCl\(_2\) 1, HEPES 10, Mg ATP 5; pH= 7.2 with KOH 48. Free Ca was calculated as 10 nmol/L. Action potentials (AP) were recorded in current-clamp mode by injection of 20% suprathreshold current for 1-2 ms. In most cases, K\(^+\) current recordings were obtained after AP measurements from the same cell by switching to voltage-clamp configuration. Since under our experimental conditions IK1 has an apparent reversal potential of \(−70 ±/− 2\) mV (n=4) when defined as the BaCl\(_2\) (0.2 mM) sensitive current, a linear leak component defined by a line
connecting the origin and the current at -70 mV was subtracted from the net current values at each potential. This procedure facilitates the analysis of the small outward component of IK1.\(^7\)

There are several factors that may have contributed to the reversal potential not being at its Nernst potential of -89 mV: (i) measurements were not corrected for junction potentials; (ii) accurate calculation of the Nernst potential assumes ion activities rather than concentrations, which are considerably lower at a pipette K\(^+\) concentration of 148 mM; (iii) native IK1 channels have some finite Na\(^+\) conductance, which would tend to lower the apparent reversal potential; and (iv), chloriding of the bath electrode may have been less than perfect. Together, these factors may account for the shift of the apparent reversal potential of IK1. It should be noted, however, these factors will similarly affect both control and transgenic cells; thus, finding a difference between the groups should be valid.

\(I_{Ca}\) was recorded at room temperature (22±0.5 °C) using external solution containing (in mmol/L): CsCl 137, CaCl\(_2\) 2, HEPES 10, MgCl\(_2\) 1, Glucose 10, pH=7.4 with CsOH. The internal pipette solution contained (in mmol/L): CsCl 80, TEA 20, Glucose 10, EGTA 14, CaCl\(_2\) 1, HEPES 10, Mg-ATP 5, pH=7.2 with CsOH.

**Measurement of intracellular [Ca\(^{2+}\)]**

Cells were incubated in Tyrodes buffer (1.2 mmol/L Ca\(^{2+}\)) containing 1 µmol/L of membrane-permeable fura-2 acetoxyethyl ester (fura-2AM, Molecular Probes, Inc., Eugene, OR Molecular Probes) for 10 minutes at room temperature. Cells were washed twice with Tyrode’s buffer containing 500 µmol/L of probenecid to prevent leakage of fura 2. Cells were incubated in the dark for 2 hours to allow for deesterification of the fura 2-AM. Cells were placed in heated chamber, superfused for 15-20 minutes with Tyrode buffer (1.8 mmol/L Ca\(^{2+}\),
Intracellular Ca\(^{2+}\) transients were measured using a rapidly alternating (1.2 kHz) dual beam excitation fluorescence photometry set-up (Vibraspec Inc., Bear Island, ME).\(^8\) Excitation wavelengths of 340 and 410 nm were used to monitor the fluorescence signals of Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free fura-2.\(^8\) After subtracting the cellular autofluorescence, [Ca\(^{2+}\)]\(_i\) is proportional to the fluorescence ratio at 340 nm and 410 excitation (F\(_{340}/F_{410}\)). Since fura-2 compartmentalizes into mitochondria,\(^9\) calculating intracellular Ca\(^{2+}\) concentrations from fura-2 fluorescence ratios can be problematic in intact cells. Thus, [Ca\(^{2+}\)]\(_i\) measurements were reported as fluorescence ratios (F\(_{340}/F_{410}\)). Ca\(^{2+}\) transients were analyzed by fitting the decay of the transient to a monoexponential curve \(y(t)=a\cdot\exp(-t/k)-b\), where \(a = \) Ca\(^{2+}\) transient amplitude, \(b = \) baseline value, and \(k = \) time constant (tau) of decay.\(^10\) After 5-10 minutes of steady-state pacing, tau, amplitude and baseline value were averaged from 3 consecutive Ca\(^{2+}\) transients for each experimental condition. The baseline value was used as a measure of diastolic [Ca\(^{2+}\)]\(_i\). A subset of cells was exposed to 5 mmol/L caffeine for 5 s using a rapid concentration clamp system. Amplitudes of caffeine-induced Ca\(^{2+}\) transients were used as estimates of SR Ca\(^{2+}\) content.

**Action potential measurements - perforated patch**

A subset of cell was used for simultaneous [Ca\(^{2+}\)]\(_i\) and membrane potential measurements using the amphotericin perforated patch method.\(^11\) Membrane voltage was measured using a bridge current-clamp amplifier (BVC-700, Dagan Corporation, Minneapolis, Minnesota) controlled by a personal computer using a Digidata 1320 acquisition board driven by pCLAMP 8.1 software (Axon Instruments, Foster City, CA). Patch electrodes with a tip resistance of 2-3 M\(\Omega\) were dipped for \(\sim\)10 s into a pipette solution containing (in mmol/L): 140 potassium
aspartate, 5 NaCl, 10 HEPES, 1 EGTA, 5 MgATP, 5 creatine phosphate, pH 7.2. The electrode was then back-filled using the same pipette solution containing 0.7 mg/ml amphotericin (Amphotericin B solubilized; SIGMA: A9528, 50 mg; Lot: K4018) dissolved in 1 ml of the internal solution added. Typically, 5 min after formation of a gigaseal under voltage-clamp configuration, access resistance was lowered to 8 – 10 M\(\Omega\), the amplifier was switched to current clamp configuration, and resting membrane potentials were recorded. Action potentials were measured at 33 °C after 5 minutes of steady-state pacing at 1 Hz and 5 Hz using field-stimulation. [\(\text{Ca}^{2+}\)], was simultaneously measured as described above.

