Ranolazine Prevents Phenotype Development in a Mouse Model of Hypertrophic Cardiomyopathy

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Background—Current therapies are ineffective in preventing the development of cardiac phenotype in young carriers of mutations associated with hypertrophic cardiomyopathy (HCM). Ranolazine, a late Na+ current blocker, reduced the electromechanical dysfunction of human HCM myocardium in vitro.

Methods and Results—To test whether long-term treatment prevents cardiomyopathy in vivo, transgenic mice harboring the R92Q troponin-T mutation and wild-type littermates received an oral lifelong treatment with ranolazine and were compared with age-matched vehicle-treated animals. In 12-months-old male R92Q mice, ranolazine at therapeutic plasma concentrations prevented the development of HCM-related cardiac phenotype, including thickening of the interventricular septum, left ventricular volume reduction, left ventricular hypercontractility, diastolic dysfunction, left-atrial enlargement and left ventricular fibrosis, as evaluated in vivo using echocardiography and magnetic resonance. Left ventricular cardiomyocytes from vehicle-treated R92Q mice showed marked excitation–contraction coupling abnormalities, including increased diastolic [Ca2+] and Ca2+ waves, whereas cells from treated mutants were indistinguishable from those from wild-type mice. Intact trabeculae from vehicle-treated mutants displayed inotropic insufficiency, increased diastolic tension, and premature contractions; ranolazine treatment counteracted the development of myocardial mechanical abnormalities. In mutant myocytes, ranolazine inhibited the enhanced late Na+ current and reduced intracellular [Na+] and diastolic [Ca2+], ultimately preventing the pathological increase of calmodulin kinase activity in treated mice.

Conclusions—Owing to the sustained reduction of intracellular Ca2+ and calmodulin kinase activity, ranolazine prevented the development of morphological and functional cardiac phenotype in mice carrying a clinically relevant HCM-related mutation. Pharmacological inhibitors of late Na+ current are promising candidates for an early preventive therapy in young phenotype-negative subjects carrying high-risk HCM-related mutations. (Circ Heart Fail. 2017;10:e003565. DOI: 10.1161/CIRCHEARTFAILURE.116.003565.)

Key Words: arrhythmias ■ cardiomyocyte ■ calcium ■ drug therapy ■ prevention ■ remodeling ■ sodium

Hypertrophic cardiomyopathy (HCM), despite being the most common inherited cardiac disease, currently does not have a disease-modifying therapeutic option.1 Beta blockers, Ca2+ channel blockers, and disopyramide are commonly used to reduce symptoms associated with left ventricular (LV) outflow tract obstruction, diastolic dysfunction, and arrhythmias in HCM.2 However, their long-term effectiveness in slowing disease progression and improving patient outcomes remains unclear and is likely to be marginal.3

See Clinical Perspective

Extensive family screening programs are identifying increasing numbers of young individuals who carry HCM-associated mutations with no or minimal phenotype and normal cardiac function.2 Although genotype–phenotype correlations in individual patients with HCM remain unreliable in predicting outcome, growing evidence suggests that certain genes or variants may be associated with adverse outcome and higher likelihood of disease progression.4 A case in point is represented by patients with thin-filament mutations, who show an increased prevalence of sudden cardiac death in the pediatric age range5 and, later in life, a higher risk of...
progression toward end-stage heart failure, when compared with the more common thick-filament mutation carriers.

To date, however, when a young individual is diagnosed with having a high-risk HCM-associated mutation, clinicians can only suggest a close follow-up strategy, for lack of robust therapeutic options capable of delaying or preventing disease onset and progression. Although gene therapy still seems distant and likely reserved to selected genotypes, an attractive alternative is represented by disease-modifying drugs interfering with the subcellular and cellular mechanisms of myocardial adverse remodeling. A growing number of preclinical studies have identified changes in intracellular Ca\(^{2+}\) handling as fundamental determinants of electromechanical dysfunction in HCM myocardium, both in transgenic animal models and in human ventricular cardiomyocytes. Indeed, elevated intracellular Ca\(^{2+}\) levels are associated with increased activation of cardiac Ca\(^{2+}\)--calmodulin-dependent protein kinase (CaMKII), a key player of adverse cellular remodeling in human HCM. HCM-related abnormalities of Ca\(^{2+}\) homeostasis seem to occur independently of the underlying mutation, thus, representing an ideal universal target.

Of note, previous studies pointed out that long-term reduction of intracellular Ca\(^{2+}\) overload with the Ca\(^{2+}\) channel blocker diltiazem is capable of delaying and mitigating phenotype expression in HCM mouse models. Following these observations, a pilot clinical study has been performed in which nonhypertrophic mutation carriers exhibiting initial diastolic abnormalities were treated with diltiazem. The results of the study revealed that early treatment with diltiazem does delay the development of diastolic dysfunction in carriers. However, the study duration was too short to measure a significant effect on the development of hypertrophy. Furthermore, diltiazem is not the ideal drug in young asymptomatic patients because of potential occurrence of bradycardia, hypotension, and exercise intolerance. Nevertheless, these observations are consistent with the idea that an early and sustained reduction in intracellular Ca\(^{2+}\) may slow the natural progression of disease phenotype.

An alternative therapeutic option to reduce intracellular Ca\(^{2+}\) overload in HCM is pharmacological inhibition of the late Na\(^+\) current (I\(_{\text{NaL}}\)) using the clinically available, prototypical inhibitor ranolazine. I\(_{\text{NaL}}\) is pathologically increased in human HCM, causing intracellular Ca\(^{2+}\) accumulation. Studies performed on human HCM cardiomyocytes showed that after I\(_{\text{NaL}}\) inhibition, reduction of action potential (AP) duration (APD) leads to decreased Ca\(^{2+}\) entry, while normalization of intracellular Na\(^+\) potentiates Ca\(^{2+}\) extrusion from the cytosol via the Na--Ca exchanger (NCX), ultimately causing a reduction of both [Ca\(^{2+}\)] and CaMKII activity.

Based on these findings, we sought to determine whether I\(_{\text{NaL}}\) inhibition with ranolazine affects long-term disease progression in HCM. Toward this goal, transgenic mice carrying the HCM-related R92Q mutation in the troponin-T (TnT) gene were subjected to a lifelong oral treatment with ranolazine and 0.03% ketoconazole (RAN group) or ketoconazole only (KET group), starting 1 to 2 days after birth. Treatment was continued till 11 to 12 months of age, when mice were used for the following experiments. Ranolazine plasma concentration at the time of killing was measured with liquid chromatography analysis coupled with tandem mass spectrometry. Echocardiography was performed on anesthetized mice as previously described to characterize LV morphology and systolic and diastolic function. Cardiac magnetic resonance was performed as previously described to assess LV and right ventricular volumes and mass, as well as to calculate the fraction of extracellular space by studying the decay of contrast signal after intravenous gadolinium compound infusion. Single cardiomyocytes were isolated from excised hearts via enzymatic dissociation and used for intracellular Ca\(^{2+}\) measurements using Ca-sensitive fluorescent dyes to evaluate the amplitude and kinetics of Ca\(^{2+}\) transients, diastolic [Ca\(^{2+}\)], and the rate of arhythmic spontaneous Ca\(^{2+}\) release during stimulation with field electrodes. In a subset of experiments, simultaneous detection of intracellular [Na\(^+\)] and [Ca\(^{2+}\)] was performed after staining single cells with Fura-2 and Natrium Green-2. Patch-clamp studies were performed to record APs and I\(_{\text{NaL}}\) in single LV cardiomyocytes. To assess T-tubule density, myocytes were stained with membrane-selective dyes and observed with a confocal microscope. Formalin-fixed LV slices were stained with picrosirius red and used to assess intramyocardial fibrosis. Fast-frozen LV myocardial samples were processed to obtain total proteins, which were used for Western blot studies to assess the expression and phosphorylation of CaMKII and other proteins involved in excitation–contraction coupling. LV and right ventricular intact trabeculae were dissected from explanted hearts and used to record isometric force during electric stimulation with different pacing protocols, aimed at evaluating twitch amplitude and kinetics, response to high-frequency pacing, prolonged pauses, as well as the effects of β-adrenergic activation. Skinned trabeculae were used to obtain force–pCa curves by transferring them manually between baths containing different pCa solutions, as previously described. Data from cells and muscles are expressed as mean±SEM (number of samples and animals are indicated in the respective figure legends). Statistical analysis was performed as previously described using SPSS 23.0 (IBM) and STATA 12.0 (StataCorp). In brief, all sets of variables were checked for normality (Shapiro–Wilks test) and for homogeneity of variances among groups (Levene’s Test). The statistical tests used to calculate P values for each data set are indicated with abbreviations in the respective figure legends. For variables where a single measurement for each mouse is included (eg, echocardiography, Western blot), the 3 different groups were compared using (1) 1-way analysis of variance with Tukey correction (for normally distributed homoscedastic data sets), (2) Kruskal–Wallis test with Dunn’s multiple comparison test (for non-Gaussian data sets) or (3) Welch’s analysis of variance with Games–Howell test (for heteroscedastic groups). For variables where measurements from an unequal number of different samples (eg, cells or trabeculae) from each mouse are included (pathock, ion fluorescence, isometric force data), we used linear mixed models to compare data groups to account for intra-subject correlation within mice. Comparisons of 2 groups, the Tukey–Kramer post hoc method was used to compute P values for all pairwise comparisons. P<0.05 was considered statistically significant.

Results

Rationale for Using Ranolazine in R92Q Mice

We first sought to determine whether and how acute ranolazine administration exerts beneficial effects in the myocardium of...
R92Q mutant mice. LV cardiomyocytes were isolated from the hearts of R92Q and WT male mice aged 4 to 6 months and used for patch clamp experiments to record \( I_{\text{NaL}} \) and APs in the absence and presence of 10 \( \mu \text{M} \) ranolazine. \( I_{\text{NaL}} \) density was 2-fold higher in cardiomyocytes from R92Q as compared with those from WT cells. In mutant cardiomyocytes, ranolazine reduced \( I_{\text{NaL}} \) down to the level of that in WT cells (Figure 1A and 1B). Accordingly, ranolazine administration led to a reduction of APD in R92Q cardiomyocytes at all investigated frequencies, while it did not affect APD in WT cells (Figure 1C and 1D). Interestingly, \( I_{\text{NaL}} \) was already increased in cardiomyocytes from R92Q mice at 1 month of age, as compared with cells from age-matched WT mice (Figure 1A in the Data Supplement), suggesting that the increase of \( I_{\text{NaL}} \) occurs early during disease development in this model. In line with previous reports,24 ranolazine (10 \( \mu \text{M} \)) shows use-dependent block of \( I_{\text{NaL}} \) with no significant effects on peak Na\(^+\) current (Figure IB and IC in the Data Supplement).

