Myofilament Ca^{2+} sensitization causes susceptibility to cardiac arrhythmia in mice

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In human cardiomyopathy, anatomical abnormalities such as hypertrophy and fibrosis contribute to the risk of ventricular arrhythmias and sudden death. Here we have shown that increased myofilament Ca^{2+} sensitivity, also a common feature in both inherited and acquired human cardiomyopathies, created arrhythmia susceptibility in mice, even in the absence of anatomical abnormalities. In mice expressing troponin T mutants that cause hypertrophic cardiomyopathy in humans, the risk of developing ventricular tachycardia was directly proportional to the degree of Ca^{2+} sensitization caused by the troponin T mutation. Arrhythmia susceptibility was reproduced with the Ca^{2+}-sensitizing agent EMD 57033 and prevented by myofilament Ca^{2+} desensitization with blebbistatin. Ca^{2+} sensitization markedly changed the shape of ventricular action potentials, resulting in shorter effective refractory periods, greater beat-to-beat variability of action potential durations, and increased dispersion of ventricular conduction velocities at fast heart rates. Together these effects created an arrhythmogenic substrate. Thus, myofilament Ca^{2+} sensitization represents a heretofore unrecognized arrhythmia mechanism. The protective effect of blebbistatin provides what we believe to be the first direct evidence that reduction of Ca^{2+} sensitivity in myofilaments is antiarrhythmic and might be beneficial to individuals with hypertrophic cardiomyopathy.

Introduction

Sudden cardiac death (SCD) as a result of cardiac arrhythmias is responsible for 10% of all deaths in the United States (1). The study of monogenetic SCD syndromes has identified a number of primary arrhythmia mechanisms in patients with structurally normal hearts (2). Much less is known about what causes arrhythmias in structurally abnormal hearts such as in hearts with hypertrophic cardiomyopathy (HCM), which is the most common cause of SCD in the young (3). HCM results from autosomal-dominant mutations in genes encoding cardiac contractile proteins (4) and is characterized by heart hypertrophy, interstitial fibrosis, and myofibrillar disarray (5). While these anatomical abnormalities provide electrical circuits for reentrant excitation and therefore could explain the high arrhythmia risk in HCM (6, 7), certain sarcomeric mutations confer a high risk of SCD, even in the absence of cardiac hypertrophy (8, 9). This suggests that other mechanisms importantly contribute to arrhythmia susceptibility in HCM, particularly for patients with mutations in troponin T (TnT) or TnI — sarcomeric proteins that are directly involved in the Ca^{2+}-dependent regulation of muscle contraction (10, 11).

In vitro studies demonstrate that TnT mutations associated with a high risk of SCD commonly sensitize myofilaments to the effect of Ca^{2+} (12). Increased myofilament Ca^{2+} sensitivity has also been found in acquired heart diseases that are characterized by a high incidence of ventricular tachycardia (VT) and SCD, such as in animals after myocardial infarction (13) and in humans with heart failure (14, 15). Hence, we hypothesized that increasing myofilament Ca^{2+} sensitivity independently contributes to arrhythmia susceptibility. To test this hypothesis, we selected HCM-linked TnT mutations that confer strong (TnT-I79N), intermediate (TnT-F110I), and no Ca^{2+} sensitization (TnT-R278C) in human cardiac fibers (16). Although the number of patients carrying these mutations is too small to draw definitive genotype-phenotype conclusions, the TnT-I79N mutation has been associated with a high rate of SCD at young age (8), whereas the nonsensitizing TnT-R278C mutation appears to carry an overall better prognosis (17, 18). To avoid any confounding effects of anatomical abnormalities, we studied transgenic mice that phenocopy the differential Ca^{2+}-sensitizing effects of the human TnT mutants (I79N > F110I > R278C) but do not exhibit cardiac hypertrophy, fibrosis, or myofibrillar disarray (16, 19, 20).

To demonstrate that Ca^{2+} sensitization is proarrhythmic irrespective of the underlying molecular mechanism, we used mice expressing the slow-skeletal isoform of TnI (ssTnI) in place of cardiac TnI (20); ssTnI lacks the phosphorylation site implicated for abnormal TnI phosphorylation in human heart failure (14, 15) and causes Ca^{2+} sensitization similar to that of TnT-I79N. We also used pharmacological agents that acutely increase (EMD 57033) (21, 22) or decrease (blebbistatin) (23) myofilament Ca^{2+} sensitivity.

Using a combination of transgenic and pharmacologic approaches both in vivo and in vitro, our study establishes myofilament Ca^{2+} sensitization as what we believe to be a novel mechanism of arrhythmogenesis and provides the first direct evidence to our knowledge that reduction of Ca^{2+} sensitivity in myofilaments is antiarrhythmic.

Nonstandard abbreviations used: AP, action potential; APD, AP duration; APD70, APD measured at 70% repolarization; CV, conduction velocity; ERP, effective refractory period; HCM, hypertrophic cardiomyopathy; MAP, monophasic AP; NTg, nontransgenic; pCa, $-\log[Ca^{2+}]$; pCa_{50}, pCa at 50% of maximal force; PCL, pacing cycle length; PVC, premature ventricular complex; SCD, sudden cardiac death; ssTnI, mice expressing the slow-skeletal isoform of TnI; TnT, troponin T; VT, ventricular tachycardia.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* doi:10.1172/JCI36642.

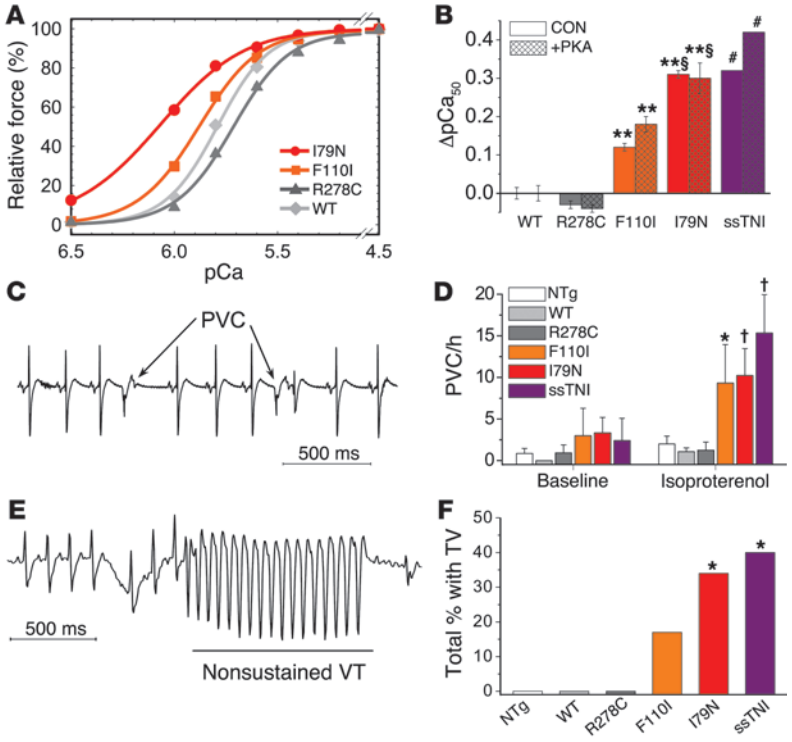

Figure 1

Isoproterenol challenge causes ventricular ectopy in transgenic mice with Ca^{2+}-sensitized myofilaments. (**A** and **B**) Effect of different TnT mutations on Ca^{2+} sensitivity of force development of skinned fiber bundles. (**B**) Average change in pCa_{50} of force development (ΔpCa_{50}) of the different groups of transgenic mice studied here. The ΔpCa_{50} values for R278C, F110I, and I79N were expressed relative to the pCa_{50} of WT before (control [CON]) and after phosphorylation with the catalytic subunit of protein kinase A. $^{**}P < 0.01$ compared with WT; $^{\S}P < 0.01$ compared with F110I; $n = 16$ each. Values for ssTNI are taken from ref. 25. In this and subsequent figures, data from mice or hearts with Ca^{2+}-sensitized myofilaments are presented in color, whereas data from mice or hearts with normal myofilament Ca^{2+} sensitivity are presented in gray. $^{\#}P < 0.05$ compared with NTg littermates, $n = 6$. (**C**–**F**) Effect of isoproterenol in anesthetized mice. (**C**) ECG recording from an F110I mouse illustrating isoproterenol-induced PVCs (arrows). (**D**) Note the significantly higher PVC rate in mice with Ca^{2+}-sensitized myofilaments (F110I, $n = 6$; I79N, $n = 9$; ssTnI, $n = 5$) compared with normal myofilament Ca^{2+} sensitivity (NTg, $n = 14$; WT, $n = 23$; R278C, $n = 13$). $^{*}P < 0.05$, $^{\dagger}P < 0.005$ compared with WT, by Mann-Whitney *U* test. (**E**) Example of nonsustained VT recorded in an I79N mouse after isoproterenol injection. (**F**) VT incidence after isoproterenol injection. $^{*}P < 0.05$ compared with WT, by Fisher's exact test; group sizes as in **D**.

