R4496C RyR2 mutation impairs atrial and ventricular contractility

Cecilia Ferrantini,1 Raffaele Coppini,1 Beatrice Scellini,1 Claudia Ferrara,1 José Manuel Pioner,1 Luca Mazzoni,1 Silvia Priori,2 Elisabetta Cerbai,1 Chiara Tesi,1 and Corrado Poggesi1

1Center for Molecular Medicine and Applied Biophysics, University of Florence, 50121 Florence, Italy
2IRCCS Fondazione Salvatore Maugeri, 27100 Pavia, Italy

Ryanodine receptor (RyR2) is the major Ca\textsuperscript{2+} channel of the cardiac sarcoplasmic reticulum (SR) and plays a crucial role in the generation of myocardial force. Changes in RyR2 gating properties and resulting increases in its open probability (P\textsubscript{o}) are associated with Ca\textsuperscript{2+} leakage from the SR and arrhythmias; however, the effects of RyR2 dysfunction on myocardial contractility are unknown. Here, we investigated the possibility that a RyR2 mutation associated with catecholaminergic polymorphic ventricular tachycardia, R4496C, affects the contractile function of atrial and ventricular myocardium. We measured isometric twitch tension in left ventricular and atrial trabeculae from wild-type mice and heterozygous transgenic mice carrying the R4496C RyR2 mutation and found that twitch force was comparable under baseline conditions (30°C, 2 mM [Ca\textsuperscript{2+}]\textsubscript{o}, 1 Hz). However, the positive inotropic response to high stimulation frequency, 0.1 µM isoproterenol, and 5 mM [Ca\textsuperscript{2+}]\textsubscript{o} were decreased in R4496C trabeculae, as was post-rest potentiation. We investigated the mechanisms underlying inotropic insufficiency in R4496C muscles in single ventricular myocytes. Under baseline conditions, the amplitude of the Ca\textsuperscript{2+} transient was normal, despite the reduced SR Ca\textsuperscript{2+} content. Under inotropic challenge, however, R4496C myocytes were unable to boost the amplitude of Ca\textsuperscript{2+} transients because they are incapable of properly increasing the amount of Ca\textsuperscript{2+} stored in the SR because of a larger SR Ca\textsuperscript{2+} leakage. Recovery of force in response to premature stimuli was faster in R4496C myocardium, despite the unchanged rates of recovery of L-type Ca\textsuperscript{2+} channel current (I\textsubscript{Ca-L}) and SR Ca\textsuperscript{2+} content in single myocytes. A faster recovery from inactivation of the mutant R4496C channels could explain this behavior. In conclusion, changes in RyR2 channel gating associated with the R4496C mutation could be directly responsible for the alterations in both ventricular and atrial contractility. The increased RyR2 P\textsubscript{o} and fractional Ca\textsuperscript{2+} release from the SR induced by the R4496C mutation preserves baseline contractility despite a slight decrease in SR Ca\textsuperscript{2+} content, but cannot compensate for the inability to increase SR Ca\textsuperscript{2+} content during inotropic challenge.

INTRODUCTION

The cardiac RyR2 is the main SR Ca\textsuperscript{2+} release channel (Franzini-Armstrong and Protsi, 1997; Bers and Fill, 1998; Lanner et al., 2010). Large amounts of SR Ca\textsuperscript{2+} are released into the cytosol through RyR2 in response to small localized elevations of cytosolic [Ca\textsuperscript{2+}]\textsubscript{i}, generated by Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels during action potentials (CICR). The SR, via RyR2, releases 70–90% of the total Ca\textsuperscript{2+} that activates contraction (Bers, 2002). The amplitude of Ca\textsuperscript{2+} transients depends directly on SR Ca\textsuperscript{2+} content (Bers, 2002) and RyR2 channel gating properties. Importantly for normal Ca\textsuperscript{2+} cycling, the SR becomes refractory after each systolic Ca\textsuperscript{2+} release, preventing spontaneous reactivation of CICR during the diastolic period (Brunello et al., 2013). RyR2 channel refractoriness plays a major role in this regulation (Kornyeyev et al., 2012; Brunello et al., 2013).

RyR2 genetic mutations or acquired defects (e.g., increased phosphorylation of the channel in heart failure) are well known to predispose individuals to arrhythmias (George et al., 2007). However, their impact on contractile function is considered of minor clinical relevance and has been poorly investigated. Mutations in the RyR2 gene were the first to be associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) (Laitinen et al., 2001), and the single amino acid substitution R4497C is one of the earliest RyR2 mutations identified in CPVT patients (Priori et al., 2002). CPVT is characterized by stress-induced syncopal episodes underlying events of ventricular tachycardia (Priori et al., 2002) with a cumulative risk of sudden death of 30–50% by age 35. No echocardiographic signs of contractile impairment are usually found in young CPVT patients, but aging or concurrent acquired disease (Kannankeril et al., 2006) may more easily promote contractile dysfunction.

Experiments on single channels or channels reexpressed in cellular vectors (Jiang et al., 2002) showed that many of the CPVT-associated mutant RyR2 (including R4497C) channels exhibit an increased sensitivity to...
cytosolic and/or SR luminal Ca\(^{2+}\) ([Ca\(^{2+}\)\text{SR}]), and thus an increased open probability (P\(_o\)) and a lower threshold for SR Ca\(^{2+}\) over spill. Consistently, in the myocardium of RyR2 mutation carriers, increase in SR Ca\(^{2+}\) content by inotropic stimuli (e.g., \(\beta\)-adrenergic stimulation, ouabain) enhances aberrant diastolic SR Ca\(^{2+}\) release initiating calcium waves (Sedej et al., 2010) and delayed afterdepolarizations (DADs) (Mohamed et al., 2007; Chen et al., 2012).