We then studied the effects of \( I_{\text{NaL}} \) inhibition on intracellular \([\text{Na}^+]\) and \([\text{Ca}^{2+}]\) by simultaneously monitoring the concentration of the 2 ions in R92Q and WT cardiomyocytes costained with the ion-sensitive fluorescent dyes Asante Natrium Green and Fura-2 (for \([\text{Na}^+]\) and \([\text{Ca}^{2+}]\), respectively). Cardiomyocytes were field-stimulated at increasing rates (1–7 Hz) in the absence and presence of ranolazine. Both intracellular \([\text{Na}^+]\) and \([\text{Ca}^{2+}]\) were higher in R92Q myocytes as compared with those in WT cells. In agreement with the observed \( I_{\text{NaL}} \) inhibition, ranolazine significantly reduced diastolic \([\text{Na}^+]\) and \([\text{Ca}^{2+}]\) in mutant cells, especially at high frequencies of stimulation (Figure 1E and 1F), while the amplitude of \([\text{Ca}^{2+}]\) transients was slightly increased (Figure ID in the Data Supplement). Contrarily, the intracellular concentration of the 2 ions was only mildly affected by the drug in WT cells (Figure 1E and 1F; Figure ID in the Data Supplement). Interestingly, only untreated R92Q cardiomyocytes show a linear correlation between intracellular \([\text{Na}^+]\) and \([\text{Ca}^{2+}]\) (Figure IE in the Data Supplement); ranolazine abolishes the correlation, suggesting that \( I_{\text{NaL}} \) inhibition is able to fully counter the pathological increase of intracellular \([\text{Ca}^{2+}]\) that depends on \([\text{Na}^+]\) overload. The remaining excess of \([\text{Ca}^{2+}]\) in R92Q cells after \( I_{\text{NaL}} \) inhibition likely depends on \([\text{Na}^+]\)-unrelated mechanisms and is unaffected by the drug at this stage. Of note, all the observed effects of ranolazine on \( I_{\text{NaL}} \), intracellular \([\text{Ca}^{2+}]\), and \([\text{Na}^+]\) were lost after 2 minutes of drug washout (Figure IF through IH in the Data Supplement).

Long-Term Treatment Protocol

To test whether ranolazine is effective in slowing or preventing the development of the pathological phenotype in mutant mice, male animals carrying the R92Q \( \text{TnT} \) mutation and WT littermates received an oral treatment with ranolazine since birth and were compared with age-matched vehicle-treated siblings. A detailed scheme of the experimental protocol is shown in Figure II in the Data Supplement. Male R92Q animals were fed with chow containing only 0.5\% ranolazine+0.03\% ketoconazole (RAN treatment). Ketoconazole was added to inhibit hepatic CYP3A4 to maintain ranolazine plasma concentration within the range of 2 to 8 \( \mu \text{mol/L} \) throughout the day, values comparable to therapeutic levels in humans (Figure III in the Data Supplement). RAN-treated animals were compared with age-matched R92Q animals fed with chow containing only 0.03\% ketoconazole (KET treatment). R92Q heterozygous mice were mated with WT females to obtain mixed litters. Treatment was started immediately after birth; each litter was assigned randomly to RAN or KET treatment group. Survival analysis did not show any difference among WT-KET, WT-RAN, R92Q-KET, and R92Q-RAN groups: the number of animal who died during the 12 months of treatment was low and comparable in the 4 groups (WT-KET=1/23, WT-RAN=1/22; R92Q-KET=2/22; R92Q-RAN=2/23; P>0.05 at \( \chi^2 \) test for all comparisons). No differences were noted between WT-KET and WT-RAN mice in terms of gross cardiac structure and function as evaluated with a specific subset of in vivo assessments (Table I in the Data Supplement). Therefore, WT-RAN mice were used as a single control group throughout the study (henceforth named WT). All animals were treated for 12 months and then used in the experiments described below. Average ranolazine concentration measured in plasma at killing was 4.41±1.01 \( \mu \text{mol/L} \) (mean±SE from 15 animals).

Echocardiography

Before echo assessments, heart rate and arterial blood pressure were measured in conscious mice: no differences were noted in the basal vital parameters among the 3 groups of animals (Table II in the Data Supplement). Echocardiographic acquisitions were performed in anesthetized 12-month-old male mice from the 3 study groups (11 WT, 11 R92Q-KET, and 11 R92Q-RAN) using a standardized protocol25; measurements were performed offline in a blind manner.

Lifelong ranolazine treatment fully prevented the development of structural LV changes in R92Q mutation carrier mice (Figure 2). The increase in interventricular septum thickness, a hallmark of HCM remodeling, was not present in ranolazine-treated mice, as evaluated from long-axis parasternal views (Figure 2A and 2C). Three short-axis views (Figure 2B) at different levels of the LV were used to estimate chamber volumes. A reduction in end-diastolic and end-systolic LV volumes, although present in all vehicle-treated mutants, was entirely prevented in R92Q-RAN mice (Figure 2D). LV hypercontractility was documented in R92Q-KET animals by an increased ejection fraction (LV ejection fraction) and an augmented fractional increase of septal thickness during contraction, as compared with WT. Remarkably, LV hypercontractility was absent in ranolazine-treated mice (Figure 2E).

No differences were noted in terms of stroke volume, cardiac output, and heart rate among the 3 groups of animals (Table III in the Data Supplement).

Doppler studies of transmural blood flow (Figure 3A) and measurements of left atrial (LA) dimensions (Figure 3C) were performed in 4-chamber views to assess diastolic function. Reduced transmural blood flow during early diastole (ie, reduced E wave amplitude) paralleled by an increased proportion of LV diastolic filling relying on atrial contraction (ie, increased A wave amplitude) is found to be consistent with impaired ventricular relaxation. LA size may increase because of elevated LV filling pressure. E/A ratio was markedly lower,
Figure 1. Acute effects of ranolazine in cardiomyocytes from R92Q-TnT mice. A, Representative superimposed traces of late Na⁺ current from wild-type (WT; left) and R92Q cardiomyocytes (right) in the absence and presence of 10 μM ranolazine. B, Average $I_{\text{Na,L}}$ density integrals, calculated from 25 to 125 ms after the onset of a 500 ms depolarization step from $-120$ to $-10$ mV. Means±SEM from 14 WT (3 mice) and 15 R92Q (3 mice) cardiomyocytes. C, Representative superimposed action potential traces from WT (left) and R92Q cardiomyocytes (right) elicited at 1 Hz in the absence and presence of 10 μM ranolazine. D, Average action potential duration at 90% of repolarization (APD₉₀) at 1 Hz stimulation rate. Means±SEM from 21 WT cells (6 mice) and 17 R92Q cells (5 mice). E, Representative simultaneous fluorescence recordings of intracellular [Ca²⁺] (Fura-2, top) and [Na⁺] (Natrium Green-2, bottom) from a WT cell (left), a R92Q cardiomyocyte (center), and the same diseased cell in the presence of 10 μM ranolazine (right). Cells were field stimulated at 1, 3, 5, and 7 Hz for $>45$ s at each frequency. F, Average intracellular diastolic [Ca²⁺] at the different frequencies of stimulation. G, Average intracellular diastolic [Na⁺]. F and G, Means±SEM from 36 WT cells (3 mice) and 27 R92Q cardiomyocytes (3 mice). A–G, Black, WT cells at baseline; gray, WT with ranolazine; red, R92Q cells at baseline; violet, R92Q cells with ranolazine. *P<0.05, **P<0.01, with linear mixed model (LMM). #P<0.05, ##P<0.01, with LMM for paired measurements.
and LA size increased in R92Q-KET mice compared with WT mice (Figure 3B and 3D). Ranolazine treatment partially prevented the impairment of diastolic function: in R92Q-RAN mice, LA dimensions were fully normalized (Figure 3D), and E/A ratio was significantly higher than that in R92Q-KET mice, though it was still lower than that in WT mice (Figure 3B).

Increased productions of ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide) from atrial and ventricular tissue are markers of the increased chamber filling pressures in the setting of diastolic dysfunction. Ranolazine treatment counteracted the increase in mRNA expression of both ANP- and BNP-coding genes (Table IV in the Data Supplement), in line with the prevention of diastolic abnormalities.

Cardiac Magnetic Resonance
A total of 8 WT, 8 R92Q-KET, and 8 R92Q-RAN mice underwent cardiac magnetic resonance tests performed using a 7-Tesla scanner (Bruker Pharmascan) optimized for small

Figure 2. Echocardiographic measurements. A, Representative parasternal long-axis views of the left ventricle at end diastole from wild-type (WT), R92Q-KET, and R92Q-RAN mice. Vertical bars mark the thickness of the interventricular septum. The length of horizontal bars equal 1 mm. B, Representative short-axis views (mid-left ventricle) at end diastole (left) and end systole (right) from mice belonging to the 3 study groups. Horizontal bars equal 1 mm. C, Thickness of the interventricular septum (IVS) measured at end diastole (left) and at end systole (right) in WT, R92Q-KET, and R92Q-RAN mice. D, Left ventricular volumes calculated using the Simpson technique at end diastole (EDV; left) and end systole (ESV; right) in mice from the 3 study groups. E, left ventricular ejection fraction (LV-EF; left) and IVS systolic thickening (right), expressed as percentage of the diastolic value, measured in mice from the 3 cohorts. C–E, Means±SEM from 11 mice per group. *P<0.05; **P<0.01; NS, P>0.05; statistical test: 1-way analysis of variance with Tukey correction. R92Q-KET indicates R92Q mutant mice fed with chow containing ketoconazole only; and R92Q-RAN, R92Q mutant mice fed with chow containing ranolazine and ketoconazole.
animals following a standardized protocol to measure chamber volumes and wall mass from sequential short-axis sections (Figure 4A). Consistent with the echocardiographic data, ranolazine prevented the reduction of LV end-diastolic volume and the increase of septal wall thickness and LV ejection fraction, which were observed in all untreated mutant mice (Figure 4B). Total LV wall mass was unchanged in R92Q-KET versus WT mice and was unaffected by ranolazine treatment (Figure 4B).

A gadolinium compound was administered intravenously, and the decay of gadolinium contrast intensity in LV myocardium was used to estimate the fraction of extracellular space as an index of the interstitial fibrosis (Figure 4C). Ranolazine treatment significantly prevented the pathological expansion of extracellular volume in R92Q mutant mice (Figure 4D).