Results

Increased incidence of ventricular arrhythmias in mice expressing Ca^{2+}-sensitizing TnT or TnI. Figure 1A shows that bundles of skinned papillary muscle fibers from transgenic mice expressing TnT-I79N exhibited increased myofilament Ca^{2+} sensitivity (indicated by the left shift in the relationship between relative force and –log[Ca^{2+}] [pCa] compared with that in the fibers expressing human WT TnT or the TnT-R278C mutant). TnT-F110I had an intermediate effect on the force-pCa relationship (Figure 1A). Figure 1B illustrates the differences in Ca^{2+} sensitivity (assessed as the pCa at which developed force was 50% of maximal force [pCa_{50}]) among the groups of mice studied here, both at baseline and after incubation, with the catalytic subunit of protein kinase A (to test the effect of β-adrenergic receptor stimulation). All transgenic TnT mice had approximately 50% of their endogenous TnT protein replaced with either WT or mutant human cardiac TnT (19, 24). Expression of ssTNI (25) increased the pCa_{50} to a similar extent as expression of TnT-I79N at baseline, and pCa_{50} increased to an even higher extent after protein kinase A phosphorylation (Figure 1B).

To examine the effect of myofilament Ca^{2+} sensitization on arrhythmia susceptibility in vivo, we continuously recorded surface ECGs in anesthetized mice at baseline conditions and after intraperitoneal injection of the β-adrenergic receptor agonist isoproterenol. As shown in Figure 1D, there was low level ventricular ectopy that did not differ among groups at baseline. After isoproterenol challenge, however, the 3 groups of mice expressing Ca^{2+}-sensitizing TnT or TnI (I79N, F110I, ssTnI) demonstrated a striking increase in the rate of premature ventricular complexes (PVCs; Figure 1C) compared with nontransgenic (NTg) littermates (Figure 1D). In contrast, the incidence of PVCs in mice expressing the nonsensitizing TnT-R278C mutant or human WT TnT was no different from that of NTg mice. Short runs of VT (Figure 1E) were observed in 17% of F110I mice, 33% of I79N mice, and 40% of ssTnI mice but not in R278C, WT, or NTg mice ($P < 0.05$ for I79N or ssTNI compared with WT mice). These results strongly support the hypothesis that mice with Ca^{2+}-sensitized myofilaments are at risk for developing ventricular tachyarrhythmias.

Fast pacing triggers sustained ventricular tachyarrhythmias in mouse hearts expressing Ca^{2+}-sensitizing TnT mutants. We quantified the effect of different HCM-linked TnT mutations on arrhythmia susceptibility ex vivo in Langendorff-perfused heart preparations. Hearts were stained with the voltage sensitive fluorescent indicator di-4-ANEPPS and imaged. Programmed stimulation using single extrasystoles with progressively shortened S1-S2 coupling intervals did not show any differences in VT induction among the groups of mice. However, there was a striking effect of heart rate on arrhythmia susceptibility assessed by progressively shortening the pacing cycle length (PCL). As illustrated in Figure 2A, mouse hearts with normal myofilament sensitivity (NTg, WT, R278C) tolerated PCLs as short as 40 ms (resulting in a pacing rate of 1,500 beats per minute) without exhibiting post-pacing VT. Rapid pacing eventually resulted in intermittent capture (Figure 2A), and sinus rhythm was restored immediately after pacing was stopped (Figure 2A). In contrast, as shown in Figure 2B, hearts expressing Ca^{2+}-sensitizing TnT mutants (I79N, F110I) exhibited VT in response to rapid pacing. Once induced, fast VT was commonly sustained (>30 s) in Ca^{2+}-sensitized transgenic hearts even when pacing was stopped (Figure 2B). For each heart, Figure 2C shows the PCL at which sustained VT was induced. Noninducible hearts are indicated by a PCL of 0 ms. Sustained VT could be induced in more than 90% of hearts expressing the Ca^{2+}-sensitizing mutants (91% and 92% of F110I and I79N hearts, respectively) but only in a much smaller fraction of hearts with normal myofilament Ca^{2+} sensitivity (20%, 13%, and 17% of WT, NTg, and R278C hearts, respectively). Among the hearts that exhibited VT, VT was induced at significantly lon-

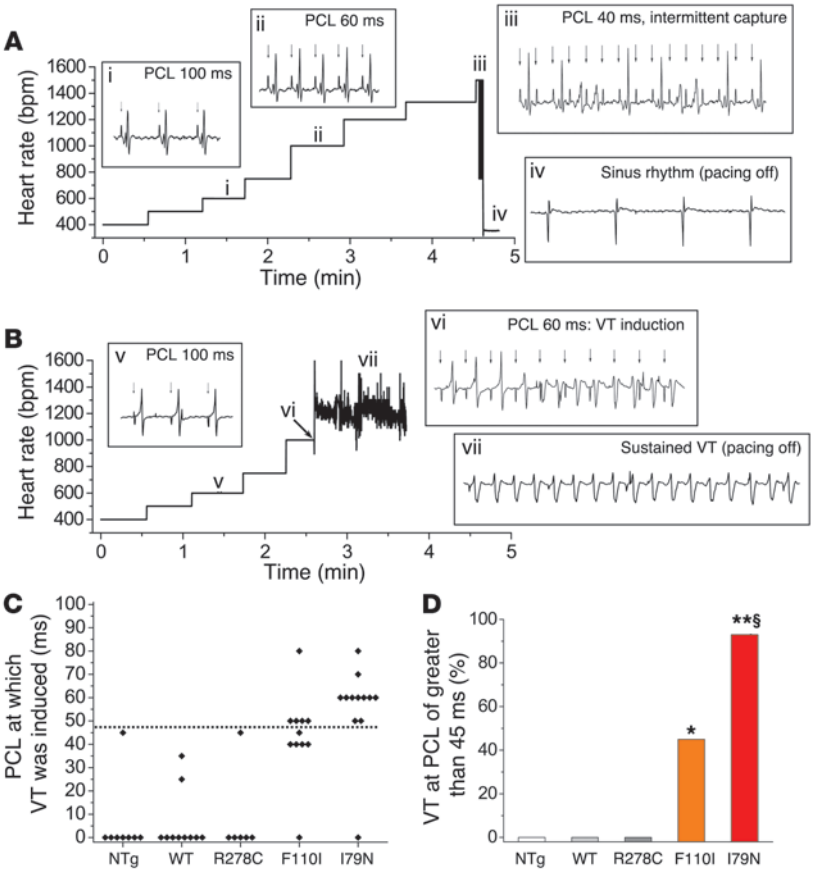

Figure 2
Fast pacing triggers sustained VT in mouse hearts expressing Ca^{2+}-sensitizing TnT mutants. Pacing challenge in isolated hearts from transgenic mice expressing TnT mutations. (**A**) Pacing protocol and heart response of a WT mouse heart, with corresponding ECG records in the insets. At longer PCLs, each pacing stimulus (arrows) was followed by a ventricular response (insets i and ii). PCL was shortened in stepwise fashion until loss of 1-to-1 capture occurred (inset iii). Cessation of pacing restored sinus rhythm (inset iv). (**B**) Heart rate response to successively shorter PCLs of an I79N mouse heart. While 1-to-1 capture was maintained at long PCLs (inset v), a PCL of 60 ms induced VT (inset vi), with a VT cycle length of approximately 50 ms. VT was sustained even after pacing was stopped (inset vii) and did not terminate until the end of the experiment. (**C**) PCLs at which sustained VT (>30 s) was induced in each heart. A PCL of 0 indicates that VT could not be induced. The dotted line marks the PCL threshold of VT induction in nonsensitized hearts. (**D**) Incidence of VT induced at PCLs above the VT threshold of normal hearts. NTg, $n = 8$; WT, $n = 10$; R278C, $n = 6$; F110I, $n = 11$; I79N, $n = 13$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with WT; $^{\S}P < 0.05$ compared with F110I, by Fisher's exact test.