Besides promoting cellular arrhythmias (Kashimura et al., 2010), SR Ca\(^{2+}\) leakage during diastole may reduce the amount of SR Ca\(^{2+}\) available for systolic release and myofilament activation. To investigate whether RyR2 mutations alter systolic Ca\(^{2+}\) release and contractile function, we used trabeculae and myocytes from transgenic mice carrying the R4496C RyR2 mutation (Cerrone et al., 2005) (equivalent to R4497C human mutation). R4496C mice show a clear arrhythmogenic phenotype under \(\beta\)-adrenergic stimulation (Liu et al., 2006), resembling the human disease. Here we aim to study whether a RyR2 mutation associated with CPVT affects atrial and ventricular contractile function. In brief, we found that atrial and ventricular myocardium from R4496C mice show impaired force responses to positive inotropic stimuli, related to blunted SR Ca\(^{2+}\) load potentiation and increased SR Ca\(^{2+}\) leakage. The altered gating properties of the RyR2 channel caused by the single amino acid substitution appear to be the sole determinants of the observed abnormalities. Collectively, our results suggest that disease-related primary or secondary modifications of the RyR2, leading to increased P\(_o\) of the channel, not only cause arrhythmias but also determine contractile impairment in the heart.

**MATERIALS AND METHODS**

**Animal model**

We used a knock-in mouse model of CPVT carrying the R4496C RyR2 gene mutation (Cerrone et al., 2005). Suitable trabeculae for mechanical experiments are found more frequently in 129SV/J than in C57BL/6 hearts. Therefore, heterozygous transgenic progenitors with a C57BL/6 genetic background were bred to 129SV/J than in C57BL/6 hearts. Therefore, heterozygous R4496C mutant mice (Charles River) to obtain crossbred mutant carriers. First-generation heterozygous crossbred mice were bred to WT 129SV/J for four generations to obtain mutant carriers bearing a 129SV/J-like phenotype.

The genotypes from the crossbred generations were determined by PCR on DNA from tail biopsy specimens. Persistence of CPVT phenotype in our crossbred strain was confirmed by electrophysiological measurements in isolated myocytes. Animals used for experiments were 16–20-sold males weighing 25–35 g. Animals were maintained and bred at the animal facility of the University of Florence. Animals were bred and used for experiments in accordance with European regulations for animal handling and care, and all experimental protocols were approved by the local committee for animal welfare.

**Intact cardiac preparations**

Right ventricular and left atrial trabeculae were isolated from mouse hearts as described previously (Stull et al., 2002). In brief, male WT (n = 20) or heterozygous R4496C mutant (n = 22) mice were heparinized and anesthetized by inhaled isoflurane. Hearts were rapidly excised and perfused retrogradely with a Krebs–Henseleit buffer containing (mmol/L): 120 NaCl, 5 KCl, 2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 20 NaHCO\(_3\), 1.8 CaCl\(_2\), and 10 glucose, pH 7.4 with 95\% O\(_2\)/5\% CO\(_2\). 20 mM BDM (2,3-butanedione-monoxime) was added to the dissection buffer to reduce cutting injury. The right ventricle was opened by cutting along the anterior interventricular junction. Thin unbranched trabeculae, running between the free wall of the right ventricle and the atrioventricular ring, were dissected by cutting through the atrioventricular ring on one extremity and removing a portion of the right ventricular wall on the other one. The left atrial appendage was gently separated from the remaining cardiac tissue and cut open. Free-running atrial trabeculae were dissected, leaving a block of tissue at both ends to facilitate mounting. Cross-sectional muscle area was calculated with the assumption of an ellipsoid shape.

**Isolation of ventricular cardiomyocytes from mouse hearts**

Left ventricular cells were isolated from male adult WT (n = 16) or heterozygous R4496C mutant (n = 18) mice as described previously (Shioya, 2007). In brief, excised hearts were immediately bathed in HEPES buffer (HB) and cannulated through the aorta for retrograde perfusion at a constant flow of 3 ml/min. The HB contained (mM): 132 NaCl, 4 KCl, 1.2 MgCl\(_2\), 10 HEPES, and 10 glucose, pH 7.35 with NaOH. We then added 0.1 mg/ml Liberase TM (Roche) to the HB, and this enzyme solution was recirculated with 0.1 mM CaCl\(_2\) and 1 mg/ml BSA. Ca\(^{2+}\) concentration was gradually raised up to 1 mM, and isolated cells were stored at room temperature (20°C).

**Mechanical measurements on intact trabeculae**

Ventricular and atrial trabeculae were mounted between the basket-shaped platinum end of a force transducer (KGA; Scientific Instruments-Heidelberg) and a motor (Aurora Scientific Inc.), 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 (Ferrantini et al., 2014). Custom-made software (LabVIEW) was used for motor control, stimulation, and force signal recording.

Muscles were allowed to stabilize at baseline conditions (30°C, 2 mmol/L [CaCl\(_2\)], 1 Hz stimulation) for at least 20–30 min and were gradually stretched to optimal initial sarcomere length (2.15 ± 0.03 µm) before starting the experimental protocol.

**Twitch force and kinetics**

Muscles were exposed to a saturating dose of isoproterenol (10 mM). Muscles were bathed in HEPES buffer (HB) and cannulated through the aorta for retrograde perfusion at a constant flow of 3 ml/min. The HB contained (mM): 132 NaCl, 4 KCl, 1.2 MgCl\(_2\), 10 HEPES, and 10 glucose, pH 7.35 with NaOH. We then added 0.1 mg/ml Liberase TM (Roche) to the HB, and this enzyme solution was recirculated with 0.1 mM CaCl\(_2\) and 1 mg/ml BSA. Ca\(^{2+}\) concentration was gradually raised up to 1 mM, and isolated cells were stored at room temperature (20°C).

**Force–frequency relationship**

Trabeculae were paced at different stimulation frequencies (0.1–8 Hz) to assess changes of sarcomere length (2.15 ± 0.03 µm) before starting the experimental protocol.

**Response to varied extracellular [Ca\(^{2+}\)] or isoproterenol**

Twitch force was measured at 1 Hz, with [Ca\(^{2+}\)\text{SR}] varying between 1 and 10 mM. Muscles were exposed to a saturating dose of isoproterenol (1 µM); recordings were started 10 min after each solution change.

**Force–frequency relationship**

Trabeculae were paced at different stimulation frequencies (0.1–8 Hz) to assess changes of steady-state twitch force.