Histology and Confocal Imaging
Formalin-fixed LV samples were embedded in paraffin, and thin sections were stained with picrosirius red to highlight myocardial collagen fibers (Figure 5A). Analysis of stained sections from 5 animals from each of the 3 groups confirmed that lifelong ranolazine treatment counteracted the increase of myocardial collagen in R92Q mice (Figure 5B), in line with contrast magnetic resonance imaging data (Figure 4D). Myocardial fibrosis in cardiac diseases is subtended by increased local synthesis of transforming growth factor β. Consistent with histological and magnetic resonance imaging data, ranolazine treatment prevented the increase in transforming growth factor β expression seen in R92Q mutants (Table IV in the Data Supplement).

Freshly isolated mouse LV cardiomyocytes were stained with membrane-selective fluorescent dye di-3-aneppdhq (Life Technologies) and were then observed with a confocal microscope (Figure 5C) to assess the structure and organization of the T-tubular system. Ranolazine treatment partially prevented the reduction of the density and the impairment of the organization of T-tubules (TTPower parameter; Figure 5D) that were much more marked in cells from untreated R92Q mice. Of note, resting sarcomere length, which was significantly reduced in untreated R92Q cardiomyocytes, was the same in R92Q-RAN and WT cells (Figure 5E).

Intracellular Ca\(^{2+}\) Handling in Single Cardiomyocytes
All experiments in cells and trabeculae were performed using experimental solutions devoid of ranolazine. Intracellular Ca\(^{2+}\) measurements were performed in isolated cardiomyocytes during electric field stimulation at 3 different frequencies (1, 3, and 5 Hz). Figure 6 shows that ranolazine treatment prevented the alterations of Ca\(^{2+}\) handling...
observed in cardiomyocytes from mutant mice. Specifically, in R92Q-RAN cardiomyocytes, the increase in diastolic intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_i]) and its frequency-dependent rise observed in untreated mutant mice were markedly decreased (Figure 6A and 6B). Additionally, ranolazine treatment fully prevented the slowing of the kinetics of Ca\(^{2+}\)-transient rise and decay (Figure 6A and 6C) and counteracted the reduction of Ca\(^{2+}\)-transient amplitude (Figure 6A and 6D). In keeping with these observations, the reduction of Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase type 2a (SERCA2a) protein and mRNA-expression of ATP2a2 gene, found in R92Q-KET hearts, was abolished in R92Q-RAN hearts (Table IV in the Data Supplement); this is likely to have contributed to the faster Ca\(^{2+}\) transient kinetics observed in R92Q-RAN versus R92Q-KET cardiomyocytes.

Cardiomyocytes were rapidly exposed to 10 mmol/L caffeine after 30 s of conditioning stimulation at 1 Hz (Figure 6E) to estimate total sarcoplasmic reticulum Ca\(^{2+}\) content and study the rate of Ca\(^{2+}\) extrusion from the cytosol via the NCX. The amplitude of caffeine-induced Ca\(^{2+}\) transients was similar in WT and R92Q-KET cardiomyocytes and was not modified by treatment with ranolazine (Figure 6E), suggesting that no major changes in sarcoplasmic reticulum Ca\(^{2+}\) content occur in this disease model at 12 months of age. However, the drug prevented the slowing of the decay rate of caffeine-induced Ca\(^{2+}\) transients, suggesting that the rate of Ca\(^{2+}\) extrusion via the NCX is normalized in R92Q-RAN cardiomyocytes (Figure 6F). Interestingly, the mRNA expression of the NCX gene was similar in the 3 study groups (Table IV in the Data Supplement).
Western Blot Studies

Fast-frozen LV myocardial samples from R92Q, R92Q-RAN, and WT animals (8 per group) were processed to obtain total proteins, which were used for Western blot studies (Figure 6G and 6H), as previously described. CaMKII autophosphorylation, a marker of CaMKII activation, was increased in R92Q hearts as compared with that in WT mice. In ranolazine-treated R92Q mice, instead, the increase of CaMKII autophosphorylation was fully prevented. In agreement with this observation, ranolazine treatment prevented the increase of the phosphorylation of specific CaMKII target site on phospholamban. Additionally, ranolazine treatment prevented the reduction of total SERCA and phospholamban (PLB) proteins in R92Q mutant hearts. Phosphorylation of protein kinase A targets was similar in WT and R92Q hearts and remained unchanged by treatment (Figure IV in the Data Supplement). Finally, ryanodine receptor’s total protein amount and phosphorylation levels were found to be similar in the 3 groups (Figure IV in the Data Supplement).

Arrhythmogenic Ca\(^{2+}\) Release

We then assessed the rate of intracellular Ca\(^{2+}\) waves and spontaneous Ca\(^{2+}\) transients occurring during pauses. Toward this goal, cardiomyocytes were stimulated at 5 Hz, and pacing was abruptly stopped for 20 s, in the absence and presence of isoproterenol 10\(^{-7}\) mol/L (Figure 7A). Treatment with...
Figure 6. Intracellular calcium in isolated cardiomyocytes and Western blot studies. A, Representative superimposed Ca\textsuperscript{2+} transients from R92-KET and R92Q-RAN myocytes elicited at 1 Hz (left), 3 Hz (middle), and 5 Hz (right). B, Diastolic Ca\textsuperscript{2+} levels, expressed as arbitrary units of fluorescence intensity, during steady state stimulation at different frequencies in cells from mice of the 3 cohorts. C, Time from stimulus to peak (peak time), time from peak to 50% decay (50% decay), and 90% decay of Ca\textsuperscript{2+} transients elicited at 1 Hz in cardiomyocytes from WT, R92Q-KET, and R92Q-RAN mouse hearts. D, Amplitude of Ca\textsuperscript{2+} transients in cardiomyocytes from the 3 study groups, elicited at stimulation rates of 1, 3, and 5 Hz. B–D, Means ± SE from 125 WT (7 mice), 180 R92Q-KET (8 mice), and 378 R92Q-RAN (10 mice) cardiomyocytes. # \(P < 0.01\) for R92Q-KET vs WT and R92Q-RAN vs R92Q-KET at all frequencies; § \(P < 0.01\) at 1 Hz, \(P < 0.05\) at 3 Hz.

E, Left, Superimposed representative fluorescence traces obtained during rapid exposure to 20 mmol/L caffeine after regular stimulation at 1 Hz, in WT, R92Q-KET, and R92Q-RAN cardiomyocytes. Right, Amplitude of caffeine-induced Ca\textsuperscript{2+} transients in mice from the 3 study groups. F, Kinetics of caffeine-induced Ca\textsuperscript{2+} transients (time from peak to 50% and 80% decay) measured from cardiomyocytes of the 3 study groups. E and F, Means ± SE from 91 WT (4 mice), 90 R92Q-KET (4 mice), and 88 R92Q-RAN (4 mice) cardiomyocytes. G, Representative Western blots for total Ca\textsuperscript{2+}-calmodulin-dependent protein kinase (CaMKI\(\delta\)), phospho-CaMKI\(\delta\) at tyrosine 287, total SERCA2, total phospholamban (PLB), phospho-PLB at tyrosine 17 (CaMKI\(\delta\) site), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). H, Average values of WT, R92Q-KET, and R92Q-RAN cardiomyocytes. Relative intensity of individual bands was quantitated and normalized to GAPDH. The ratio for control was assigned a value of 1. CAMK=CaMKI\(\delta\) B–D, F and H, \(*P < 0.05; **P < 0.01; NS, P > 0.05\); statistical tests: Linear mixed models with Tukey-Kramer correction for heteroscedastic groups in B; LMMT in D–F; Welch’s analysis of variance (WA) in H.
ranolazine counteracted the increase of spontaneous Ca\(^{2+}\) release seen in untreated R92Q cardiomyocytes. Indeed, the frequencies of spontaneous Ca\(^{2+}\) waves and Ca\(^{2+}\) transients in cells from R92Q-RAN mice were markedly reduced compared with those from R92Q-KET mice, both at baseline and during β-adrenergic stimulation (Figure 7A and 7B), that is, they were in the range of those observed in cardiomyocytes from WT mice.

Mechanical Function in Ventricular Trabeculae

Isometric force was measured from LV and right ventricular trabeculae or thin papillary muscles during field stimulation with different pacing protocols (Figure 8A and 8F). The response to stimulation frequencies (0.1–6 Hz) and the effects of β-adrenergic stimulation were evaluated, as well as the occurrence of spontaneous contractions during prolonged pauses (Figure 8B through 8G). Ranolazine treatment restored the positive inotropic responses that were markedly blunted in R92Q myocardium. In R92Q-KET trabeculae, we observed a significant reduction of twitch tension compared with that in WT, both at fast pacing rates (≥3 Hz) and in response to isoproterenol, while the positive inotropic effects in response to fast stimulation and isoproterenol in R92Q-RAN muscles were similar to those of WT (Figure 8B and 8C). Ranolazine treatment markedly mitigated the frequency-dependent increase of diastolic tension in R92Q trabeculae (Figure 8D). Moreover, the kinetics of contraction and relaxation at low pacing frequencies (0.1–1 Hz) were faster in R92Q-RAN trabeculae, as compared with those in vehicle-treated mutants (Figure 8E). Ranolazine, however, did not prevent the prolongation of twitch relaxation at higher frequencies (>1 Hz). Interestingly, treatment reduced the occurrence of spontaneous contractions during pauses to values similar to those observed in WT trabeculae (Figure 8F), a finding consistent with the reduction of intracellular Ca\(^{2+}\) waves and Ca\(^{2+}\) transients detected in isolated cardiomyocytes from R92Q-RAN hearts.

Finally, as previously reported\(^\text{23}\) the R92Q cTnT mutation is associated with a marked increase in myofilament Ca\(^{2+}\) sensitivity that was assessed in skinned ventricular trabeculae (Figure 8G). Ranolazine treatment did not affect the increase in myofilament Ca\(^{2+}\) sensitivity associated with the mutation. The pCa\(_{50}\) of force generation was higher in both untreated and treated R92Q skinned preparations versus WT and was similar in R92Q-KET and R92Q-RAN mice.

Discussion

R92Q-TnT Mouse Recapitulates the Main Features of Human HCM

In this work, we used a comprehensive, multilevel approach (from single cells to the whole heart) to demonstrate that the I\(_{\text{NaL}}\) blocker ranolazine slows and attenuates the development and progression of morphological and functional HCM phenotypes in mutant mice carrying the R92Q mutation in the cardiac TnT gene.