ger PCLs in I79N hearts (64 ± 14 ms, $n = 12$) compared with F110I hearts (49 ± 12 ms, $n = 10$; $P < 0.05$ vs. I79N) or nonsensitized hearts (NTg, WT, R278C, 38 ± 10 ms, $n = 4$; $P < 0.01$ vs. I79N). VT was never induced in nonsensitized hearts with PCLs longer than 45 ms (VT threshold of normal hearts is indicated by the dotted line; Figure 2C). The incidence of sustained VT induced at PCLs above the VT threshold of normal hearts was in 92% of I79N hearts, 50% of F110I hearts, and 0% of R278C hearts (Figure 2D). Hence, the VT incidence corresponded to the degree of Ca^{2+} sensitization of the respective mutation (I79N > F110I > R278C = WT = NTg; Figure 1B and Figure 2D).

Ca^{2+}-sensitizing TnT mutants increase the spatial dispersion of ventricular activation during fast pacing rates and generate a substrate for functional reentry. To explore the mechanism responsible for VT induction, we analyzed the epifluorescent images obtained at each pacing rate during the experiments shown in Figure 2. Figure 3A shows examples of isochronal voltage maps of an R278C heart and an I79N heart for each pacing rate. Compared with the heart expressing the nonsensitizing R278C mutant, the I79N heart exhibited an increasingly greater spatial variability in activation times with faster pacing. To quantify this effect, we calculated the conduction velocity (CV) along 10 equally spaced radial lines (Figure 3B) for each heart at each PCL. Spatial dispersion of ventricular activation (presented as CV dispersion) was assessed using the relative SD of the 10 CV determinations. Increasing the pacing rate (resulting in shorter PCL) decreased the averaged CV. There were no significant differences in the averaged CV at any pacing rate between the TnT mutants (Figure 3C). In contrast, faster pacing progressively increased CV dispersion of Ca^{2+}-sensitized hearts (I79N, F110I), whereas the CV dispersion did not change significantly with increasing pacing frequency in nonsensitized hearts (R278C) over a comparable PCL range. As a result, CV dispersion was significantly greater in hearts expressing I79N or F110I compared with hearts expressing R278C with normal myofilament sensitivity (Figure 3D). Optical maps recorded during sustained VT revealed stationary repetitive activation patterns, with rotors partially exposed on the surface of the left ventricle (Figure 3E and Supplemental Video 1; supplemental material available online with this article; doi:10.1172/JCI36642DS1). Taken together, our data demonstrate that the Ca^{2+}-sensitizing I79N and F110I mutants caused a regional slowing of conduction during rapid pacing and rendered hearts susceptible to functional reentry.

Acute myofilament Ca^{2+} sensitization reproduces the arrhythmia promoting effects of Ca^{2+}-sensitizing TnT mutants. We used the Ca^{2+} sensitizer EMD 57033 to test whether acute myofilament Ca^{2+} sensitization is sufficient to reproduce the arrhythmia promoting effects of TnT mutants. In skinned cardiac fibers from mouse hearts, EMD, at a concentration of 3 μM, increased the pCa_{50} of force development to a similar degree as the I79N mutation (Figure 4A). In isolated ventricular mouse myocytes, EMD had no effect on K^+ or Ca^{2+} currents but shortened the ventricular action potential duration (APD). The APD shortening could be prevented by blocking myofilament activation and contraction (Supplemental Figure 1).The effect of EMD is analogous to that previously reported for myocytes from I79N mice (26). NTg hearts were subjected to the pacing protocol described in Figure 2 at baseline, in the presence of 3 μM