**Post-rest potentiation**

Stimulation pauses of different duration (2–60 s) were inserted after the last contraction of a steady series at 1 Hz to evaluate post-rest potentiation of the first stimulated beat after the pause.

**Mechanical restitution and post-extrasystolic potentiation**

After 30 s of stimulation at 1 Hz, a premature stimulus was given at...
different premature intervals (from 50 to 900 ms), determining a premature contraction (extrasystole) that was reduced in amplitude. The relative amplitudes of premature beats were plotted against the corresponding stimulus intervals, and the restitution curve was fitted using a single exponential to calculate the restitution rate. Stimulation was resumed 1 s after the premature beat, and the force of the first subsequent beat was measured to evaluate postextrasystolic potentiation.

Electrophysiological and intracellular Ca2+ measurements in intact cardiomyocytes

Freshly isolated cardiomyocytes were used for action potentials, ionic currents, and intracellular Ca2+ measurements. Action potentials or ionic currents were measured under current- and voltage-clamp conditions, respectively, using either the whole-cell perforated-patch or ruptured-patch configurations of the patch-clamp technique. Myocytes were loaded with Ca2+ indicator by a 30-min incubation with 10 μmol/L FluoroForte (Enzo Life Sciences). Cell suspension was then transferred to a temperature-controlled recording chamber with a continuous flow rate of 0.5 ml/min (30 ± 0.5°C), mounted on the stage of an inverted microscope. A rapid solution-change system (Warner Instruments) with a multi-barreled pipette allowed fast (<3-ms) exchange of the solution bathing the cell under study.

For perforated-patch experiments, we used the amphotericin method. For action potential recordings, the pipette solution contained (mM): 115 potassium methanesulfonate, 25 KCl, 3 MgCl2. The standard Tyrode’s buffer contained (mM): 115 potassium methanesulfonate, 25 KCl, 10 glucose, 10 HEPES, and 3 MgCl2. The standard Tyrode’s buffer contained (mM): 115 potassium methanesulfonate, 25 KCl, 10 glucose, 10 HEPES, and 3 MgCl2. For action potential recordings, the pipette solution contained (mM): 110 K+-aspartate, 23 KCl, 0.4 CaCl2 (calculated free Ca2+ = 10-7 M), 3 MgCl2, 5 HEPES-KOH, 1 EGTA-KOH, 0.4 GTP-Na+, 5 ATP-Na+, and 5 creatine phosphate, pH 7.3 (KOH).

The bathing solution contained (mM/L): 140 NaCl, 6 CsCl, 10 glucose, 10 HEPES, 1 MgCl2, 2 CaCl2, 0.02 niflumic acid, and 0.02 TTX, pH 7.4 (CsOH). Ruptured-patch experiments were used to record SR calcium content. The pipette solution contained (mM): 110 K-aspartate, 23 KCl, 0.4 CaCl2 (calculated free Ca2+ = 10-7 M), 3 MgCl2, 5 HEPES-KOH, 1 EGTA-KOH, 0.4 GTP-Na+, 5 ATP-Na+, and 5 creatine phosphate, pH 7.3 (KOH).

Stimulation was resumed 1 s after the premature beat, and the force of the first subsequent beat was measured to evaluate postextrasystolic potentiation.

3-Hz conditioning stimulation in the presence of isoproterenol (30 nmol/L). DADs were scored when spontaneous intracellular Ca2+ oscillations generated a >20-mV membrane depolarization.

Voltage-clamp protocols. ICa-L current–voltage relationship was recorded by imposing 200-ms depolarizing steps from −50 to 50 mV from a holding potential of −80 mV. ICa-L restitution was evaluated by inserting a premature 50-ms depolarizing step to 0 mV after 15 conditioning depolarizing steps at 1 Hz, at variable intervals ranging from 30 to 950 ms. Finally, SR Ca2+ content was studied as follows: after 30–50-msec conditioning steps to 0 mV at 1 Hz (holding = 80 mV) in normal Tyrode’s solution, bathing solution was rapidly switched to expose the cell to 10 mmol/L caffeine for 10 s. Inward Na+-Ca2+-exchanger (NCX)-mediated current during the 10 s of caffeine exposure was integrated to calculate the total amount of charge crossing the membrane (Cout).

Total SR Ca2+ content (expressed as millimole per liter of cytosol) was calculated as follows: [Ca2+]s = [(1 + 0.12)(Cout/F × 1000)]/(Cm*8.31), where Cm = membrane capacitance and F = Faraday’s number (96,500 C/M).

The process was repeated using 2- or 3-Hz conditioning steps (see also Fig. 6). Timing of caffeine exposure was adjusted to get premature or longer intervals from the last conditioning steps: 100–900-ms premature intervals and 2–8-s resting intervals were tested, respectively, assess SR Ca2+ content recovery and SR Ca2+ accumulation during pauses. Baseline frequency was 1 Hz for the recovery protocol and 3 Hz for the pauses protocol. The recovery of SR Ca2+ content was also analyzed by quantifying the relative amplitude of premature caffeine-induced Ca2+ transients with respect to the caffeine-transient evoked 1 s after the last paced beat. At earlier intervals (100–300 ms), the onset of caffeine transient occurred before the end of the last regular transient; therefore, the shape of the average of 10 regular 1-Hz transients (recorded during the conditioning train of stimuli) was subtracted from the fused trace (see Fig. 8 for details).

Statistical analysis

Data are expressed and plotted as the mean ± SEM obtained from several independent determinations on different myocytes or trabeculae. Number of cells/trabeculae (n) and number of animals (N) are indicated in the figure legends for each set of measurements. Unpaired Student’s t test is used for comparisons. A p-value of <0.05 is considered statistically significant. All measurements aimed at comparing WT to R4496C preparations are based on the following assumptions: (a) the variance of different preparations derived from single animals is comparable with the variance found among different animals, and (b) the number of preparations derived from each animal is comparable.