The mouse model we used in this study\(^\text{14}\) recapitulates the main pathophysiological features of HCM in patients, both at whole heart and single cardiomyocyte level. In
Figure 8. Mechanics of intact and skinned trabeculae. A, Representative force traces from left ventricular (LV) trabeculae of wild-type (WT), R92Q-KET, and R92Q-RAN mice, stimulated at 4 Hz. B, Relationship between active twitch force and stimulation frequency (0.1–7 Hz) in trabeculae from the 3 groups. C, Twitch tension measured in the presence of isoproterenol 10^{-7} mol/L (Iso). D, Diastolic (passive) tension measured during steady state stimulation at different frequencies (0.5–7 Hz). B–D, #P<0.05 for frequencies 1 to 7 Hz; §§P<0.05 for frequencies 4 to 7 Hz. E, Time from stimulus to peak contraction (peak time, left) and time from peak to 50% relaxation (RT50%, right), measured during stimulation at different frequencies. ‡P<0.05 for frequencies 0.1 to 1 Hz; †P<0.05, 0.1 to 0.5 Hz; ||P<0.05, 0.1 to 3 Hz. F, Top, Representative traces from R92Q-KET (above) and R92Q-RAN (below) trabeculae displaying spontaneous beats during pauses. Gray lines indicate electric stimuli. Bottom, Percent of trabeculae showing spontaneous contractions during pauses (>2 in a 20 s pause) in intact trabeculae. Errors were calculated by approximating the binomial distribution with a normal distribution (central limit theorem). B–F, Means±SE from 9 WT (6 mice), 11 R92Q-KET (8 mice), and 9 R92Q-RAN (6 mice) trabeculae. G, Top, Average tension/pCa relationship in skinned trabeculae; bottom, pCa at 50% of maximal tension (pCa50). Means±SE from 5 WT (2 mice), 5 R92Q-KET (2 mice), and 5 R92Q-RAN (2 mice) trabeculae. C, F, G, *P<0.05; **P<0.01; NS, P>0.05; statistical tests: Linear mixed models with Tukey-Kramer correction in B–E; Welch’s analysis of variance in F and G.
particular, our echocardiographic measurements showed a marked diastolic dysfunction with LA enlargement in mutant mice (Figure 3), paralleled by slower twitch kinetics and increased diastolic tension of trabeculae (Figure 8).

At single cell level, alterations in Ca\textsuperscript{2+} handling subtended such diastolic abnormalities, including delayed Ca\textsuperscript{2+} transient decay, diastolic Ca\textsuperscript{2+} accumulation, lower SERCA expression, and reduced NCX activity (Figure 6). Interestingly, increased \( I_{\text{NaL}} \) was a major contributor to the observed alterations of intracellular Ca\textsuperscript{2+} handling in R92Q cardiomyocytes. All these functional cellular abnormalities are qualitatively similar to those we previously observed in human HCM cardiomyocytes.\textsuperscript{10} The only remarkable difference between the R92Q mouse model and the human HCM cells is the absence of a prolonged ventricular AP in mouse myocytes, despite the observed increase of \( I_{\text{NaL}} \).

On the contrary, when the excess of \( I_{\text{NaL}} \) is inhibited by ranolazine, APD is significantly shorter in R92Q cardiomyocytes, as compared with WT cells. Previous work provided a possible explanation for this observation.\textsuperscript{26,29} In a transgenic mouse model carrying the I\textsubscript{79N} mutation of cTnT (whose effects on sarcomeric function are similar to R92Q), the higher myofilament Ca sensitivity, by increasing the total cytosolic Ca-buffering capacity of the cardiomyocyte, led to lower Ca-transient amplitude. The reduced size of Ca\textsuperscript{2+} transients in I\textsubscript{79N} myocytes (as in R92Q cells, see Figure 6D and Supplementary Figure 1 in the Data Supplement), led to partial suppression of the Ca\textsuperscript{2+} extrusion mode of the exchanger, thereby, resulting in a smaller inward NCX-mediated current (\( I_{\text{NCX}} \)) during the repolarization phase, ultimately leading to shorter APD in mutant myocytes. An additional possible mechanism leading to AP shortening in R92Q cells is the activation of \( I_{\text{K,ATP}} \) channels because of reduction of ATP concentration caused by the reduced energy efficiency of mutant myocardium.\textsuperscript{15}

In addition to the functional cellular abnormalities, the R92Q transgenic line showed a marked increase in endomyocardial fibrosis (Figures 4 and 5), a hallmark of HCM in patients, particularly in those carrying thin-filament gene mutations.\textsuperscript{6} Finally, a clear propensity to develop ventricular arrhythmias in response to adrenergic stimulation has been previously shown in this transgenic line\textsuperscript{30} and was confirmed by our studies in single cells and intact trabeculae (Figures 7 and 8). The functional and structural remodeling of mutant myocardium was associated with increased activation of CaMKII, a nodal protein in the regulation of cardiac physiology and pathology, whose enhanced activity is notably associated with cardiac hypertrophy and heart failure.\textsuperscript{31} Interestingly, a similar increase in the phosphorylation of CaMKII and its downstream targets was also consistently found in cardiac samples from HCM patients.\textsuperscript{21} Of note, despite an increased septal thickness measured with echo and magnetic resonance imaging, total LV wall mass was similar in R92Q versus WT hearts, in line with earlier findings in mice from this lineage.\textsuperscript{14} HCM in patients is diagnosed in the presence of a maximal LV diastolic thickness >15 mm and not by the presence of an increased LV total mass.\textsuperscript{2} Indeed, regional distribution of hypertrophy is common in HCM patients, and >35% of them show no or mild increase of total LV mass.\textsuperscript{32} Moreover, the absence of global LV hypertrophy and the restrictive physiology, far from representing a limitation of this model, are typical of thin-filament HCM and are often observed in patients carrying similar high-risk mutations.\textsuperscript{3,5} All in all, the cardiac pathological phenotype observed in R92Q transgenic mice provides a better approximation of the wide spectrum of myocardial and cellular abnormalities observed in human pathology, as compared with other currently available HCM mouse models carrying mutations in Myosin Heavy Chain\textsuperscript{11} or Myosin-binding Protein-C (MyBPC),\textsuperscript{33} increasing the translational value of our study.

**Rationale for the Use of Ranolazine as a Disease-Modifying Strategy.**

The choice of ranolazine as a lifelong preventive treatment was driven by the positive acute effects that this drug exerted in cells and trabeculae isolated from patients with HCM\textsuperscript{10} and by its safety profile and tolerability in patients. The mechanisms by which ranolazine is capable of normalizing intracellular Ca\textsuperscript{2+} are directly related to its effect to inhibit cardiac \( I_{\text{NaL}} \). LV cardiomyocytes from R92Q hearts show a significantly increased density of \( I_{\text{NaL}} \) when compared with WT cells (Figure 1); interestingly, the increase of \( I_{\text{NaL}} \) is an early feature of cellular remodeling in R92Q myocardium, as it is already present in cells from 1-month-old mutant mice. Early hyperactivation of CaMKII (because of the onset of intracellular Ca\textsuperscript{2+} overload) increases the phosphorylation of NaV1.5 channels at specific sites determining impaired current inactivation and may, thus, be the cause of the early increase of \( I_{\text{NaL}} \) in mutant hearts. Indeed, increased intracellular [Ca\textsuperscript{2+}]\textsuperscript{15} is widely considered among the first common consequences of HCM-causing sarcomeric mutations. Increased myofilament Ca\textsuperscript{2+} sensitivity (Figure 8) leads to increased diastolic [Ca\textsuperscript{2+}] as a consequence of the larger cytosolic Ca-buffering capacity.\textsuperscript{28,29} The increased ATP consumption by the mutant sarcomere\textsuperscript{1,5,33} may reduce the amount of ATP available to fuel SERCA-mediated Ca-reuptake into the sarcoplasmic reticulum, ultimately contributing to cytosolic Ca\textsuperscript{2+} overload, the main determinant of CaMKII activation and pathological \( I_{\text{NaL}} \) enhancement.

The increased \( I_{\text{NaL}} \) results into a marked intracellular Na\textsuperscript{+} accumulation, especially at high pacing rates (Figure 1), which in turn contributes to increase intracellular diastolic [Ca\textsuperscript{2+}] by impairing the forward function of the NCX (Figure 6). As in human HCM,\textsuperscript{10} acute exposure to ranolazine, that markedly inhibited \( I_{\text{NaL}} \), reduced both intracellular [Na\textsuperscript{+}] and diastolic [Ca\textsuperscript{2+}] in R92Q cardiomyocytes (Figure 1). The normalization of diastolic Ca\textsuperscript{2+} by ranolazine highlights the possibility of additional benefits in the long-term administration because increased diastolic Ca\textsuperscript{2+} accumulation is one of the main subcellular determinants of myocardial adverse remodeling and disease progressions in animal models of HCM irrespective of genotype.\textsuperscript{11,36} Long-term treatment with ranolazine was effective in reducing the magnitude of cellular remodeling in other models of hypertrophy.\textsuperscript{37,38} In spontaneously hypertensive rats, chronic ranolazine treatment started before
the onset of hypertrophy prevented the development of T-tubules disruption and significantly attenuated the degree of alterations of Ca\textsuperscript{2+} handling.\textsuperscript{37} In a model of deoxycortico
tosterone acetate salt–induced hypertension in rats, ranolazine treatment prevented the development of secondary modifications of the myocardial contractile apparatus, thus, reducing the severity of diastolic dysfunction.\textsuperscript{38} However, long-term treatment with ranolazine has not been previously attempted in models of genetic heart disease.

**Lifelong Ranolazine Treatment Prevents the Development of Diastolic and Systolic Dysfunction**

In the present work, transgenic mice carrying the R92Q mutation of TnT were treated with ranolazine since birth and proved safe in this setting. Early initiation of treatment is likely essential because HCM-related alterations of myocardial structure and function are thought to develop early in life, possibly starting in the prenatal phase.\textsuperscript{39} Ranolazine treatment effectively countered the development of diastolic dysfunction in our HCM mice, as evaluated by Doppler echocardiography (Figure 3); in keeping with the improved diastolic function, a lower diastolic tension was observed in trabeculae from treated mice (Figure 8). The effect of the treatment on diastolic function was associated with the prevention of pathological Excitation-Contraction-coupling changes: myocytes from treated R92Q mice lacked the increase in diastolic Ca\textsuperscript{2+} seen in vehicle-treated mice and showed a faster decay rate in Ca\textsuperscript{2+} transient because of maintained near-normal SERCA and NCX function. Preservation of SERCA function seems particularly relevant to the outcome of ranolazine treatment; indeed, SERCA overexpression via gene transfer was capable of reducing phenotypic expression and disease progression in a murine model of HCM carrying a tropomyosin mutation.\textsuperscript{36}

Although abnormalities of Ca\textsuperscript{2+} transient kinetics were fully prevented in R92Q-RAN cardiomyocytes, the prolongation of twitch relaxation kinetics was not entirely prevented in trabeculae from ranolazine-treated mice. The interplay between Ca\textsuperscript{2+} removal from the cytosol and the intrinsic ability of myofilaments to switch off contraction contributes to determine the velocity of twitch relaxation. In line with previous studies,\textsuperscript{14} skinned muscle from R92Q mice exhibited increased Ca\textsuperscript{2+} sensitivity (Figure 8). Because this feature is a direct consequence of the mutation altering the thin-filament structure\textsuperscript{40} and occurs upstream from the site of action of ranolazine, it was not surprising that Ca\textsuperscript{2+} sensitivity was not affected by the treatment. Notably, this rules out any effect of ranolazine on transgene expression. The persistence of sarcomeric abnormalities explains why altered contraction kinetics are still present in R92Q-RAN myocardium, despite the normalization of intracellular Ca\textsuperscript{2+} dynamics.