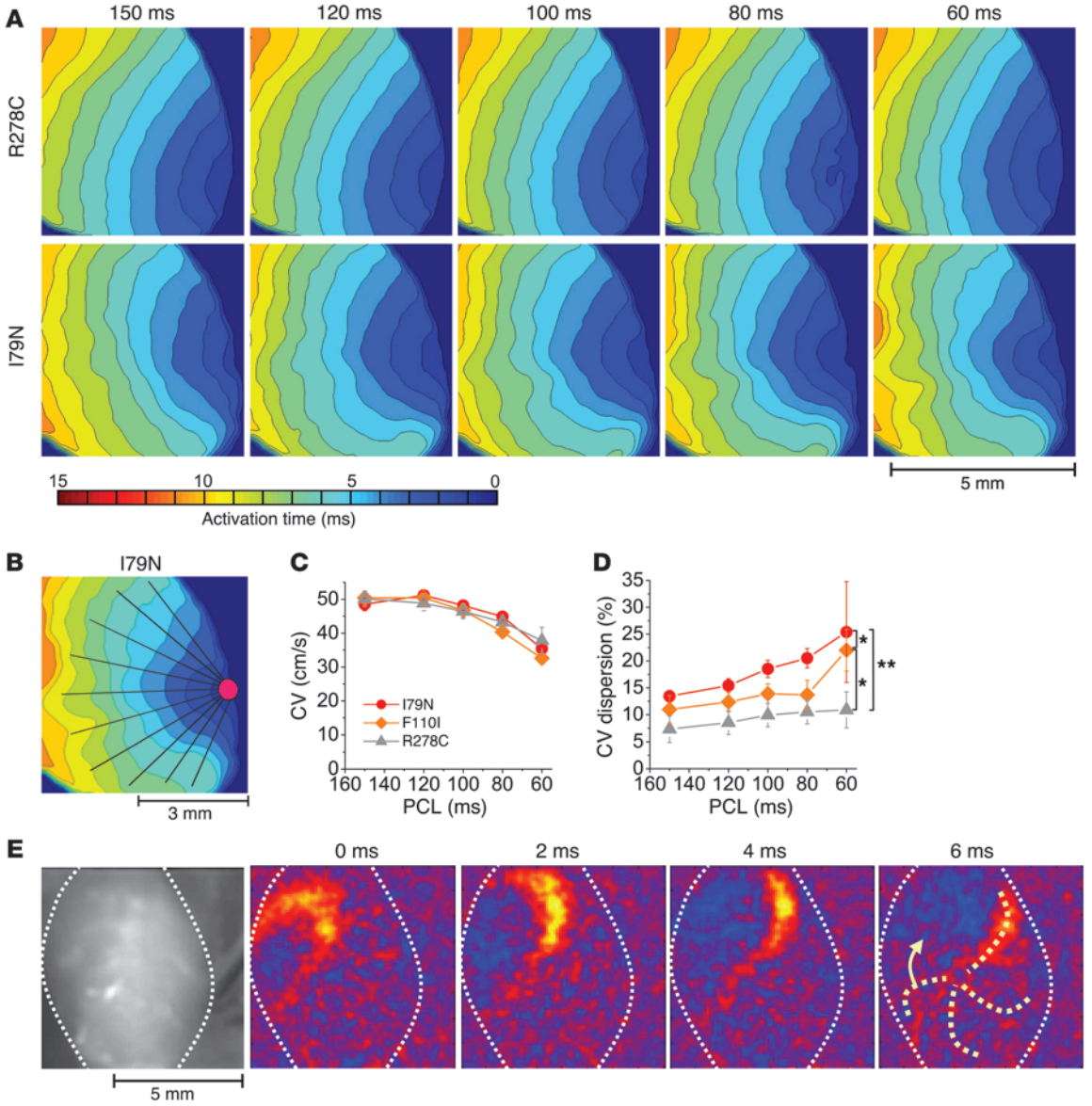

Figure 3

Ca^{2+}-sensitizing TnT mutants increase the spatial dispersion of electrical activation during fast pacing and create a substrate for functional reentry. (**A**) Examples of isochronal activation maps of mouse hearts at different pacing rates from an R278C and an I79N heart. Hearts were stained with the voltage-sensitive fluorescent indicator di-4-ANEPPS, and epicardial images were obtained from the anterior aspect of the left ventricle at the PCL indicated above each image. (**B**–**D**) Effect of TnT mutants on CV and CV dispersion. CVs were calculated along 10 equally spaced radial lines from the stimulus side (red circle) near the apex as shown in **B** and were averaged for each heart. CV dispersion was quantified as the SD of the 10 CV determinations and expressed as percentage of average CV per heart. (**C**) Average CV decreased with shorter PCLs (shorter PCLs result in faster pacing) in all groups regardless of genotype. (**D**) Spatial CV dispersion was significantly increased in Ca^{2+}-sensitized transgenic hearts. $^*P < 0.05$, $^{**}P < 0.01$ between groups, by 2-way ANOVA with repeated measures; I79N, $n = 7$; F110I, $n = 7$; R278C, $n = 5$. (**E**) Optically recorded repetitive activation pattern during VT. Fluorescence difference images of successive frames taken from the anterior left ventricle epicardial surface of an I79N heart during sustained VT. The time stamp is referenced to the first image. The activation pattern during VT shows typically a spiral wave rotating around a fixed center, forming a reentrant excitation (see also Supplemental Video 1).

EMD, and after EMD was washed out. As summarized in Figure 4B, EMD, like I79N, rendered hearts susceptible to induction of sustained VT during rapid pacing; the effect was completely reversible upon washout. Analysis of isochronal voltage maps (Figure 4, C and D) showed that, similar to the F110I and the I79N experiments, EMD did not affect average CV (Figure 4E) but caused a significant, increased CV dispersion at fast pacing rates (Figure 4F). These data further support the concept that myofilament Ca^{2+} sensitization renders hearts susceptible to ventricular tachyarrhythmias, even in the absence of an anatomical substrate.

Myofilament Ca^{2+} sensitization changes the shape of the ventricular action potential. To start exploring the mechanisms responsible for the

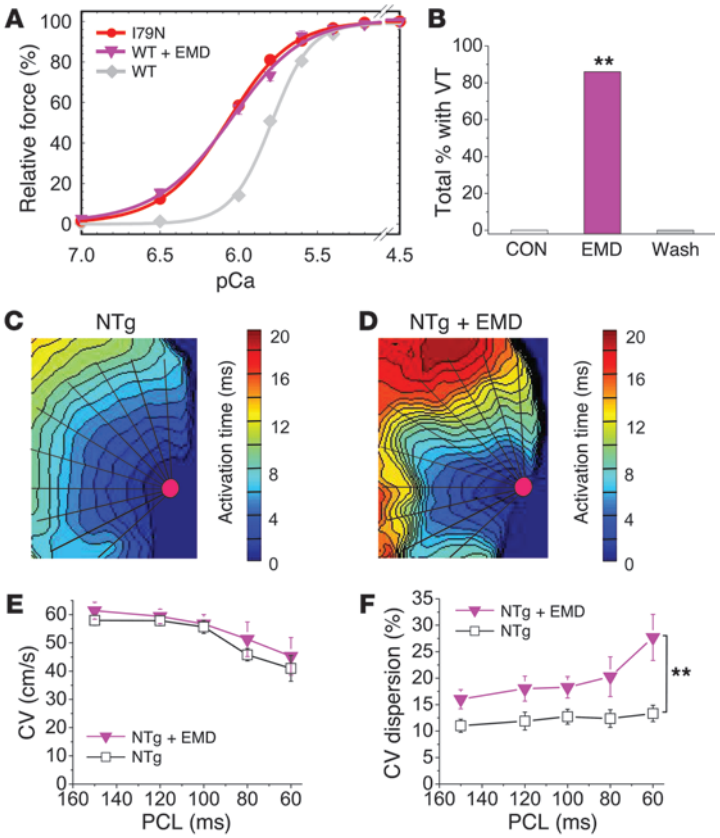

Figure 4
Acute challenge with the Ca^{2+}-sensitizing compound EMD 57033 reproduces the effects of Ca^{2+}-sensitizing troponin mutants in NTg mouse hearts. (**A** and **B**) Effect of EMD 57033 on myofilament Ca^{2+} sensitivity and VT susceptibility. (**A**) Normalized force-pCa relationships of skinned cardiac fibers. EMD at 3 μM increased the Ca^{2+} sensitivity of WT fibers to a similar degree as transgenic expression of TnT-I79N. (**B**) Incidence of sustained VT of NTg hearts before treatment, in the presence of EMD, and 5 minutes after washout of EMD (Wash), using the pacing protocol described in Figure 2. $^{**}P < 0.01$ compared with CON or washout, by Fisher's exact test, $n = 7$. (**C**–**F**) Effect of EMD 57033 on ventricular activation, CV, and CV dispersion. Examples of isochronal activation maps obtained at a PCL of 60 ms from an NTg heart before (**C**) and during exposure to 3 μM EMD (**D**). Note the increased spatial variability of activation times in the presence of EMD. Average CV and CV dispersion were calculated for each heart as described in Figure 3. (**E**) Average CV plotted as a function of PCL. (**F**) Spatial CV dispersion was significantly increased by EMD exposure. $^{**}P < 0.01$, by 2-way ANOVA with repeated measures; NTg, $n = 8$.

increased CV dispersion and reentrant VT, we examined the effect of myofilament Ca^{2+} sensitization on the ventricular action potential (AP), using the monophasic AP (MAP) method (27). The optical measurements were not suited for a detailed AP analysis because of prominent motion artifacts during repolarization. As shown in Figure 5A, the Ca^{2+}-sensitizing I79N mutant changed the shape of the ventricular repolarization, resulting in a shallower repolarization slope. APD measured at 70% repolarization (APD70) was significantly shortened, whereas terminal repolarization (APD90) was not significantly different from WT hearts or hearts expressing the nonsensitizing R278C mutant. Ca^{2+}-sensitized ssTnI hearts exhibited similar APD70 shortening. Average data are summarized in Figure 5B. The changes in AP waveform of I79N and ssTnI hearts were evident already at relatively slow pacing rates (PCL, 300 ms) and persisted with fast pacing (Supplemental Figure 2). Acute application of EMD reproduced the AP changes observed in hearts expressing I79N and ssTnI (Figure 5, C and D), which were completely reversible upon washout of EMD. In concordance with the shortened APD70, Ca^{2+} sensitization by EMD or I79N significantly shortened the effective refractory period (ERP) (WT, 50.2 ± 1.2 ms; I79N, 38.0 ± 1.2 ms; R278C, 48.0 ± 1.5 ms; $n = 7$–11 hearts; $P < 0.05$; control, 42.7 ± 2.6 ms; EMD, 32.7 ± 1.8 ms; washout, 40.9 ± 4.0 ms; $n = 12$ hearts; $P < 0.01$). We next tested EMD in isolated cat hearts, which have ventricular AP shapes, resembling more closely that of human APs. EMD significantly shortened APD30, APD50, and APD70, resulting in a "triangular" AP shape (Figure 5, E and F). The ERP was also significantly shortened (control, 169.0 ± 3.3 ms; EMD, 150.5 ± 5.4 ms; washout 173.3 ± 3.3; $n = 4$ hearts; $P < 0.01$). VT was induced in 1 out of 4 hearts. Moreover, the beat-to-beat variability of AP repolarization was significantly increased in the presence of EMD, frequently resulting in an APD alternans pattern (Figure 5, G and H, and Supplemental Figure 3). APD variability was also significantly increased in Ca^{2+}-sensitized mouse hearts (Supplemental Figure 3). Taken together, these data suggest that the AP triangulation, ERP shortening, and beat-to-beat APD alternans contributed to the arrhythmia susceptibility of Ca^{2+}-sensitized hearts.