Mathematical modeling

Using the COR CellML platform (Garny et al., 2009), we developed a comprehensive mathematical model of WT and R4496C ventricular cardiomyocytes. A RyR2 gating function featuring channel modulation by cytosolic and luminal [Ca2+]i (Iyer et al., 2007) was inserted into a cardiomyocyte model, derived from Shannon et al. (2004), adapted to the mouse (Bondarenko et al., 2004), and included a function of isometric force generation (Rice et al., 1999). Changes of RyR2 gating caused by R4496C mutation were simulated in the model through appropriate adjustments of RyR2 gating parameters to fit previous data from single mutant RsR2 channels (Jiang et al., 2002) and our experimental results. The model could emulate the experimental protocols performed in cells and trabeculae.

Online supplemental material

The online supplement includes a detailed description of the mathematical model, including an account of the kinetic model of RyR2 gating (Fig. S1) and of the composition of the mouse...
cardiomyocyte model, an overview of the experiments performed to validate the model, as well as an explanation on how the constants describing the behavior of mutant modeled cardiomyocytes were derived (Fig. S2). The ability of the model to predict experimental results is shown in Fig. S3. The complete model file comprising the full set of equations can be found as an online supplement (R4496C_model.cellml) and can be run using freely available CellML programs such as COR or OpenCell (http://cellml.org/tools/modeling-environments). The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201511450/DC1.

RESULTS

Blunted inotropic responses in R4496C myocardium

Under basal experimental conditions (30°C, 1-Hz stimulation rate, 2 mM [Ca²⁺]ₜ₉₉), no changes in isometric twitch tension and kinetics were found between R4496C and WT atrial and ventricular trabeculae (Table 1). Notably, atrial trabeculae display lower tension and faster twitch kinetics when compared with corresponding ventricular trabeculae, in line with previous comparisons of atrial and ventricular myocardium from different mammalian species (Ásgrímsson et al., 1995).

Differences in peak twitch tension between R4496C and WT trabeculae became evident after inotropic stimulation of atrial and ventricular myocardium. Peak tension was lower in R4496C than in WT preparations in response to interventions aimed to markedly increase isometric twitch force, i.e., 10⁻⁷ M isoproterenol (Iso) and 5 mM [Ca²⁺]ₜ₉₉ (Fig. 1 and Table 1). Despite these blunted positive inotropic responses, changes in contraction kinetics caused by the inotropic interventions were still present and comparable to those found in WT trabeculae. In particular, Iso accelerated twitch force generation and relaxation, whereas the application of 5 mM [Ca²⁺]ₜ₉₉ determined a slight prolongation of twitch duration in both WT and R4496C ventricular preparations (Fig. 1 and Table 1). Notably, negligible changes of contraction kinetics in response to Iso or 5 mM [Ca²⁺]ₜ₉₉ were observed in atrial trabeculae from both WT and R4496C mice.

Despite a similar twitch amplitude at 1 Hz, when stimulation frequency was increased to 5 Hz, the physiological rate-dependent increase of contractile force was significantly blunted in R4496C atrial and ventricular preparations as compared with WT trabeculae (Fig. 2 A).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n = 12)</th>
<th>R4496C (n = 10)</th>
<th>P value</th>
<th>WT (n = 12)</th>
<th>R4496C (n = 11)</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Peak tension (mN/mm²)</td>
<td>11.3 ± 1.3</td>
<td>12.9 ± 2.4</td>
<td>NS</td>
<td>26.0 ± 4.4</td>
<td>26.8 ± 4.5</td>
<td>NS</td>
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<td>Time to peak (ms)</td>
<td>44.9 ± 2.4</td>
<td>44.7 ± 1.7</td>
<td>NS</td>
<td>63.4 ± 4.2</td>
<td>64.9 ± 3.7</td>
<td>NS</td>
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<tr>
<td>Time to 50% relaxation (ms)</td>
<td>24.1 ± 1.1</td>
<td>24.8 ± 2.2</td>
<td>NS</td>
<td>42.3 ± 1.7</td>
<td>46.1 ± 1.8</td>
<td>NS</td>
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<td>Peak tension (mN/mm²)</td>
<td>49.3 ± 8.3</td>
<td>22.9 ± 6.4</td>
<td>&lt;0.01</td>
<td>66.3 ± 12.4</td>
<td>41.8 ± 8.5</td>
<td>&lt;0.05</td>
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<td>Time to peak (ms)</td>
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<td>37.7 ± 3.7</td>
<td>NS</td>
<td>43.7 ± 3.6</td>
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<td>22.3 ± 1.9</td>
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<td>Peak tension (mN/mm²)</td>
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<td>25.2 ± 5.6</td>
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<td>65.4 ± 12.2</td>
<td>49.1 ± 9.0</td>
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<td>Time to peak (ms)</td>
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<tr>
<td>Time to 50% relaxation (ms)</td>
<td>25.8 ± 2.7</td>
<td>27.1 ± 3.1</td>
<td>NS</td>
<td>45.3 ± 4.8</td>
<td>46.5 ± 2.3</td>
<td>NS</td>
</tr>
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</table>

Values are expressed as means ± SEM. The p-value is estimated by the Student’s t test. The number in parentheses is the number of preparations. Time to peak is the time from stimulus to peak contraction. Time to 50% relaxation is the time from peak to 50% decay of force.
In spite of some different reports in isolated myocytes (Fernández-Velasco et al., 2009), the relation between tension and stimulation frequency is biphasic in intact mouse myocardium, with a descending limb (0.1–1 Hz at 30°C) and an ascending limb (1–10 Hz) (Stull et al., 2002; Stuyvers et al., 2002). The slope of the ascending limb of the force–frequency relationship was depressed in R4496C atrial and ventricular trabeculae compared with WT preparations (Fig. 2 B). Rate adaptation of twitch duration (i.e., acceleration of contraction kinetics with increase in stimulation frequency) was preserved in R4496C ventricular myocardium (Fig. 2 B). Interestingly, both WT and R4496C atrial trabeculae show no or minimal rate adaptation of twitch duration.