The link between fibrosis and diastolic impairment is critically relevant for the pathophysiology of HCM, particularly for thin-filament mutations.\textsuperscript{5} Treatment with spironolactone (an aldosterone receptor blocker) and N-acetyl-cysteine (an antioxidant) attenuated fibrosis in transgenic mice carrying the R92Q-TnT mutation, and the reduction of fibrosis alone led to improvement in diastolic function.\textsuperscript{41,42} It is, therefore, notable that ranolazine treatment counteracted the expansion of extracellular volume in the ventricular myocardium of R92Q mice, as evaluated with contrast magnetic resonance imaging, reduced the proliferation of intramyocardial fibrosis in histological sections (Figures 4 and 5), and prevented the increase of transforming growth factor β expression.

In addition to preventing diastolic dysfunction, life-
long ranolazine treatment also prevented the development of systolic abnormalities and rescued the blunted inotropic responses in mutant mice. Ventricular trabeculae from R92Q mice showed a slight reduction in twitch amplitude under baseline conditions but displayed significantly reduced responses to a variety of positive inotropic challenges mimicking exercise conditions, including high-frequency stimulation and β-adrenergic activation with isoproterenol. The reduction of inotropic reserve in R92Q mice was completely abolished by ranolazine (Figure 8). The clinical relevance of this effect is also important because progression toward systolic dysfunction and the so-called end stage occurs in 8% to 10% of patients with HCM and in ≤20% of patients carrying thin-filament mutations.\textsuperscript{6} Of note, ranolazine prevented the disruption and disorganization of T-tubules, as evaluated with confocal microscopy using fluorescent membrane staining (Figure 5), an effect that likely contributed to the preservation of LV diastolic and systolic function.\textsuperscript{22}

**Ranolazine Treatment Prevents the Development of Arrhythmogenic Substrates**

Our results suggest that long-term ranolazine treatment is able to counteract the development of an arrhythmogenic substrate in the myocardium of mutant mice with HCM. Although we did not directly evaluate the frequency of ventricular ectopy nor the inducibility of arrhythmias in living animals, cardiomyocytes from treated mice showed a marked reduction in frequency of Ca\textsuperscript{2+} waves and spontaneous Ca\textsuperscript{2+} transients during pauses, both at baseline and during challenge with isoproterenol. Likewise, spontaneous contractions in intact trabeculae from R92Q-RAN mice were nearly absent, while they occurred with relatively high frequency in vehicle-treated mice. The reduction of Ca\textsuperscript{2+}-dependent arrhythmogenesis is likely subtended by the normalization of intracellular Ca\textsuperscript{2+} handling by ranolazine,\textsuperscript{10} although other factors, such as the prevention of transcriptional changes in ion channel genes, such as I\textsubscript{Kr}, may have contributed to the effect of the treatment (Table IV in the Data Supplement). CaMKII can acutely regulate ion channels (I\textsubscript{Na}, I\textsubscript{Ca}, I\textsubscript{K}) and Ca\textsuperscript{2+}-handling proteins (RyR, IP3R, PLB), directly contributing to triggered arrhythmias (such as Early After-depolarizations and Delayed After-depolarizations).\textsuperscript{43} Reduction of CaMKII activity by ranolazine treatment is likely to have contributed to the reduction of arrhythmias in treated R92Q mice. Finally, at the whole heart level, the reduction of cellular arrhythmic triggers is paralleled by a reduced intramyocardial fibrosis (a substrate for re-entry circuits), potentially lowering the risk of sustained ventricular arrhythmias.
Mechanisms Underlying the Efficacy of Ranolazine in R92Q-TnT Mice

The CaMKII-I_{nat} vicious cycle is a well-established phenomenon occurring in several cardiac diseases, including human HCM. In brief, increased I_{nat} leads to intracellular Na⁺ overload, which impairs NCX-mediated extrusion of Ca⁺, thus, contributing to Ca⁺ overload, which in turn potentiates CaMKII activity via calmodulin binding; in addition, Na⁺ overload increases mitochondrial reactive oxygen species production and oxidation of CaMKII, leading to its constitutive activation. The hyperfunctional CaMKII, via direct phosphorylation of NaV1.5 Na⁺ channels, contributes to further increase in I_{nat}. Although increased I_{nat} is never the primary cause of disease (with the exception of LQT3), it is a common feature of cardiac cell remodeling in several conditions, including mice carrying the R92Q TnT mutation (Figure 1). Inhibition of I_{nat} is likely to thwart the establishment of the aforementioned vicious cycle, leading to a sustained reduction of CaMKII activity. Indeed, we have found that the degree of CaMKII autophosphorylation at Thr286 remained reduction of CaMKII activity. Indeed, we have found enhancer factor-2 (MEF2)-controlled genes. In addition, relieving the inhibition of the transcription of myocyte enhancer factor-2 (MEF2)-controlled genes.45 In addition, CaMKII hyperactivity in cardiac diseases is associated with changes of gene expression program that ultimately contribute to drive myocardial remodeling both at cellular level (hypertrophy, changes of cellular substructures, expression of ion channels, and EC-coupling proteins) and at extracellular level (intra-myocardial fibrosis). Activated CaMKII can migrate to the nucleus and phosphorylate hystone deacetylase (HDAC4), thus, relieving the inhibition of the transcription of myocyte enhancer factor-2 (MEF2)-controlled genes.46 In addition, Ca-activated calmodulin may also bind to calcineurin and dephosphorylate Nuclear factor of activated T cells (NFAT), which in turn may increase GATA-dependent transcription.43 Interestingly, MEF2 and GATA-controlled genes are involved in the development of pathological cardiac disease hypertrophy, as well as in the often associated extracellular matrix remodeling and fibrosis. Therefore, the reduction of CaMKII activity after I_{nat} inhibition in ranolazine-treated R92Q mice is likely to be the main mechanism that prevented disease-related structural and functional myocardial changes in treated mice.

In a recent work by Flenner et al., mice carrying an MyBPC mutation related to HCM were treated with ranolazine, and most of the observed effects of the drug in that model were found to be related with a significant inhibition of β-adrenergic receptors. Ranolazine has a significant β-blocking effect when the plasma concentration is >13 μmol/L, much higher than the clinically relevant concentration range (from 2 to 5 μmol/L). Indeed, Flenner et al.46 reached very high plasma ranolazine concentrations in their treated mice (>20 μmol/L), so a β-blocking effect observed with their protocol is expected, but scarcely clinically relevant. In our ranolazine-treated male mice, plasma ranolazine concentration ranged from 2.5 to 5.5 μmol/L (average 4 μmol/L). Moreover, they do not show lower heart rate and blood pressure when compared with vehicle-treated mice (Table II in the Data Supplement). Furthermore, phosphorylation of protein kinase A targets is not reduced in the hearts from treated versus untreated R92Q mice (Figure IV in the Data Supplement). Therefore, we can reliably exclude that a significant β-blocking action plays a role in determining the effects of ranolazine in R92Q-TnT mice. Finally, despite their widespread use in HCM patients, β-blocking agents have never been proven to be capable of slowing or preventing the development or progression of disease in murine HCM models or in patients on long-term administration.

Implications for Clinical Management

Taken together, our data support the role of ranolazine or other I_{nat} inhibitors to prevent, delay, or attenuate the onset and severity of disease in subjects carrying mutations associated with severe forms of HCM.44 The feasibility of preventive treatment strategies in young HCM mutation carriers has been recently ascertained by Ho et al.,12 who recently completed a trial with diltiazem and are currently performing a trial with valsartan in this group of patients (VANISH Trial [Valsartan for Attenuating Disease Evolution In Early Sarcomeric HCM]).

Although the present results support the use of ranolazine in subjects carrying high-risk thin-filament mutations, whether its efficacy extends to other genotypes remains to be determined. A recent study by Flenner et al.46 showed that a 6-month treatment with ranolazine did not reduce cardiac hypertrophy or diastolic dysfunction in an MyBPC-mutant HCM mouse model. The lack of effects of ranolazine in that work stems from the absence of a significant increase of I_{nat} in cardiomyocytes from MyBPC-mutant mice as compared with WT littermates, at variance with the R92Q-TnT model. In human cardiomyocytes from adult patients with HCM, however, we previously showed that similar electric abnormalities (including increased I_{nat}) are also present in cardiomyocytes from patients carrying MyBPC mutations, and the benefits of acute ranolazine administration seemed independent from the presence of a specific mutation. The cardiac pathological phenotype of the MyBPC mouse model in the work by Flenner et al.46 was rather mild, with slight diastolic dysfunction, minimal LV hypertrophy, and little cellular abnormalities, and provided a lesser approximation of advanced human disease as observed in patient samples, as compared with the R92Q model. Moreover, Flenner et al started treatment in young adult mice of 2 months of age when disease phenotype was already fully expressed, while we initiated treatment right after birth, before HCM phenotypic expression. This suggests that initiation of treatment before the onset of cardiac pathological changes is preferable to achieve a disease-modifying effect in HCM. Indeed, other supposedly disease-modifying agents, such as sartans or statins, have failed to reduce established hypertrophy and fibrosis in adult HCM patients with overt disease, while early treatment of young mutation carriers with diltiazem has somewhat succeeded.

Based on the results of the present work, we think that it would be essential for a preventive treatment with I_{nat} inhibitors to be started before the onset of pathological phenotype, that is, during early to late childhood (5–14 years). We do not think that initiation of treatment at birth in humans would be necessary to achieve the expected results. The reason why we
decided to treat cTnT-R92Q mice since birth is because pathological cardiac changes (including increased \(I_{\text{Na}}\)) start developing early in R92Q mice and are mostly present at 1 month of age. Because it was impossible for us to define the exact time of the onset of disease-related changes during the first month of life, we decided to initiate treatment right after birth. Comparing the development of HCM in transgenic mice with that occurring in human children and young adults is nearly impossible, given the remarkable differences in the physiology of heart maturation and growth. At birth, the functional maturity of cardiomyocytes in the mouse heart is relatively higher than that in newborn humans. From a translational standpoint, we think that starting treatment in a newborn mouse would be the equivalent of starting treatment in a 6-year-old child. This strategy could be feasible in individuals carrying high-risk mutations, such as thin-filament mutations, identified in familial screening programs. Our work highlights the role of ranolazine as an agent to treat young carriers of high-risk mutations (eg, thin-filament mutations or aggressive MYH7 mutations). These subjects have a relatively high risk of lethal arrhythmias during adolescence and early adulthood and an increased likelihood of developing terminal heart failure later in life, with either hypococontractile or restrictive phenotype. These patients are currently orphan of effective therapies because current strategies are unable to prevent arrhythmias or heart failure; thus, early prevention of phenotype development is the only possible therapeutic option.