Contractile uncoupling and myofilament desensitization with blebbistatin prevents the proarrhythmic effects of Ca^{2+} sensitization. If myofilament Ca^{2+} sensitization is responsible for increasing arrhythmia susceptibility, then normalizing myofilament function should be antiarrhythmic. While a specific Ca^{2+} desensitizing compound does not exist, the myosin ATPase inhibitor blebbistatin has been used as a contractile uncoupling agent for electrophysiological studies and has been reported to have either minimal or no significant effects on cardiac ion channels and APs (28). Given that blebbistatin inhibits force generation of strongly attached crossbridges (23) and based upon the indirect interaction between troponin C and crossbridges (29), we hypothesized that blebbistatin would also decrease myofilament Ca^{2+} sensitivity.

Blebbistatin reduced Ca^{2+} sensitivity (pCa_{50}) in a concentration-dependent manner in all skinned fibers from TnT transgenic hearts (Figure 6A). The concentration dependence of blebbistatin's reduction in pCa_{50} (Figure 6B) paralleled its effect on developed force (Figure 6C). Note that a blebbistatin concentration of 3 μM was required to reduce the pCa_{50} of I79N fibers to that of WT fibers (Figure 6B, dashed line), whereas for F110I fibers, which exhibited a significantly smaller pCa_{50} increase than I79N fibers (Figure 1B), 0.1 μM blebbistatin was sufficient. Blebbistatin at

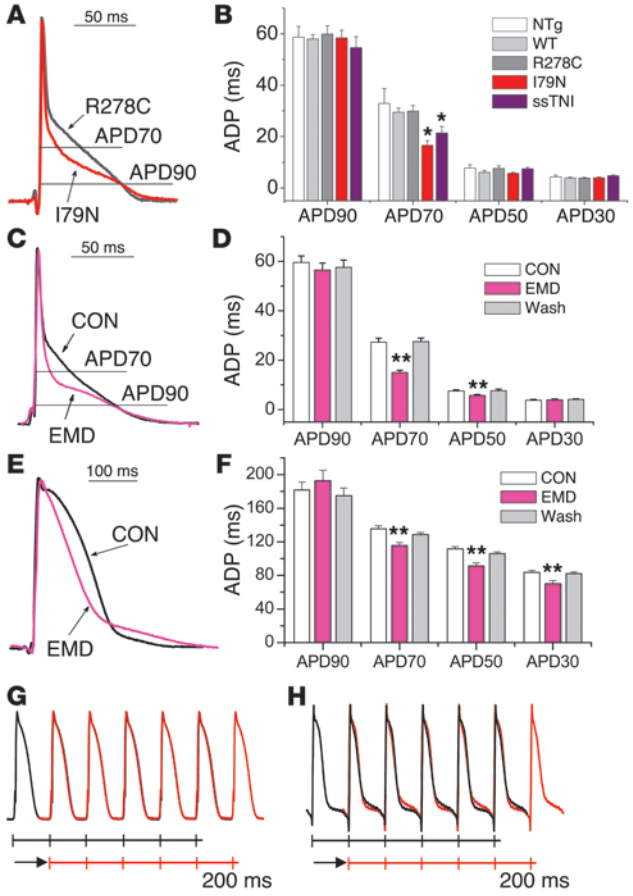

Figure 5

Ca^{2+} sensitization changes the ventricular AP in isolated mouse and cat hearts. (**A**–**D**) Effect of TnT mutants or EMD on the mouse ventricular AP. (**A**) Superimposed MAP records comparing the effect of the Ca^{2+}-sensitizing I79N and the nonsensitizing R278C mutation on mouse ventricular AP. PCL, 150 ms. While the overall APD is unchanged, I79N hearts have a lower terminal repolarization phase. (**B**) Average MAP durations measured at 90%, 70%, 50%, and 30% repolarization levels. PCL, 150 ms; n = 7–18 mice, $^{*}P < 0.05$ compared with NTg, by Student's t test. (**C** and **D**) The effect of acute Ca^{2+} sensitization with EMD 57033 on mouse ventricular AP. EMD shortened APD70, analogous to the effect of Ca^{2+}-sensitizing mutants. PCL, 150 ms; n = 9 mice; $^{**}P < 0.01$ compared with CON or washout, by Student's t test. (**E** and **F**) Effect of EMD 57033 on the cat ventricular AP. (**E**) Superimposed MAP recording demonstrating the changes in the ventricular AP wave shape induced by EMD 57033 in cats. (**F**) Average MAP durations measured at 90%, 70%, 50%, and 30% repolarization levels. PCL, 400 ms; n = 4 cats; $^{**}P < 0.01$ compared with CON or washout, by Student's t test. (**G** and **H**) EMD causes beat-to-beat variability of AP repolarization in cat hearts. Example MAP records at baseline (**G**) and in the presence of EMD (**H**). The red trace represents the same MAP record shifted by 1 PCL (200 ms) and superimposed to illustrate the repolarization alternans.

3 μM also antagonized the Ca^{2+}-sensitizing effect of 3 μM EMD in skinned fibers (Figure 6D). Once blebbistatin abolished the Ca^{2+}-sensitizing effect of EMD, EMD was unable to match the same maximal force seen in the absence of both chemical compounds (Figure 6E), suggesting that blebbistatin and EMD have distinct mechanisms of action.

We examined the effect of 3 μM blebbistatin on ventricular APs, ventricular activation, and arrhythmia susceptibility, using the pacing protocol described in Figure 2. Pretreatment with blebbistatin completely prevented the effect of EMD on the ventricular AP shape (Figure 7, A and B) and abolished the differences in APD beat-to-beat variability among the groups (data not shown). Blebbistatin alone had no effect on ventricular activation and CV dispersion of NTg hearts (Figure 7C). However, blebbistatin prevented the EMD-induced increase in CV dispersion (compare Figure 3C and Figure 7D). On average, treatment with blebbistatin significantly decreased the spatial CV dispersion of Ca^{2+}-sensitized hearts at maximal PCLs (I79N, F110I, EMD) but had no effect on the CV dispersion of nonsensitized hearts (NTg, WT, R278C; Figure 7E). Blebbistatin also prevented the induction of VT in hearts expressing the Ca^{2+}-sensitizing TnT mutants and EMD-treated hearts (Figure 7F). These data demonstrate that reduction of contractile force and normalization of myofilament Ca^{2+} sensitivity can prevent the increased arrhythmia risk resulting from myofilament Ca^{2+} sensitization by HCM-linked TnT mutants.

Discussion

We have shown that Ca^{2+}-sensitizing TnT or TnI mutants create a myocardial substrate that is susceptible to ventricular arrhythmia. The underlying mechanism appears to be increased spatial dispersion of activation times during rapid heart rates, which is capable of generating functional reentry. Importantly, these effects were evident even in the absence of anatomical abnormalities, commonly invoked as the cause of arrhythmias in HCM. We demonstrated that the proarrhythmic action of TnT mutants depended on their Ca^{2+}-sensitizing effects: Arrhythmia induction could be reproduced in NTg hearts by perfusion with the Ca^{2+}-sensitizing compound EMD and prevented by Ca^{2+} desensitization and decreasing contractile force with blebbistatin. The underlying mechanism appeared to be related to a modification of the ventricular AP repolarization shape, which resulted in a shortened ERP and increased beat-to-beat variability of APD. The results with blebbistatin identify modulation of myofilament function as potential antiarrhythmic intervention for patients with HCM with sarcomeric mutations that increase myofilament Ca^{2+} sensitivity.