**ECG and action potential data analysis**

All data were analyzed in blinded fashion without knowledge of the genotype. Telemetric ECG recordings were continuously analyzed during defined periods of normal activity (1 hr in the evening, 5 minutes prior to the stress test), during stress tests, and during one hour after the test. For ECG recordings from anesthetized mice and isolated hearts, the entire experimental protocol was reviewed from digitally stored files, using time- and event-logged annotations. Data were manually analyzed for heart rate, PR, and QT interval as described.\(^1\) The entire recording periods were scrutinized for ventricular arrhythmias, which were classified as premature ventricular beat (PVB), couplet (2 consecutive PVBs), or non-sustained VT (\(\geq 3\) consecutive PVB). Two independent experienced electrophysiologists confirmed all episodes of ventricular arrhythmias. The number and type of arrhythmia was recorded during each experimental period, and the occurrence of arrhythmias was compared between I79N-Tg and controls. Effective
refractory period was measured as the maximum extrastimulus interval (S1-S2) at twice-diastolic threshold strength that failed to evoke a propagated response.

TAP, MAP and single-cell AP data were analyzed using a custom-built software program written in LabVIEW (National Instruments Austin, TX) as previously described. Action potential duration was measured at repolarization levels of 30, 50, 70, and 90% (APD$_{30}$ to APD$_{90}$), with 0% defined as the peak amplitude of the action potential upstroke and 100% as the diastolic potential. For AP data obtained using the ruptured patch method, 0% was defined as 30 mV overshoot potential to allow comparison with the TAP and perforated patch APs.

**Detection and confirmation of arrhythmias in telemetric ECG recordings**

Detection of ventricular arrhythmias in telemetric ECG recordings is a challenge, due to potential superimposition of electrical artifacts that may be caused by movements of the recording system or by recording of muscle activity. These problems are more relevant when the animals exercise, as was the case in the stress protocols in our study. Furthermore, telemetric ECG recordings in mice are usually limited to one recording channel, thereby precluding comparative analysis of activations in different recordings leads. Nonetheless, there is no computerized algorithm to discern arrhythmias from artifact, and such an analysis therefore solely relies on the careful eye of an experienced, unbiased observer. To achieve a valid measure of arrhythmia incidence despite these non-modifiable limitations in our study, we established the following, somewhat lengthy analysis procedure to avoid a systematic bias in the analysis of arrhythmias in the telemetric ECG recordings:
a) All potential disturbances of the cardiac rhythm were identified and printed on paper at a high paper speed.

b) All printouts of potential arrhythmias were independently analyzed by two observers (PK and another electrophysiologist from the Department of Cardiology, University Hospital Münster, Germany, who was not otherwise involved in this study). The second observer always had experience in clinical and experimental electrophysiology. These two observers classified the arrhythmia as A) artifact, B) ventricular arrhythmia, C) supraventricular arrhythmia, or D) not determinable. If both observers agreed, the classification was accepted. If there was a mismatch in classification, they met with a third electrophysiologist and decided on the final classification.

c) For classification of potential arrhythmias, several criteria were pre-specified. These included:

i) Quality of the recording before and after the potential arrhythmia

ii) Detection of the normal rhythm (QRS complexes) within the arrhythmia episode

iii) Continuation of the normal rhythm after the end of the arrhythmia

iv) Plausibility of successive ventricular activations (e. g. cycle length > 30-35 ms, the refractory period in the mouse ventricle) and morphology of the “QRST complexes” within the arrhythmia episodes. In our experience with telemetric ECG recordings in mice, muscle artifacts tend to be shorter in duration and more rapid in upstroke than ventricular beats.
In addition to these criteria, the clinical experience in identifying artifacts in Holter ECG recordings helped the observers in their individual judgment. All analyses were performed blinded to genotype.

References


10. Lim CC, Apstein CS, Colucci WS, Liao R. Impaired cell shortening and relengthening with increased pacing frequency are intrinsic to the senescent mouse cardiomyocyte. *J Mol Cell Cardiol*. 2000;32:2075-82.

Online figure 1. Heart rate of a conscious control mouse during warm air jet stress test. Heart rate is plotted continuously versus time (start of stress test = 0 min). Arrows indicate the beginning of each exposure to warm air jets. Each exposure lasted 15 seconds. After an initial period of a relatively sustained increase in heart rate, lasting from the first to the 5th exposure, warm air jets increased heart rate only transiently.