Ranolazine is already available in the clinic for angina treatment and has been recently assessed in patients with symptomatic HCM (RESTYLE-HCM study [Ranolazine in Symptomatic Hypertrophic Cardiomyopathy]; EudraCT number, 2011-004507-20); in addition, eleclazine, a novel selective NaL inhibitor with improved efficacy and safety, is currently under clinical development and is being trialed in HCM patients (LIBERTY-HCM study [Effect of Eleclazine (GS-6615) on Exercise Capacity in Subjects With Symptomatic Hypertrophic Cardiomyopathy]; EudraCT number, 2013-004429-97). The results of these studies may provide clinical evidence in support to our observations and answer the question whether the strategy of \(I_{\text{Na}}\) inhibition is effective in ameliorating the clinical profile of adult patients with overt HCM. Whatever the results of these studies, however, the present work strongly supports a rationale for ranolazine or other \(I_{\text{Na}}\) inhibitors as promising preventive therapy in carriers of HCM mutations at risk of developing a severe form of the disease.

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Disclosures

None.

References


14. Tardiff JC, Hewett TE, Palmer BM, Olsson C, Factor SM, Moore RL, Robbins J, Leinwand LA. Cardiac troponin T mutations result in

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Coppini et al

16 Ramolane Prevents Cardiac Remodeling in HCM


CLINICAL PERSPECTIVE

Family screening programs are detecting increasing numbers of young individuals who carry mutations associated with hypertrophic cardiomyopathy (HCM) before the onset of cardiac hypertrophy and dysfunction. Recent advances in HCM patient phenotyping led to the identification of several high-risk mutations (eg, thin-filament mutations) associated with early sudden death and progression toward heart failure. To date, however, when a young individual is diagnosed with having a high-risk mutation, clinicians can only suggest a close follow-up strategy, for lack of robust therapeutic options capable of preventing disease onset and progression. Although gene therapy still seems distant, an attractive alternative is represented by disease-modifying drugs interfering with the cellular mechanisms of myocardial adverse remodeling. Our previous studies in surgical samples from HCM patients showed that intracellular calcium overload and increased late-sodium current are key determinants of the electric and mechanical dysfunction of HCM myocardium, which were reverted by the late-sodium current inhibitor ranolazine. In a clinically relevant mouse model carrying the high-risk R92Q mutation of TnT gene, we here show that lifelong treatment with ranolazine, initiated before the onset of cardiac phenotype, is capable of preventing structural and functional cardiac changes associated with HCM (LV hypertrophy, intramyocardial fibrosis, diastolic dysfunction, and arrhythmogenesis). In ranolazine-treated mice, reduction of calcium overload prevents the activation of calmodulin kinase 2, thereby stopping the pathway of myocardial hypertrophic remodeling. Ranolazine is currently in clinical use for angina and has a remarkable safety profile. The present work supports a rationale for the rigorous evaluation of ranolazine as preventive therapy in young carriers of high-risk HCM mutations.
Ranolazine Prevents Phenotype Development in a Mouse Model of Hypertrophic Cardiomyopathy

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SUPPLEMENTAL MATERIAL

Ranolazine Prevents Phenotype Development in a Mouse Model of Hypertrophic Cardiomyopathy

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Expanded Methods

Study approval

Animals were bred and used for experiments in accordance with Italian and European regulations for animal handling and care and all experimental protocols were approved by the local committee for animal welfare. In agreement with current regulations, the protocol was submitted to the Committee for Animal Welfare of the Italian Ministry of Health on February 2014 and approved on March 2014.

Animal model

The knock-in mouse-model carrying the R92Q TNNT2 mutation was generated in Dr. Tardiff’s Lab on C57BL/6N genetic background. Heterozygous progenitors were bred with C57BL/6NCrl females (Charler River, Strain Code: 027) and the genotypes or the offspring determined by PCR on DNA from tail biopsy specimens. Animals were maintained and bred at the animal facility of the University of Florence, Italy.

Long-term treatment of murine models with Ranolazine

Mice were fed with chow containing 0.5% ranolazine and 0.03% ketoconazole (Research Diets Inc.); ketoconazole was added as a CYP2D6 inhibitor, in order to reduce the metabolic breakdown of the drug and get the desired plasma levels of the drug (3-11 µM) in males across the whole day. Drug dosage was set up using a separate set of animals fed with ranolazine containing chow for 7 days and then sacrificed at different times of the day (7:30 AM and 2:00 PM) to quantify plasma levels of ranolazine (Supplementary Fig. 3). Treatment started right after birth and each litter was randomized (50%-50%) to chow containing ranolazine and ketoconazole (RAN) or chow containing ketoconazole (0.03%) only (KET). Given the physicochemical characteristics of ranolazine and the relatively high plasma concentrations obtained in female mice with our treatment regimen (Supplementary Fig.3),
the distribution of drug into milk was assumed to be sufficient to achieve an effective dosage in breastfed new-borns until weaning is complete. Litters were genotyped at 1 month of age and R92Q were separated from WT mice. Only male mice were kept on treatment after genotyping.

Determination of ranolazine concentration in plasma and heart samples
A liquid-chromatography analysis coupled with tandem-mass spectrometry (LC-MS/MS) was carried out using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA). Calibration solutions with different ranolazine concentrations were analyzed six times by LC-MS/MS method to obtain a calibration curve, using a linear regression analysis. An aliquot of 20 μl of each plasma sample was used for drug quantification. Calculated precision (relative standard deviation -RSD%- of replicates) was 3.0%. Limit of detection and limit of quantitation were 0.01μM and 0.03μM, respectively. In a separate set of experiments, the same procedure was performed on lates obtained from heart samples using a standard method2.

Echocardiography
Echocardiographic tests were performed using Visualsonics Vevo 2100 echography platform. Mice were anesthetized with isoflurane (induction, 4%–5%; maintenance, 1%–2.5% in oxygen-enriched air) in an induction chamber and then positioned prone on a heated bed. Parasternal long-axis view was used to measure septum thickness and LV diameter. Three different short-axis views were acquired to allow LV volume reconstruction with Simpson’s method. Apical 4-chamber view was used to measure atrial size and to assess transmitral blood flow with pulsed-wave Doppler. All measurements on echo images were performed offline by the same operator in a blind manner.

Cardiac Magnetic Resonance Imaging (MRI)
Cardiac magnetic resonance imaging was performed using a Brucker Pharmascan 7T small animal MRI scanner, equipped with a 740 mT/m gradient coil. Mice were anesthetized with isoflurane (induction, 4%–5%; maintenance, 1%–2.5% in oxygen) and positioned supine on a heated bed in the MRI scanner. CMR images were acquired with ECG and respiratory gating. For LV/RV size and function, short-axis cine gradient-echo images were obtained with full LV coverage (repetition time, 5.9 ms; echo time, 2.2 ms; temporal resolution, 20–30 ms; in-plane spatial resolution, 100–120×180–210 μm; slice thickness, 1 mm; no gap). Manually traced epicardial and endocardial contours at end-systole and end-diastole were used to determine the LV/RV end-diastolic and end-systolic volumes, ejection fraction and myocardial mass.

*Contrast MRI*

Gadolinium diethylenetriaminepentacetic acid was injected intravenously (tail vein) in a single bolus dose of 0.5 mmol/kg. Myocardial T1 was measured in a mid-LV slice once before contrast and 35-40 minutes after contrast using a Look-Locker technique (repetition time, 2.5 ms; echo time, 1.8 ms; flip angle, 10°; in-plane resolution, 190 μm; slice thickness, 1 mm). For 6 myocardial segments and the blood pool, signal intensity was plotted versus time after inversion and T1 values were obtained by nonlinear least-squares fitting of the intensity curves. The reciprocal of T1 (R1 = 1/T1) was used to calculate extracellular volume (ECV). ECV was calculated using the following expression: ECV = (1 – hematocrit) * (R1_MusclePost – R1_MusclePre) / (R1_BloodPost – R1_BloodPre). For the purposes of this analysis, hematocrit was considered to be 0.45.

*Confocal Microscopy*

Isolated cardiomyocytes were incubated for 20 minutes with 5 μM of Di-3-ANEPPDHQ (Thermo-Fisher) membrane dye, resuspended in Tyrode buffer, placed atop a glass-bottomed chamber (MaTek) and observed using a 63x oil immersion objective on a Leica TCS5 inverted microscope.
The excitation wavelength was 488 nm and the emission filter was set at 504-649 nm. A single image was recorded from each cell by choosing a plane comprising the central portion of the cell. Images were then analyzed offline with ImageJ, using an automatic analysis plugin (TTorg)³.

**Dissection of ventricular trabeculae**

LV and RV trabeculae were dissected as previously described ⁴, ⁵. In brief, mice were heparinized and anesthetized by isoflurane, the heart was rapidly excised, placed beneath a binocular microscope and perfused retrogradely with a Krebs-Henseleit solution (KH), containing (mM): 120 NaCl, 5 KCl, 2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 0.5 CaCl₂ and 10 glucose; pH 7.4 equilibrated with 95%O₂/5%CO₂. BDM (20 mM) and KCl (15mM) were added to the initial solution to minimize contractures following cutting damage. RV and LV were opened and thin unbranched trabeculae were carefully dissected. Trabecula diameters were measured and cross-sectional area was calculated with the assumption of an ellipsoid shape.