Rapid heart rate frequently precedes arrhythmia induction in patients with HCM. With the wider use of implantable cardiac defibrillators in patients with HCM, data are becoming available on the heart rhythm preceding the ventricular tachyarrhythmia (VT or ventricular fibrillation) responsible for SCD in patients with HCM. In a study of 54 adult patients with HCM that were recipients of implantable cardiac defibrillators for primary prevention, sinus tachycardia or atrial fibrillation with rapid ventricular rates were the initiating rhythms in over 90% of arrhythmic events requiring defibrillation (43 of 51 episodes) (30). Similarly, a study of 8 patients with HCM documented atrial fibrillation that degenerated into ventricular fibrillation in 4 patients, requiring defibrillation. Notably, these 4 patients were significantly younger than the remainder of the cohort (31). Importantly, a recent study in children with HCM demonstrated that over 90% (10 of 11) of episodes of ventricular fibrillation were preceded by sinus tachycardia, which occurred even during drug therapy intended to reduce heart rates (32). None of these 3 studies provided genotype data. However, TnT mutations occurred in 4 out 6 cases of confirmed VT and SCD in a small cohort of young patients with HCM (33). Consistent with these results, TnT mutations, which commonly

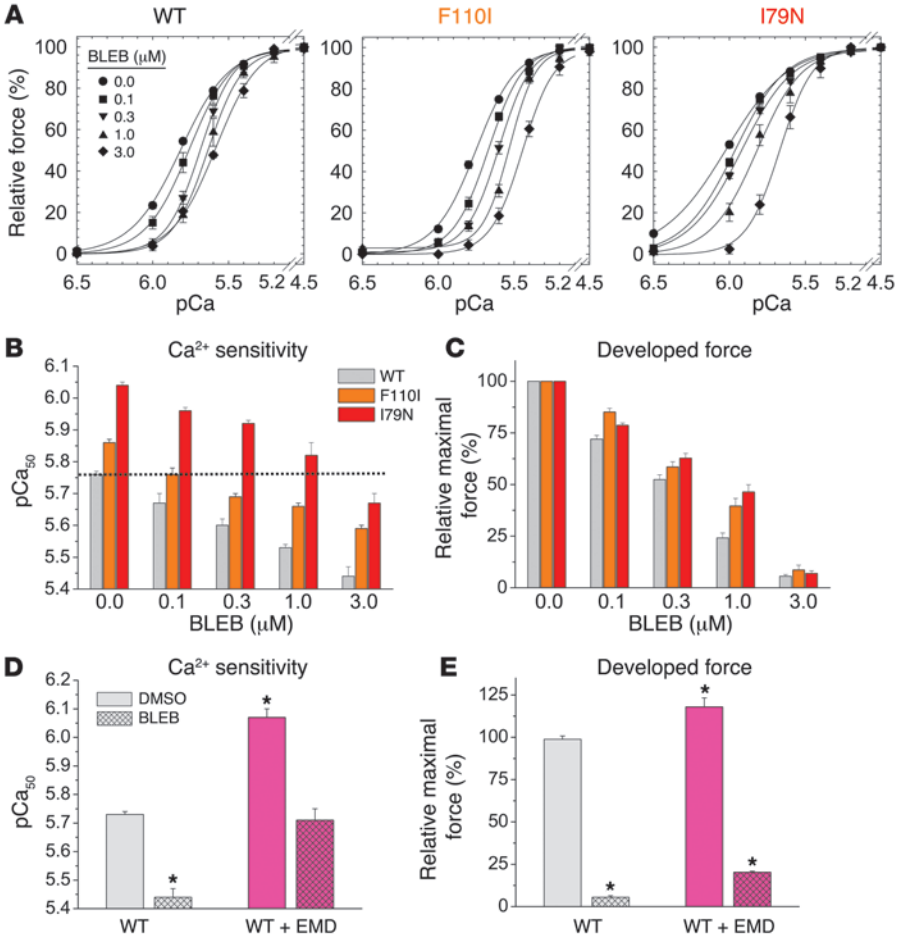

Figure 6
Blebbistatin (BLEB) reduces myofilament Ca^{2+} sensitivity in mouse skinned cardiac muscle. (**A**–**C**) Effect of blebbistatin on the Ca^{2+} sensitivity of force development and relative maximal force of mouse skinned cardiac muscle expressing different TnT mutants. (**A**) Normalized force-pCa relationships of skinned cardiac fibers expressing WT, F110I, or I79N in the presence of blebbistatin (0.0–3.0 μM) as shown. Ca^{2+} sensitivity of force development was assessed by the pCa_{50}. Maximal force was determined at pCa 4.0. Blebbistatin concentration-response relationships compared with vehicle (DMSO) for Ca^{2+} sensitivity (**B**) and developed force (**C**) in WT, F110I, and I79N fibers. Note that 3 μM blebbistatin was needed to reverse the pCa_{50} increase of I79N fibers (dashed line). n = 4 independent experiments per blebbistatin concentration. (**D** and **E**) Effect of blebbistatin in the presence of EMD 57033. Average effect of EMD 57033 (3 μM) and/or blebbistatin (3 μM) on Ca^{2+} sensitivity (**D**) and developed force (**E**) in WT mouse skinned cardiac fibers. Blebbistatin (3 μM) reversed the pCa_{50} increase induced by EMD. n = 4 independent experiments per group; $^*P < 0.05$ compared with DMSO.

increase myofilament Ca^{2+} sensitivity (12), have been linked to a high risk for SCD and historically are found in a larger proportion of young patients referred for tertiary care (8, 34) than in the adult HCM outpatient population (18, 35). Our data from the transgenic mouse models identify a mechanism whereby fast heart rates can be particularly proarrhythmic in patients with Ca^{2+}-sensitizing TnT mutations.

Myofilament Ca^{2+} sensitization creates an arrhythmogenic substrate in the absence of anatomical defects. One of our key findings is that myofilament Ca^{2+} sensitization increased the spatial dispersion of ventricular activation at fast pacing rates, which resulted in reentry arrhythmias, even in the absence of an anatomical substrates. Since the transgenic models studied here do not exhibit interstitial fibrosis and myocyte disarray (16, 19, 20), our data indicate that the Ca^{2+}-sensitizing action of TnT mutations independently contributes to the risk for VT induction. Consistent with this hypothesis, the nonsensitizing TnT-R278C mutation appears to carry a better prognosis of mild to moderate hypertrophy late in life (17, 18), although the clinical phenotype can be quite variable in different families, suggesting that modifier genes contribute importantly to the human phenotype (36).

We cannot exclude that in previous findings the previous histopathological examinations (16, 19, 20) missed subtle changes in myocyte architecture, which could have contributed to the high rate of VT in the transgenic hearts. For example, we cannot exclude that in a previous finding electrical activation from the discrete anatomical locations of transmural Purkinje tissue (37) was altered and may have contributed to the spatial CV dispersion in the transgenic mice. However, pharmacological Ca^{2+} sensitization with EMD reproduced the proarrhythmic effects of the Ca^{2+}-sensitizing TnT mutants. Furthermore, the increased arrhythmia risk conferred by the Ca^{2+}-sensitizing TnT mutants could be prevented by blebbistatin, which causes significant Ca^{2+} myofilament desensitization. Our results are in accordance with Dou et al. (23), who observed a decrease of 0.34 log units in the pCa_{50} of mouse cardiac skinned fibers treated with 10 μM of racemic blebbistatin. It remains to be determined whether blebbistatin is equally effective in reducing arrhythmia risk in hearts with anatomical lesions.