When a stimulation pause was applied during a series of regularly paced twitches at 1 Hz, the increase in amplitude of the first twitch after the pause was significantly blunted in R4496C preparations compared with WT (Fig. 3 A). In all preparations, post-rest potentiation of twitch tension depended on the duration of the rest pause (Fig. 3 B). Maximum post-rest potentiation was markedly lower in R4496C than in WT atrial and ventricular trabeculae and was reached at shorter rest intervals. Accordingly, steady-state twitches elicited at low pacing frequencies (0.1–0.5 Hz) were blunted in R4496C myocardium (Fig. 2 B). These results confirm the decreased ability of R4496C myocardium to increase force in response to positive inotropic interventions. Of note, in WT myocardium, all positive inotropic interventions (isoproterenol, pauses, high frequency) lead to a larger fractional increase of force in the atria, suggesting a higher inotropic reserve in atrial versus ventricular myocardium, as described previously (Ásgrímsson et al., 1995).

Faster mechanical restitution in R4496C myocardium

Mechanical restitution was studied by introducing a premature stimulus into a regular stimulus sequence: the associated contraction (extrasystole) was reduced in amplitude (Fig. 4 A). The force of the extrasystolic beat increases with increasing the interval preceding the premature stimulus, until the steady-state force is again established (mechanical restitution) (Cooper and Fry, 1990). Mechanical restitution curves display the fractional recovery of force (premature twitch force/steady-state twitch force) plotted against the premature stimulus interval. Mechanical restitution was faster in R4496C

![Figure 2. Steady-state force–frequency relationship. Representative traces (A) and mean (±SE) twitch tension (B; top) show a flattened force–frequency relationship in R4496C atrial and ventricular trabeculae when compared with WT preparations. Tension is normalized to 1-Hz twitch amplitude. § = P < 0.05 for 4–7-Hz frequencies. In atrial preparations, relative force at 0.5 Hz was also significantly lower in R4496C versus WT trabeculae. (Bottom) No differences were found in the kinetics of twitches at different frequencies of stimulation (Peak, time to peak contraction; RT50, time to 50% relaxation). Error bars represent mean ± SEM.](image)

![Figure 3. Post-rest potentiation. (A) Traces showing reduced maximal post-rest force in a typical R4496C ventricular trabecula compared with WT. (B) Average percent increase of twitch force after different intervals of stimulation pauses from a basal frequency of 1 Hz in atrial (left) and ventricular (right) R4496C versus WT trabeculae. In atrial trabeculae, absolute maximal post-rest twitch force at a 60-s rest interval was 53.3 ± 9.6 mN/mm² in WT versus 19.9 ± 4.6 mN/mm² in R4496C (P < 0.01). In ventricular trabeculae, maximal post-rest twitch force at a 60-s rest interval was 76.3 ± 15.6 mN/mm² in WT versus 44.3 ± 8.6 mN/mm² in R4496C (P < 0.01). Error bars represent mean ± SEM.](image)
β-Adrenergic stimulation, by maximally speeding up SR Ca\(^{2+}\) uptake, accelerated mechanical restitution in both WT and R4496C preparations (Fig. 4 B, inset). However, recovery of isometric force in the presence of isoproterenol was still significantly faster in R4496C trabeculae as compared with WT muscles. Notably,
post-extrasystolic potentiation was reduced in R4496C versus WT atrial trabeculae (Fig. 4 C), in line with the observed reduction of post-rest potentiation in R4496C myocardium.

Mechanisms underlying the blunted inotropic response of R4496C myocardium

To better understand the mechanisms underlying the blunted inotropic response of R4496C trabeculae, we investigated excitation–contraction coupling of single ventricular cardiomyocytes from R4496C and WT hearts by simultaneous patch clamp and intracellular Ca2+ measurements. Patch-clamp experiments showed that no significant alterations of basal membrane electrical activity were present in R4496C ventricular myocytes. Duration of action potentials was comparable at all frequencies investigated in WT and R4496C myocytes (Fig. 5, A and B), and no differences were observed in the amplitude and voltage dependency of L-type Ca2+ current (Fig. 5, C and D).

Under the same basal conditions used in trabeculae (30°C, 2 mM [Ca2+]o), Ca2+-transient amplitude and kinetics did not differ between R4496C and WT cardiomyocytes (Fig. 5, E and F) at a 1-Hz stimulation rate, in line with the absence of contractile impairment observed under basal conditions in intact R4496C trabeculae. However, when cardiomyocytes were paced at higher frequencies (2–3 Hz), Ca2+-transient amplitude of R4496 cardiomyocytes was reduced with respect to WT (Fig. 5, E and F).

R4496C and WT myocytes were then rapidly exposed to 10 mM caffeine after a train of conditioning depolarizing steps at 1, 2, and 3 Hz, to study SR Ca2+ content, as calculated from the integral of NCX current (Fig. 6 A). SR Ca2+ content in R4496C myocytes was slightly reduced compared with WT at 1 Hz (−16%), in agreement with previous observations on the same transgenic model (Kashimura et al., 2010). The difference in SR Ca2+ content between R4496C and WT myocytes became larger at higher stimulation rates, reaching −34% at 3 Hz (Fig. 6 B). These results indicate that R4496C cardiomyocytes are able to maintain Ca2+-transient amplitude despite a lower SR Ca2+ content at low inotropic levels, i.e., 1 Hz. However, the reduced ability to store Ca2+ in the SR causes a blunted Ca2+-transient amplitude at higher pacing rates.

When stimulation pauses were introduced in a series of regularly paced Ca2+ transients at 3 Hz in current clamp, the post-rest increase of Ca2+-transient amplitude was blunted in R4496C versus WT myocytes (Fig. 7, A and C), in agreement with the reduced post-rest potentiation of force observed in the mutant trabeculae. Interestingly, when SR Ca2+ content was measured by suddenly exposing the cell to caffeine after pauses of different durations, the physiological increase of SR Ca2+ content after the pause was absent in R4496C myocytes (Fig. 7, B and D). As an example, after a pause of 8 s, SR Ca2+ content increased by 21 ± 4% in WT, whereas it tended to decrease in R4496C cardiomyocytes (P < 0.05; Fig. 7 D). These results suggest that a reduced ability to accumulate Ca2+ in the SR during rest intervals underlies the blunted response to stimulation pauses. During stimulation pauses, diastolic intracellular Ca2+ oscillations (Fig. 7 A) were observed in 73% of R4496C (n = 16/22) versus 5% of WT (n = 1/20) myocytes (P < 0.01; Fisher’s exact test). Consistently, spontaneous contractions were observed during long (60-s) rest intervals in 33% of R4496C trabeculae (n = 4/12) but were never seen in WT preparations (n = 0/13) (P < 0.01; Fisher’s exact test). An increased rate of diastolic intracellular Ca2+ oscillations suggests enhanced spontaneous Ca2+ releases from the SR, which likely contribute to store depletion in R4496C myocytes and explain post-extrasystolic potentiation was reduced in R4496C versus WT atrial trabeculae (Fig. 4 C), in line with the observed reduction of post-rest potentiation in R4496C myocardium.