**Mechanical measurements on intact trabeculae**

Ventricular trabeculae were mounted between the basket-shaped platinum end of a force transducer (KG7A, WPI) and a motor (Aurora Scientific), both connected to micromanipulators, in a glass-bottomed heated horizontal tissue bath with platinum wires for field-stimulation. Sarcomere length was measured by laser diffraction ⁴. Muscles were allowed to stabilize at baseline conditions (30°C, 2mM [Ca²⁺], 1Hz) in KH solution for at least 20-30 min and were stretched to optimal initial sarcomere length (2.15±0.03µm) before starting experiments. Twitch force and kinetics were measured during steady state-state stimulation at different frequencies (0.1 to 7Hz), in the absence and presence of isoproterenol (0.1µM). Additionally, pauses of different duration (2 to 60s) were inserted after a steady series at 3Hz to evaluate post-rest potentiation of the first stimulated beat after the pause, as well as the occurrence of spontaneous beats during the pause.
Mechanical measurements on skinned trabeculae

Force-PCa curves were obtained as previously described 6. Briefly, trabeculae were skinned by overnight incubation in a relaxing solution containing 0.5% Triton X100. The skinned preparations were mounted horizontally between a force transducer and a motor by means of T-clips. Muscles were activated by transferring them manually from a relaxing solution to baths containing different pCa solutions and the pCa-force relationship was determined.

Isolation of single cardiomyocytes from mouse hearts

Ventricular cardiomyocytes were isolated by enzymatic dissociation as previously described 5. In brief, the excised heart was perfused retrogradely for 15 minutes at a constant flow of 3 mL/min with heated (37°C) cell-isolation buffer containing (mM) 120 NaCl, 1.2 MgCl2, 10 KCl, 1.2 KH2PO4, 10 glucose, 10 HEPES, 20 taurine, 5 pyruvate; pH 7.2 (NaOH). Of note, 15 minutes of perfusion with ranolazine-free buffer was sufficient to wash-out all ranolazine from the hearts of RAN-treated mice: indeed, ranolazine was undetectable in lisates from samples cut from those hearts after 15 minutes of perfusion and subjected to LC-MS/MS analysis. Afterwards, 0.1 mg/mL Liberase TM (Roche) was added to the perfusing solution and recirculated for 8-10 minutes. The ventricles were then excised and cut into small pieces in enzyme-free buffer containing 1mg/ml BSA and gently stirred for 5 minutes to facilitate myocyte dissociation. Cells were let to settle for 15 minutes and resuspended in a modified Tyrode buffer with 0.05mM CaCl2 with 1 mg/ml BSA. [Ca2+] was gradually raised to 1.0 mM in 6 subsequent steps over a total time of 1 hour. The standard Tyrode bath solution contained (in mmol/L) 136 NaCl, 5.4 KCl, 0.33 Na2PO4, 1.8 CaCl2, 1 MgCl2, 10 dextrose, and 10 HEPES-NaOH; pH was adjusted to 7.35 with NaOH.
**Intracellular Ca\textsuperscript{2+}-transient measurements**

Freshly isolated cardiomyocytes were incubated for 30 minutes with the Ca\textsuperscript{2+}-sensitive dye FluoForte (Enzo), resuspended in Tyrode buffer and set aside for 30 minutes to allow for full dye de-esterification. Cardiomyocytes were then plated on a Tyrode-perfused heated (37°C) glass-bottomed dish with platinum electrodes for field stimulation, mounted on an inverted microscope, and perfused with Tyrode buffer for 10 minutes before starting recordings. The total time from heart excision to the first recordings in myocytes was 3 hours. We measured fluorescence emitted at 505-525 nm with a fast camera (Photometrics Cascade 128+) during fixed excitation at 490 nm with a monochrome LED source, while stimulating the cells at 1Hz, 3Hz and 5Hz using <3ms square pulses. Measurements were performed at steady state by averaging 20-50 subsequent Ca\textsuperscript{2+} transients.

In order to quantify the occurrence of arrhythmic events, cells were paced at 5Hz for at least 30 seconds and then stimulation was abruptly stopped for 15s, while recording intracellular Ca\textsuperscript{2+} movements. We quantified the occurrence of Ca\textsuperscript{2+} waves (slow raise kinetics, amplitude 20-50% of a regular transient) and spontaneous Ca\textsuperscript{2+} transients (fast upstroke, amplitude comparable to a regular transient). The protocol was repeated in the presence of Isoproterenol (0.1µM).

**Simultaneous [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] measurements**

Cardiomyocytes were incubated at room temperature with the Na\textsuperscript{+}-sensitive dye Asante Natrium Green for 40 minutes (Teflabs) and with the Ca\textsuperscript{2+}-sensitive dye Fura-2 for 15 minutes (Thermo-Fisher). Cells were resuspended in Tyrode buffer and set aside for 30 minutes to allow full de-esterification of the dyes. Fluorescence emission was collected at 505-525 nm while excitation wavelength was rapidly cycled among 340nm, 380nm and 490nm (one cycle at each time-point, final time resolution = 9ms). Cells were field-stimulated at at 1Hz, 3Hz, 5Hz and 7Hz in the absence and presence of ranolazine. At the end of the experiment, the selective Na\textsuperscript{+} ionophore Sqi-Pr (Teflabs) was added to bathing solution (5µM) to obtain maximal Asante Natrium Green fluorescence.
**Patch-clamp studies**

Patch-clamp studies were performed as previously described. Action potentials (APs) were measured using the perforated-patch configuration (amphotericin-B method), while ruptured-patch was used to measure I_{NaL}. For AP recordings, the pipette solution contained (in mM) 115 K methanesulfonate, 25 KCl, 10 HEPES, 3MgCl₂ and cells were superfused with Tyrode buffer. APs were elicited with short depolarizing stimuli (<3ms) at different frequency of stimulation (1Hz, 3Hz and 5Hz, >30s at each frequency). AP measurements were performed at 36±1 °C. For I_{NaL} measurements, isolated ventricular cardiomyocytes were superfused with an extracellular solution containing (in mM) 135.0 NaCl, 4.6 CsCl, 1.1 MgSO₄, 10 glucose, 10.0 HEPES, and 0.01 nitrendipine, pH 7.4, at 25±1 °C. Pipette solution contained (in mM) 120 l-aspartic acid, 20 CsCl, 1 MgSO₄, 4 Na₂ATP, 0.1 Na₃GTP, and 10 HEPES; pH 7.3 (with CsOH). To measure late I_{Na} and the extent of block by ranolazine, 500-ms depolarizing steps to −10 mV from a holding potential of −120 mV were applied to cells at a rate of 0.2 Hz. In a subset of experiments, 150 ms depolarizing steps were applied at a rate of 5Hz. The magnitude of I_{NaL} was determined by integration of the area of the current between 25 and 125 ms after onset of the −10 mV clamp pulse, using the integration (area) feature of the pCLAMP program (Molecular Devices).

**Western Blot**

Western blot analysis was performed by a standard method on proteins isolated from LV specimens from WT, R92Q-KET and R92Q-RAN mice. The following antibodies were used: phospho-CaMKII at threonine 287, RyR2 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); CaMKIIδ (Abcam, Cambridge, MA); phospho-PLB at serine 16 (Millipore, Billerica, MA); threonine 17, SERCA2a, phospho-RyR2 at serine 2808 and serine 2814 (Badrilla, Leeds, UK) and PLB (Thermo-
Fisher, Waltham, MA). Relative intensity of individual bands from Western blots was quantitated using ImageJ software and normalized to GAPDH. The ratio for control was assigned a value of 1.

Quantification of mRNA expression of myocardial proteins

Left ventricular tissue samples were cleaned of blood and freeze-dried in liquid nitrogen within 5 minutes from excision and stored at -80°C for further use. Total RNA from each frozen cardiac sample was isolated and DNase-treated with the RNeasy Fibrous Tissue Mini Kit (Qiagen) and single-stranded cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The real-time RT-PCR reactions were performed using TaqMan® Gene Expression Master Mix and predesigned TaqMan® Gene Expression assays (Applied BioSystems, Thermo-Fisher). All reactions were carried out using an ABI Prism 7500 Sequence Detection System (Applied Biosystems), were performed in triplicate and included a negative control and. Relative quantification of the mRNA level for the different genes was determined using the comparative method ($\Delta\Delta CT$).

The threshold cycle (Ct) difference of the index gene and the reference gene, calculated from each specimen, is subtracted from the average Ct of the control group; this value is used as the exponent of 2 to calculate $\Delta\Delta Ct$ for each specimen. For all mRNA quantification assay, GAPDH was used as reference gene. In order to validate GAPDH as a reference gene, GAPDH mRNA was compared with ribosomal RNA 18S and expression level calculated as $\Delta\Delta Ct$ (not shown). TaqMan® Gene Expression assays part numbers: ATP2a2= Mm01201431_m1; PLN= Mm00452263_m1; NCX= Mm01232254_m1; RyR2= Mm00465877_m1; KChIP2= Mm00518914_m1; Kv4.3= Mm01302126_m1; CaCNA1= Mm01188822_m1; MYH6= Mm00440359_m1; MYH7= Mm01319006_g1; TGF-β= Mm01178820_m1; BNP= Mm01255770_g1; ANP= Mm01255747_g1.
Statistical Analysis

Data from cells and muscles are expressed as mean±SEM (Standard Error of Mean) values obtained from a number of independent determinations on different samples: number of samples and mice for each set of measurements is indicated in figure legends. To faithfully compare different sets of measurements, sensitivity analysis was performed for each statistical comparison using SPSS 22.0 (IBM, USA), in order to account for:

1- Non-gaussian distribution (Shapiro-Wilk test).

2- Heteroscedasticity (Levene’s test).

The statistical tests used to calculate P-values for each dataset were different depending on the properties of individual datasets and on the number of groups to compare. The specific tests used for each comparison are indicated with abbreviations in the respective figure legends.

1- For sets variables where a single measurement from each mouse is included (e.g. echocardiography data, western blot results), the three different groups (WT, R92Q-KET and R92Q-RAN) were compared using one of the following tests, using SPSS 22.0:

(a) One-Way ANOVA with Tukey correction (abbreviation: OWAT), for normally-distributed homoscedastic datasets.

(b) Kruskal-Wallis test with Dunn’s multiple comparison test (abbreviation: KWD), for non-Gaussian-distributed datasets (as identified with the Shapiro-Wilk test)

(c) Welch’s ANOVA with Games-Howell test (abbreviation: WA), when there was a significant difference among the variances of the different groups heteroscedastic groups (identified by Levene’s test).