Mechanisms responsible for the AP changes and induction of VT. Ca^{2+}-sensitized hearts exhibited shorter ERP, increased beat-to-beat APD variability, and increased spatial CV dispersion, all of which have previously been shown to be proarrhythmic mechanisms (38–41). There are several possibilities to explain how the Ca^{2+} sensitization of myofilaments may increase spatial dispersion of conduction velocities. Likely possibilities include spatially discordant APDs and/or Ca^{2+} transients in an alternans (39) or other pattern. Unfortunately, neither can be readily measured with standard optical mapping approaches, since contractile uncoupling prevented the arrhythmias (Figure 7) and hence is not an option here. Nevertheless, our MAP data demonstrate that myofilament sensitization caused AP beat-to-beat instability and APD alternans in hearts of

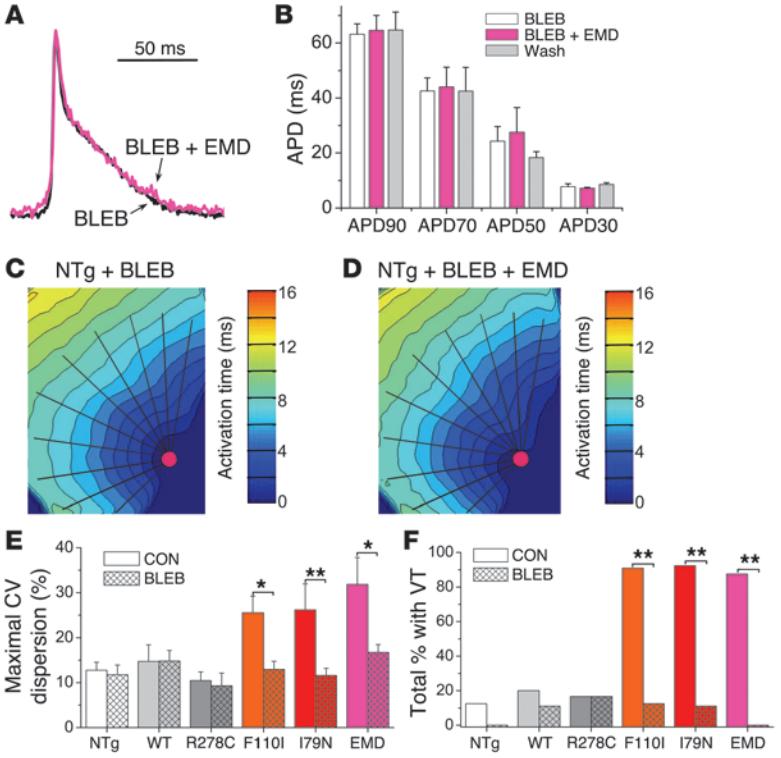

Figure 7

Blebbistatin prevents AP triangulation, normalizes CV dispersion, and prevents VT induction in Ca^{2+}-sensitized mouse hearts. (**A**) Optical AP records from an NTg heart perfused with solutions containing 3 μM blebbistatin before and after 3 μM EMD. Note that blebbistatin prevents the EMD-induced AP changes shown in Figure 5. (**B**) Average effect of EMD on murine ventricular APD in the presence of blebbistatin. *n* = 4 hearts; PCL, 150 ms. (**C** and **D**) Isochronal activation maps obtained at a PCL of 60 ms from an NTg heart perfused with solutions containing 3 μM blebbistatin before (**C**) and after (**D**) addition of 3 μM EMD to the perfusate. Isolated mouse hearts were stained with the voltage-sensitive fluorescent indicator di-4-ANEPPS and paced at stepwise-shorter PCLs as described in Figure 2. Note that in the presence of blebbistatin, EMD no longer altered ventricular isochronal activation maps. (**E**) Maximal CV dispersion before and after treatment with blebbistatin. For control conditions, the TnT mutations and the group size follow: NTg, *n* = 7; WT, *n* = 6; R278C, *n* = 6; F110I, *n* = 8; I79N, *n* = 8; EMD, *n* = 6. The group sizes of TnT mutations in the presence of 3 μM blebbistatin follow: NTg, *n* = 3; WT, *n* = 7; R278C, *n* = 5; F110I, *n* = 7; I79N, *n* = 7; EMD, *n* = 3; $^*P < 0.05$, $^{**}P < 0.01$ by Mann-Whitney *U* test. (**F**) Average incidence of sustained VT (>30 s duration) in the different transgenic mice under control conditions and in the presence of 3 μM blebbistatin. For control conditions, the TnT mutations and the group size follow: NTg, *n* = 8; WT, *n* = 10; R278C, *n* = 6; F110I, *n* = 11; I79N, *n* = 13; EMD, *n* = 7. The group sizes of TnT mutations in the presence of blebbistatin follow: NTg, *n* = 4; WT, *n* = 8; R278C, *n* = 5; F110I, *n* = 7; I79N, *n* = 8; EMD, *n* = 4; $^{**}P < 0.01$, by Fisher's exact test.

mice and cats. The APD alternans could be a direct consequence of the AP triangulation, resulting in a shallow terminal repolarization slope, which could lead to incomplete recovery of Na^+ channel at fast pacing rates. AP triangulation and beat-to-beat instability has been demonstrated as a predictor of proarrhythmia (40). Furthermore, the APD alternans may contribute to the regional slowing in CV observed in Ca^{2+}-sensitized hearts (41). While not directly measured here, increased myofilament Ca^{2+} sensitivity could cause AP triangulation and/or APD alternans by several mechanisms: (a) Increased Ca^{2+} binding to the Ca^{2+}-sensitized troponin complex (42) may contribute to decreased Ca^{2+} transients, with a slower decay rates that were responsible for the shorter APDs of I79N myocytes (26). Ca^{2+} transients with slower decay rates can cause Ca^{2+} transient alternans at fast heart rates (39, 43). (b) Independent of any changes in intracellular Ca^{2+} handling, increased end-diastolic pressure due to the impaired myocardial relaxation can also cause APD shortening (44). (c) Regional differences in ATP utilization due to the increased myofilament activation and reduced energy efficiency caused by the TnT mutations (45, 46) may cause regional differences in mitochondrial substrate utilization and activation of K_{ATP} channels. The extent to which these or other mechanisms are operational will have to be determined in future studies.

Myofilament Ca^{2+} sensitization in acquired heart disease. Substantial evidence exists for increased myofilament Ca^{2+} sensitivity in animals after myocardial infarction (13) and in humans with heart failure (14, 15). Both diseases are also characterized by a high incidence of VT and SCD. Interestingly, exercise training after myocardial infarction, which normalizes the increased myofilament Ca^{2+} sensitivity (47), also has been shown to reduce mortality and the rate of ventricular arrhythmias in animal (48, 49) and human studies (50). Further, treatment with the Ca^{2+} sensitizer levosimendan significantly increased the incidence of VT in patients with heart failure (51). Together with the experimental data reported here, these studies support what we believe to be the novel concept that myofilament Ca^{2+} sensitization renders hearts susceptible to ventricular tachyarrhythmias. The protective effect of blebbistatin raises the prospect of normalizing myofilament Ca^{2+} sensitivity as an antiarrhythmic approach in HCM and other disorders of altered myofilament Ca^{2+} sensitivity.

Methods

Animal model. The use of animals was approved by the Animal Care and Use Committees of Georgetown University, Vanderbilt University, and the University of Miami. A total of 240 age-matched (3–6 months) and sex-matched mice (bred in-house at Vanderbilt University and the University of Miami) and 4 adult female cats (Harlan Sprague Dawley Inc.) were used for the experiments. The generation of the transgenic mouse models used here has been reported previously (16, 19, 20). To model the autosomal-dominant human disease, we chose transgenic TnT lines (WT line 3, I79N line 8, F110I line 1, and R278C line 5) that have comparable TnT protein expression levels, whereby approximately 50% of the endogenous mouse TnT protein is replaced with WT or mutant human cardiac TnT (16, 19). NTg littermates from the same background mouse strain (BL6SJF1/J) were used as NTg controls wherever indicated. Hearts of transgenic mice were examined in blinded fashion by experienced cardiac pathologists and had no evidence of regional hypertrophy, fibrosis, and myofibrillar disarray as described in detail in our previous reports (16, 19, 20).

In vivo ECG studies. Surface ECG recordings were done under general anesthesia as described (26). ECGs were recorded for 5 minutes at baseline, followed 15 minutes after with intraperitoneal administration of isopro-

terenol (0.4 mg/kg), and ventricular ectopy was quantified as described (26). Average heart rates after isoproterenol injection were not significantly different among the groups of mice and ranged from 450 to 500 beats per minute, which was significantly lower than those of conscious mice (26), likely due to the effect of general anesthesia.