Mechanisms underlying the blunted inotropic response of R4496C myocardium

To better understand the mechanisms underlying the blunted inotropic response of R4496C trabeculae, we investigated excitation–contraction coupling of single ventricular cardiomyocytes from R4496C and WT hearts by simultaneous patch clamp and intracellular Ca2+ measurements. Patch-clamp experiments showed that no significant alterations of basal membrane electrical activity were present in R4496C ventricular myocytes. Duration of action potentials was comparable at all frequencies investigated in WT and R4496C myocytes (Fig. 5, A and B), and no differences were observed in the amplitude and voltage dependency of L-type Ca2+ current (Fig. 5, C and D).

Under the same basal conditions used in trabeculae (30°C, 2 mM [Ca2+]o), Ca2+-transient amplitude and kinetics did not differ between R4496C and WT cardiomyocytes (Fig. 5, E and F) at a 1-Hz stimulation rate, in line with the absence of contractile impairment observed under basal conditions in intact R4496C trabeculae. However, when cardiomyocytes were paced at higher frequencies (2–3 Hz), Ca2+-transient amplitude of R4496 cardiomyocytes was reduced with respect to WT (Fig. 5, E and F).

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the blunted increase in SR Ca\textsuperscript{2+} load during resting pauses (Fig. 7 D).

In agreement with previous findings (Liu et al., 2006; Fernández-Velasco et al., 2009; Kashimura et al., 2010), an increased occurrence of spontaneous diastolic intracellular [Ca\textsuperscript{2+}], oscillations was also observed in R4496C versus WT ventricular cardiomyocytes under Iso (96% of R4496C myocytes vs. 38% of WT myocytes; P < 0.01; Fisher’s exact test).

Mechanisms underlying the faster mechanical restitution of R4496C myocardium
Mechanical restitution of cardiac muscle reflects the interplay between sarcolemma and SR refractoriness. Specifically, for very premature beats, e.g., <100 ms, electrical refractoriness and I\textsubscript{Ca,L} recovery are the major determinants of the mechanical phenomenon (Franz, 2003); recovery of calcium release (dictated by RyR gating properties; Banijamali et al., 1991; Kornyeyev et al., 2012; Brunello et al., 2013) and the rate of SR Ca\textsuperscript{2+} refilling (Poggesi et al., 1987) affect the later stages, when sarcolemma recovery is already completed. To understand the mechanisms underlying the faster mechanical restitution observed in R4496C intact myocardium, we evaluated the restitution rates of I\textsubscript{Ca,L}, Ca\textsuperscript{2+} transient, and SR Ca\textsuperscript{2+} content in ventricular myocytes using specific patch-clamp protocols. All restitution curves display the fractional recovery of the studied parameter plotted against the duration of the premature interval. As expected, the faster mechanical restitution (Fig. 8 A) was paralleled by a quicker recovery of Ca\textsuperscript{2+}-transient amplitude in R4496C ventricular myocytes (Fig. 8 B). No differences were present between R4496C and WT cardiomyocytes in terms of membrane electrical refactoriness (54 ± 6 ms in R4496C vs. 57 ± 7 ms in WT; P = 0.45) and the recovery properties of I\textsubscript{Ca,L} (Fig. 8 A). Furthermore, we evaluated the rate of SR Ca\textsuperscript{2+} content recovery by rapidly exposing regularly stimulated cardiomyocytes to caffeine at different premature intervals (Fig. 8 C). SR Ca\textsuperscript{2+} recovery rate was similar in R4496C and WT cells (Fig. 8 D). This supports the hypothesis that R4496C mutation speeds up restitution by altering the gating properties of the RyR2 and not by increasing the rate of SR refilling.

Mathematical model
Experimental data from intact trabeculae and isolated cardiomyocytes were fitted into a validated cardiomyocyte computational model (Shannon et al., 2005; Garny et al., 2009; see supplemental file), where RyR2 gating is modulated by cytosolic [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}]\textsubscript{i}) as well as by SR luminal [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}]\textsubscript{SR}). To best simulate the features of the R4496C mutation, the rate constants of both activation and deactivation of RyR2 channels were modified, as detailed in the supplemental file. In line

Figure 7. Effects of pauses on Ca\textsuperscript{2+} transients and SR Ca\textsuperscript{2+} load. (A) Representative traces of Ca\textsuperscript{2+} transients from R4496C and WT ventricular myocytes during stimulation pauses imposed after a conditioning stimulation at 3 Hz. Arrows mark the first beats triggered by external stimulation after the resting period. The amplitude of the first peak is analyzed to assess post-rest potentiation of Ca\textsuperscript{2+}-transient amplitude. Diastolic intracellular Ca\textsuperscript{2+} oscillations and spontaneous Ca\textsuperscript{2+} transients occur in the R4496C ventricular myocyte during the stimulation pause. (B) Simultaneous Ca\textsuperscript{2+} fluorescence traces recorded during the application of caffeine at different resting intervals, after a conditioning stimulation at 3 Hz in WT (left) and R4496C (right) myocytes. Traces where caffeine is delivered after pauses of different duration (1.5–3.5 s; conditioning activation train at 3 Hz) are shown superimposed. (C and D) Average percent increase of Ca\textsuperscript{2+}-transient amplitude (C) and SR Ca\textsuperscript{2+} load (D) after stimulation pauses of different duration in R4496C (n = 22 and 5) versus WT (n = 20 and 4) ventricular myocytes. Error bars represent mean ± SEM.
knock-in mouse model carrying the R4496C mutation (Cerrone et al., 2005; Liu et al., 2006; Fernández-Velasco et al., 2009). We compared heterozygous R4496C mice with WT siblings. Previous work on these mutant mice was focused on the pro-arrhythmogenic significance of the R4496C mutation, demonstrating enhanced diastolic SR Ca²⁺ leakage (Kashimura et al., 2010) and DADs at the cellular level (Liu et al., 2006), paralleled by ventricular and supraventricular arrhythmias in the whole heart (Cerrone et al., 2005, 2007). Here, we studied the R4496C mutation from the mechanical perspective, recording twitch tension from intact trabeculae in isometric, load-controlled conditions. We found that the R4496C mutation impairs contractility of both atrial and ventricular myocardium, consistently with ubiquitous RyR2 expression in the heart. Although steady-state systolic Ca²⁺ release and force development under basal experimental conditions are normal, upon inotropic stimulations, which resemble stress or exercise conditions (i.e., β-adrenergic activation or high beating rates), atrial and ventricular myocardium of R4496C mice show impaired ability to raise SR Ca²⁺ load, increase the