2- In some datasets, average data derives from multiple samples (myocytes, trabeculae) from different mice. The intrinsic correlation among different cells or trabeculae from the same mice (within-subject correlation) could influence the outcome of the results, especially when a different number of samples from each mouse is included in the final means. We estimated within-subject correlation for each
variable Multi-level one-way analysis-of-variance (ANOVA). In brief, the variance among the cells/trabeculae from each single subject is compared with the overall variance. When the estimated intra-class correlation exceeds 0.10 it is considered to have a significant effect on the results, requiring multi-level analysis for comparison. In order to account for intra-subject correlation, we used linear mixed models to compare data groups, using the *xtmixed* function of the STATA 12.0 program (StataCorp, USA). When Levene’s test reported unequal variances in unpaired comparisons, linear mixed models were adjusted for heteroscedasticity (via independent calculation of residuals in the two groups). Linear mixed models were also used to compare paired measurements (effect of ranolazine), by adding an additional level to data hierarchy (the two repeated measurements within the same cell/trabecula, in the absence and presence of the drug). The Probability (*P*) values were calculated from linear mixed models. For multiple comparisons, we used the Tukey post-hoc test. *P*<0.05 was considered statistically significant.
Supplementary Figure 1

Late Na-current in young mice and acute effects of ranolazine. Legend on next page.
Late Na-current in young mice and acute effects of ranolazine (additional data). (A) Representative superimposed traces of late Na$^+$ current (elicited at 0.2 Hz) from cardiomyocytes of 1 month-old WT and R92Q mice (left) and from a R92Q cardiomyocyte in the absence and presence of 10µM ranolazine (center); on the right, average I$_{NaL}$ density integrals, calculated from 25 to 125 ms after the onset of a 500 ms depolarization step from -120mV to -10mV. Means ± S.E.M. from 15 WT (2 mice) and 16 R92Q (2 mice) cardiomyocytes. (B) Left: representative superimposed traces of late Na$^+$ current (elicited at 5 Hz) from a R92Q cell in the absence and presence of RAN. Center: average I$_{NaL}$ density integrals, calculated from 25 to 125 ms after the onset of 150 ms depolarizing steps applied at 5Hz. Right: percentage change of peak Na$^+$ current and I$_{NaL}$-interval by ranolazine in R92Q cardiomyocyte by eliciting the current at 0.2Hz and at 5Hz: notably, the effect at 5Hz is greater. (C) Left: representative superimposed action potential traces from WT (left) and R92Q cardiomyocytes (right) elicited at 5Hz, in the absence and presence of 10µM ranolazine. Center: average action potential duration at 90% of repolarization (APD90% at 5Hz stimulation rate). Means ± S.E.M. from 21 WT cells (6 mice) and 17 R92Q cells (5 mice); Right: percentage change of APD90 by RAN in R92Q cells at 1Hz and at 5Hz: the effect is significantly greater at 5Hz. (D) Scatter graph showing the relationship between diastolic intracellular [Ca$^{2+}$] and [Na$^+$] at 7Hz pacing rate in WT, R92Q cells and the same R92Q cardiomyocytes in the presence of ranolazine. There is a significant linear correlation of [Ca$^{2+}$] and [Na$^+$] only in untreated R92Q cardiomyocytes. When the excess of I$_{NaL}$ in R92Q cells is inhibited using ranolazine, the dependency of intracellular diastolic [Ca$^{2+}$] on intracellular [Na$^+$] is abolished. (E) Average [Ca$^{2+}$] transient amplitude at the different frequencies of stimulation. R92Q cells show reduced CaT amplitude when compared with WT cells. Ranolazine increases CaT amplitude at fast rates in R92Q cardiomyocytes. (D-E) Data from 36 WT cells (3 mice) and 27 R92Q cardiomyocytes (3 mice). (F-H) Wash-out experiments in R92Q myocytes: measurements at 1, 2 and 5 minutes after switching to ranolazine-free solution. (F) Wash-out of Ran effects on I$_{NaL}$; data from 9 R92Q cells (2 mice) (G) Wash-out of Ran effects on diastolic [Ca$^{2+}$]. (H) Wash-out of Ran effects on [Na$^+$]. (G-H) Data from 17 R92Q cells (2 mice). (A-H) Black= WT cells at baseline; Grey= WT with ranolazine; Red= R92Q cells at baseline; Violet= R92Q cells with ranolazine. *=P<0.05, **=P<0.01, calculated with LMM. #=P<0.05, ##=P<0.01, LMM adjusted for paired data groups.
**Supplementary Figure 2**

**Lifelong ranolazine treatment layout in R92Q and WT mice.** Each litter was randomized immediately after birth into one of the two treatment groups on a 1:1 basis. Mice were genotyped at 1 month of age. After genotyping, male mice were fed with the selected chow for a total of 11 to 12 months. At the end of the treatment period, male mice were employed for *in vivo* evaluations or sacrificed for *ex vivo* experiments on explanted hearts.
Ranolazine dosing in mice: Plasma ranolazine concentration was measured in a subgroup of 3-4 months-old WT C57-BL6 mice after feeding with chow containing 0.5% ranolazine and 0.03% ketoconazole for 7 days. A total of 16 animals (50% females) was used for this purpose (4 per group). Male or female animals were sacrificed either at 7:30 AM or at 1:00 PM and the blood extracted. Feeding occurs mainly during nighttime, therefore ranolazine levels at 7:00 AM were higher than at 1:00 PM.
Supplementary Figure 4. Additional western blot studies. (A) Representative western blots for phosphor-PLB at serine 16 (PκA site), total ryanodine receptor (RYR2), phospho-RYR2 at serine 2808 (PκA site) and 2814 (CaMKII site), and GAPDH. (B) Average values WT, R92Q-KET and R92Q-RAN hearts (8 samples each). Relative intensity of individual bands was quantitated and normalized to GAPDH. The ratio for control was assigned a value of 1. Statistical test: OWAT.
## Supplementary Table 1

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>WT-RAN</th>
<th>WT-KET</th>
<th>P WT-KET vs.WT-RAN</th>
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<tr>
<td><strong>TAIL-CUFF MEASUREMENTS IN CONSCIOUS ANIMALS</strong></td>
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<tr>
<td>HEART RATE (BPM)</td>
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<td>673±31</td>
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<td>SYSTOLIC BLOOD PRESSURE</td>
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<td>109±4</td>
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<td>66±3</td>
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<td>MEAN BLOOD PRESSURE</td>
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<td>91±4</td>
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<tr>
<td><strong>ECHOCARDIOGRAPHY IN ANESTHETIZED ANIMALS</strong></td>
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<tr>
<td>DIASTOLIC SEPTAL THICKNESS</td>
<td>0.82±0.05</td>
<td>0.83±0.06</td>
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<tr>
<td>END-DIASTOLIC LV VOLUME</td>
<td>61.5±4.3</td>
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<td>LV-EF (%)</td>
<td>59.2±3.4</td>
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<td>E/A RATIO</td>
<td>1.41±0.08</td>
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<td>LEFT ATRIUM AREA (mm²)</td>
<td>4.32±0.23</td>
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<td>HEART RATE (BPM) FROM ECG</td>
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Comparison between WT-KET and WT-RAN mice: Table shows vital parameters (acquired from conscious animals) and echocardiographic data (from anesthetized animals) recorded from WT mice that were treated with ranolazine-containing chow (RAN) or with vehicle-containing food (KET). Of note, no changes are noted between the two groups, suggesting that lifelong ranolazine treatment does not have any effect in healthy animals. Statistical test: OWAT.
Supplementary Table 2

<table>
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<tr>
<th>PARAMETER</th>
<th>WT-RAN</th>
<th>R92Q-KET</th>
<th>R92Q-RAN</th>
<th>P WT vs. R92Q-KET</th>
<th>P KET vs. RAN</th>
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<tr>
<td>HEART RATE (BPM)</td>
<td>666±41</td>
<td>606±29</td>
<td>600±30</td>
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<td>SYSTOLIC BLOOD PRESSURE (mmHg)</td>
<td>108±4</td>
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<td>DIASTOLIC BLOOD PRESSURE (mmHg)</td>
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<td>MEAN BLOOD PRESSURE (mmHg)</td>
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<td>82±2</td>
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Vital parameters: Table shows vital parameters acquired in conscious animals from the three study groups (WT, R92Q-KET, R92Q-RAN), using a tail cuff pressure/pulse recorder. Statistical test: OWAT.
## Supplementary Table 3

<table>
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<th>PARAMETER</th>
<th>WT-RAN</th>
<th>R92Q-KET</th>
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<th>P KET vs. RAN</th>
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<tr>
<td>Stroke Volume (µL)</td>
<td>37±2</td>
<td>36±2</td>
<td>34±3</td>
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<td>Cardiac Output (mL/min)</td>
<td>20±1</td>
<td>19±1</td>
<td>18±2</td>
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<tr>
<td>Heart Rate (BPM)</td>
<td>487±10</td>
<td>484±9</td>
<td>481±9</td>
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<td>&gt;0.05</td>
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<td>Diastolic LV length (mitral v. to apex, mm)</td>
<td>7.02±0.12</td>
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<td>6.82±0.09</td>
<td><strong>0.008</strong></td>
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<td>LA diameter (mm)</td>
<td>2.30±0.21</td>
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<td><strong>0.045</strong></td>
<td><strong>0.027</strong></td>
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<td>IVRT (LV Isovolumic relaxation time, ms)</td>
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<td>16.8±1.1</td>
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Additional echocardiographic parameters: Table shows additional echocardiographic data recorded from WT, R92Q-KET and R92Q-RAN mice. Statistical test: OWAT.
**Supplementary Table 4**

<table>
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<tr>
<th>GENE</th>
<th>WT-RAN</th>
<th>R92Q-KET</th>
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<th>P WT vs. R92Q-KET</th>
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<td>Kv4.3</td>
<td>1.12±0.14</td>
<td>1.88±0.27</td>
<td>1.21±0.14</td>
<td>0.0224</td>
<td>0.0409</td>
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<tr>
<td>CaCNA1</td>
<td>0.99±0.09</td>
<td>0.87±0.05</td>
<td>0.85±0.08</td>
<td>0.2590</td>
<td>0.8345</td>
</tr>
<tr>
<td>MYH6</td>
<td>1.02±0.07</td>
<td>1.06±0.06</td>
<td>1.44±0.14</td>
<td>0.6695</td>
<td>0.0225</td>
</tr>
<tr>
<td>MYH7</td>
<td>1.10±0.14</td>
<td>5.15±1.48</td>
<td>7.15±2.05</td>
<td>0.0122</td>
<td>0.4392</td>
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<td>TGF-β</td>
<td>0.97±0.17</td>
<td>4.16±1.16</td>
<td>1.16±0.21</td>
<td>0.0140</td>
<td>0.0203</td>
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<tr>
<td>BNP</td>
<td>1.13±0.15</td>
<td>3.26±0.55</td>
<td>1.81±0.29</td>
<td>0.0018</td>
<td>0.0315</td>
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<tr>
<td>ANP</td>
<td>1.06±0.14</td>
<td>13.2±3.27</td>
<td>5.47±1.37</td>
<td>0.0016</td>
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<tr>
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</table>

**mRNA expression:** Table shows semi-quantitative mRNA expression of selected genes in the myocardium of mice from the three study groups (WT, R92Q-KET, R92Q-RAN). Analysis was performed using RT-PCR. Statistical test: OWAT.
References.