Isolated perfused heart studies. Mice were anesthetized with 20 ml/kg 2.5% tribromoethanol (Avertin) via intraperitoneal injection. After a surgical level of anesthesia was achieved, a thoracotomy was performed, the heart was removed, and the animal was euthanized by exsanguination. Hearts were perfused in the Langendorff mode as we described previously for mouse hearts (26). In brief, retrograde perfusion via the aorta was carried out at a constant perfusion pressure of 70 mmHg at 36°C. The flow of Thebesian veins was drained via a thin incision through the apex of the left ventricle. Krebs-Henseleit buffer containing 133 mM NaCl, 4.0 mM KCl, 1.5 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.0005 mM *S*-propranolol, and 10 mM glucose was prepared at the time of the experiment. Cats were sedated with 22 mg/kg ketamine and 0.2 mg/kg acepromazine via intramuscular injection. Heparin (500 IU) and 25 mg/kg phenobarbital was applied through an intravenous access. When a surgical level of anaesthesia was reached, a median sternotomy was performed, and the hearts were isolated and perfused as described above, with a perfusion pressure of 90 ± 10 mmHg.

To maintain a constant heart rate, the atrioventricular node was thermally ablated, and the hearts were paced at different pacing rates via a unipolar platinum electrode placed on the epicardium near the apex of the left ventricle. Pacing current was maintained at 2 times threshold, and volume conducted ECGs were recorded continuously throughout the experiment as previously described (26). For the recording of MAP signals in mice, we used a custom-build miniature silver/silver chloride catheter, which we previously have validated against conventional microelectrode recordings (27). For cat hearts, we used a commercially available standard MAP catheter (EP Technologies Inc.). To test acute myofilament Ca^{2+} sensitization in WT hearts, we used the Ca^{2+}-sensitizing compound EMD 57033, which has strongly specific myofilament Ca^{2+}-sensitizing properties (21, 22). Phosphodiesterase inhibition, a common effect among sensitizing agents, is not observed at the concentration (3 μM) used here (22). EMD reverses the inhibition of actin-myosin interactions by troponin-tropomyosin and also promotes transition of crossbridges from weak to strong force-generating states (21). There is also evidence that EMD binds to the C-lobe of troponin C in a region that interacts with TnI as well as TnT (52). Thus, EMD acts by enhancing thick filament and thin filament interaction. This makes EMD attractive to model the effect of Ca^{2+}-sensitizing mutations in TnT or TnI. EMD 57033 was a gift of Merck KGaA, and all other chemicals were purchased from Sigma-Aldrich.

Optical mapping studies. After staining with 10–15 μl of di-4-ANEPPS stock solution (0.5 mg/ml dimethyl sulfoxide) slowly injected through a port on the bubble trap above the perfusion cannula, hearts were illuminated using a coherent diode-pumped, solid-state Coherent Verdi laser (532 nm). The fluorescence emitted from the heart was collected with a RedShirt charge-coupled device camera (14-bit, 80 × 80 pixels, 1,000 fps, CardioCCD-SMQ; RedShirt Imaging), equipped with a 52-mm standard lens in combination with a magnifying lens (+4; Tiffen), and passed through a cutoff filter (No. 25 Red, 607 nm; Tiffen). The magnification was adjusted to result in an imaging area of approximately 9 × 9 mm^2. Custom-developed "C"-based software controlled data acquisition, external stimulation, and laser illumination as described by us (53). Optical data were recorded at frame rates of 1,000 per second for periods of 2–3 seconds. For each experiment, the heart was paced from an epicardial stimulation side near the apex for at least 90 seconds at constant cycle lengths prior to the optical recordings. APs were computed from inverted fluorescence data corresponding to a single pixel. The location of the pixel was the center of the left ventricle. Conduction velocities of the propagating wave were calculated from isochrone maps along 10 equally spaced radial lines distributed along the cross section of the initial stimulus activation. The activation time, and therefore the isochrones, were defined as the time of maximum upstroke velocity of the filtered, averaged, and inverted fluorescence signal relative to the time of the stimulus. A temporal filter, with a running average over a period of 6 frames and a rotationally symmetric Gaussian low-pass filter with a SD of 1 applied to a 5 × 5 matrix, was used to filter the time averaged fluorescence data. The dispersion in CV was defined as the SD of the 10 CV measurements across the heart.

Measurement of the Ca^{2+} sensitivity of force development and maximal force in mouse skinned cardiac fibers. Skinned papillary muscles were prepared from the left ventricle of freshly obtained mouse hearts as previously described (19, 24). Small bundles of fibers were isolated and placed in a pCa 8.0 relaxing solution (10^{-8} M $[Ca^{2+}]_{free}$, 1 mM $[Mg^{2+}]_{free}$, 7 mM EGTA, 2.5 mM MgATP, 20 mM MOPS [pH 7.0], 20 mM creatine phosphate, and 15 U/ml creatine phosphokinase, ionic strength [*I*] = 150 mM) containing 50% glycerol at 4°C for approximately 4–6 hours. Fibers were then transferred and stored at –20°C for up to 4 days. Mouse muscle fiber bundles with a diameter varying between 75 and 125 μm and an average length of 1.3 mm were attached to tweezer clips connected to a force transducer. To ensure complete membrane removal and complete access to the myofilament, the fibers were treated with pCa 8.0 containing 1% Triton X-100 for 30 minutes before the beginning of the experiment. To remove the excess Triton X-100 from the fibers, extensive washing was carried out with pCa 8.0, and then the Ca^{2+} sensitivity of force development was evaluated. To determine the Ca^{2+} sensitivity of force development, the fibers were gradually exposed to solutions with increasing Ca^{2+} concentrations (pCa 8.0–4.0). Data were fitted using the following equation: % change in force = $100 \times [Ca^{2+}]^n/([Ca^{2+}]^n + [Ca^{2+}_{50}]^n)$, where $[Ca^{2+}_{50}]$ is the free $[Ca^{2+}]$ that produces 50% force and *n* is the Hill coefficient. The various pCa solutions were calculated using the computer program (pCa Calculator), recently developed in our laboratory (54). EMD and the (–/–) isomer of blebbistatin were dissolved in DMSO (100%) at a concentration of 30 mM. The stock concentration was adjusted with DMSO for each experimental drug concentration to achieve a final DMSO concentration of 0.05% in all experiments. All fiber experiments and handling of blebbistatin were carried out in a dark room using a dimmed red light. For each mounted fiber, several Ca^{2+} sensitivity curves were run: native (in the absence of DMSO and blebbistatin), control (in the presence of 0.05% DMSO), and experimental (in the presence of blebbistatin and/or EMD). Before running the control and experimental curves, fibers were incubated in pCa 8.0 in the presence of DMSO or blebbistatin for 20 minutes. To confirm the blebbistatin effect, after the experiment the fiber was exposed to white light for 30 minutes and the maximal force was recovered at pCa 4.0. Phosphorylation experiments were carried out by incubation the cardiac skinned fibers with 500 U/ml of protein kinase A catalytic subunit (bovine heart catalytic subunit; Sigma-Aldrich) in pCa 8.0 solution for 30 minutes at room temperature.

Statistics. All experiments were done in random sequence with respect to the genotype, and measurements were taken by a single observer who was blinded to the genotype. Differences between groups were assessed using ANOVA (for normally-distributed parameters) or by Kruskal-Wallis test (for incidence of PVCs). If statistically significant differences were found, individual groups were compared with Student's *t* tests or by nonparametric tests as indicated in the text. Results were considered statistically significant if the *P* value was less than 0.05. Unless otherwise indicated, results are expressed as arithmetic mean ± SEM.

Acknowledgments

This work was supported in part by NIH grants HL88635, HL71670, and HL46681 (to B.C. Knollmann), HL88635-S1 (to F. Hilliard), HL67415 and HL42325 (to J.D. Potter), HL62426 and HL64035 (to R.J. Solaro) and by American Heart Association Established Investigator Award 840071N (to B.C. Knollmann), Scientist Development Grant 635037N (to V.Y. Sidorov), and Postdoctoral Award 0825368E (to J.R. Pinto). We thank Tao Yang, Jiang-Sheng Liang, and Silviu Diaconu for their valuable technical assistance.

Received for publication July 1, 2008, and accepted in revised form September 10, 2008.

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