**DISCUSSION**

The impact of CPVT-related RyR2 mutations on cardiac muscle contractility was investigated using a validated

![Diagram](https://example.com/diagram.png)

**Figure 8.** Restitution of twitches, L-type Ca²⁺ current, Ca²⁺ transients, and SR Ca²⁺ load in ventricular myocardium. All restitution curves are fitted by a single exponential to obtain restitution rates (1/τ). (A) Average mechanical restitution curves from nine WT (n = 7) and nine R4496C (n = 8) ventricular trabeculae and L-type Ca²⁺ current recovery curves from 15 WT (n = 3) and 14 R4496C (n = 3) myocytes are shown superimposed. Rates for mechanical restitution: 1.96 ± 0.11 s⁻¹ in WT and 2.63 ± 0.34 s⁻¹ in R4496C; P < 0.01. Rates for L-type Ca²⁺ current recovery: 8.68 ± 0.35 s⁻¹ in WT and 8.79 ± 0.41 s⁻¹ in R4496C; P = 0.51. (B) Average curves showing the restitution of Ca²⁺-transient amplitude in 21 WT (n = 4) and 23 R4496C (n = 5) ventricular myocytes. Rates: 3.72 ± 0.34 s⁻¹ in WT and 5.33 ± 0.64 s⁻¹ in R4496C; P < 0.05. (C) Representative examples of simultaneous intracellular Ca²⁺-transient and membrane current recordings during rapid application of 20 mM caffeine at different premature intervals. (Inset) Caffeine-induced Ca²⁺ transient obtained by subtracting the average shape of the preceding regular Ca²⁺ transients from the fused traces. (D) SR Ca²⁺ load recovery curves calculated from both the amplitude of caffeine-induced Ca²⁺ transient and the integration of inward NCX current. Data are derived from 13 WT (n = 3) and 15 R4496C (n = 3) myocytes. Rates calculated from caffeine-induced Ca²⁺ transient: 7.15 ± 0.21 s⁻¹ in WT and 7.71 ± 0.29 s⁻¹ in R4496C; P = 0.48. Rates calculated from integration of the inward NCX current: 8.63 ± 0.23 s⁻¹ in WT and 8.74 ± 0.31 s⁻¹ in R4496C; P = 0.48. Error bars represent mean ± SEM.
amount of Ca\(^{2+}\) released from the SR, and enhance contractile force. Positive inotropic interventions, such as high [Ca\(^{2+}\)]\(_{\text{out}}\) and long stimulation pauses, produced similar results. Consistently, we found enhanced occurrence of spontaneous Ca\(^{2+}\) oscillations during pauses and β-adrenergic stimulation in single cells, which suggests increased diastolic SR Ca\(^{2+}\) losses. Consistently, Fernández-Velasco et al. (2009) reported enhanced occurrence of Ca\(^{2+}\) sparks in unstimulated R4496C myocytes (resembling long stimulation pauses); Sedej et al. (2010) showed a larger increase in Ca\(^{2+}\) wave amplitude and frequency in R4496C myocytes by raising SR Ca\(^{2+}\) load with ouabain. Interestingly, the kinetics of Ca\(^{2+}\) transients and force twitches were always preserved in R4496C myocardium, both at baseline and under different inotropic challenges.

Of note, the intrinsic limitations of single cardiomyocyte studies prevented us from testing pacing frequencies higher than 3 Hz in cells. Intact trabeculae, however, were paced up to 7 Hz, which is well within the physiological range of mouse heart rates. At 7 Hz, mechanical impairment in R4496C atrial and ventricular trabeculae was larger than that seen at 3 Hz; therefore, we can predict that the underlying changes of Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load would also be larger in the physiological range of frequencies (7–10 Hz).

Although the R4496C mutation is the initiating element, secondary changes, perhaps compensatory, may contribute to determining the observed SR Ca\(^{2+}\) load depletion and contractile alterations. The following arguments exclude a role of changes other than those introduced by the mutation itself. A relative reduction of SR Ca\(^{2+}\) ATPase (SERCA) versus NCX function could lead to reduced SR Ca\(^{2+}\) load. However, in R4496C, there is no increase in NCX activity as estimated from caffeine-induced Ca\(^{2+}\)-transient decay rate and no hints of reduced SERCA function (e.g., no slower Ca\(^{2+}\)-transient decay), phospholamban expression, or phosphorylation (Fernández-Velasco et al., 2009; Kashimura et al., 2010). Reduced sarcolemmal Ca\(^{2+}\) influx could impair Ca\(^{2+}\) accumulation in R4496C myocytes, but no difference between R4496C and WT myocytes was found in the amplitude, voltage dependence, and recovery of I_{Ca-L}. Ca\(^{2+}\)-transient and force decay rates are similarly accelerated by isoproterenol in R4496C and WT ventricular preparations. This rules out major alterations of the β-adrenergic receptor response in R4496C mice. Finally, previous work has shown that in R4496C myocardium, RyR2 expression and phosphorylation levels are unchanged (Fernández-Velasco et al., 2009).

We conclude that all contractile alterations observed in cardiac muscle from R4496C mice can be accounted for by changes in RyR2 channel gating. Single-channel recordings on HEK293 cells re-expressing RyR2 mutant channels have shown that the R4496C mutation increases RyR2 P_{o} by approximately three times by destabilizing the closed state of the channel; mean closed time was several times shorter in R4496C RyR2 compared with WT channel, whereas mean opening time was mildly affected (Jiang et al., 2002, 2004). Because of the increased sensitivity of mutant RyR2 activation to luminal [Ca\(^{2+}\)], the threshold SR Ca\(^{2+}\) load at which spontaneous SR Ca\(^{2+}\) release and Ca\(^{2+}\) waves occur is lower in R4496C myocytes compared with WT; as a consequence, positive inotropic interventions are less effective at increasing SR Ca\(^{2+}\) load (Kashimura et al., 2010) caused by increased loss of SR Ca\(^{2+}\) during diastole. At baseline conditions, the increased SR fractional release caused by the altered channel gating of mutant RyR2 helps maintain Ca\(^{2+}\)-transient and twitch amplitudes despite a slightly reduced SR Ca\(^{2+}\) load (Jiang et al., 2002; Eisner et al., 2009). According to our results, under
Implications for human pathology

The failure of R4496C myocardium to increase force upon inotropic challenges contrasts with the apparent lack of functional impairment and/or cardiomyopathy in CPVT mutant mice and patients. Although intact trabeculae are a good approximation of the whole myocardium, the isometric conditions may somewhat enhance the difference between mutants and WT: a relatively large difference of peak twitch force in trabeculae could translate in a smaller decrease of cardiac output in the whole heart. Indeed, numerous hemodynamic adaptations may occur in vivo and ameliorate the effects of a reduced SR Ca²⁺ load reserve, thus avoiding development of overt cardiomyopathy. For example, in mice and patients with RyR2 mutations, resting bradycardia is commonly observed (Leenhardt et al., 1995; Priori et al., 2002; Neco et al., 2012). Lower heart rate may help maintain stroke volume via prolonged diastolic filling and increased end-diastolic volumes, despite mildly reduced contractility. Stress echocardiography on patients with R4497C RyR2 mutation could shed light on possible contractile impairment during exercise but, unfortunately, no such detailed assessments are available (Leenhardt et al., 1995). Indeed, as physical exertion and stress are the main triggers for arrhythmias in CPVT, diagnosed patients are instructed to forgo sport activity and symptom-limited ECG stress tests are performed only at the time of diagnosis and are avoided during clinical follow-up (Hayashi et al., 2009; van der Werf et al., 2012a; Wangüemert et al., 2015). Furthermore, the majority of CPVT patients are under β-blocker therapy, usually with potent, unselective agents such as
propranolol or nadolol (van der Werf et al., 2012b), or are subjected to cardiac left sympathetic denervation (De Ferrari et al., 2015); both strategies cause a marked limitation to the exercise capacity of patients. Therefore, it is hard to determine whether exercise limitation caused by contractile dysfunction is present in CPVT patients, and further investigation on selected patient cohorts is warranted.

The stress-induced contractile dysfunction caused by RyR2 mutations may contribute to the induction of arrhythmias during exercise in CPVT patients. The insufficient increase of cardiac output may create a positive feedback on the sympathetic system leading to hyper-activation of the adrenergic response. In addition, a marked contractile dysfunction leading to reduced cardiac output at high heart rates may cause myocardial perfusion deficit in the sub-endocardium, increasing the dispersion of repolarization, thereby promoting the degeneration of ventricular tachycardia to reentry-based ventricular fibrillation (Viitasalo et al., 2008).

A reduced contractile reserve, that scarcely affects contractile function of young patients, may significantly reduce tolerance to a sustained high mechanical load, e.g., in the presence of hypertension. Recent work on a RyR2 mutant mouse model showed that the mutation favors the development of cardiac hypertrophy and heart failure in response to chronic pressure overload (van Oort et al., 2010) or overexpression of CaMKIIβ (Dybkova et al., 2011). Because of its relatively poor prognosis, CPVT is usually diagnosed in adolescents and young adults; thus, all patient cohorts described in the literature are mainly comprised of young patients (Hayashi et al., 2009; Wangiémert et al., 2015). This makes it hard to predict how CPVT patients would respond to aging and aging-related cardiac illnesses, such as hypertension or coronary heart disease. This aspect may become clearer in the near future, as patient survival has significantly increased thanks to the use of implantable cardiac defibrillators and the introduction of new therapeutic strategies in addition to β blockers (e.g., flecainide, cardiac sympathetic denervation) (van der Werf et al., 2011; De Ferrari et al., 2015).

RyR2 mutations can directly cause arrhythmogenic right ventricular cardiomyopathy (Milting et al., 2006), where stress and exercise-induced arrhythmias are associated with a marked mechanical impairment of the right ventricle; the mechanisms shown here may explain the development of right-ventricular dysfunction and dilatation in RyR2-linked arrhythmogenic right ventricular cardiomyopathy.

Besides the rare disease-causing RyR2 mutations, a larger number of more common RyR2 polymorphisms may similarly affect RyR2 gating properties, although to a minor extent. These RyR2 polymorphisms, although benign in healthy patients, may modify the phenotypic expression of primary and secondary cardiomyopathies by altering the mechanical properties of myocardium under stress.

Finally, increased phosphorylation of RyR2, either by PKA or by CaMKII, has been found in human and animal models of genetic cardiomyopathies (i.e., hypertrophic cardiomyopathy; Coppini et al., 2013) and acquired cardiac diseases (i.e., ischemic heart failure; Fischer et al., 2013), leading to changes of RyR2 gating that resemble CPVT-related mutations. In line with our results, we can speculate that changes of RyR2 gating caused by hyperphosphorylation may significantly contribute to contractile dysfunction in many acquired cardiac diseases. Pharmacological modulation of RyR2 gating in cardiac diseases may therefore be beneficial not only for arrhythmia reduction but also for contractile improvement.

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Mathematical model

1—RyR2 gating model

To test the effects of an altered sensitivity to luminal calcium on the average kinetic properties of the channel, a simple model of RyR2 function was developed, written in Labview 2009 (National Instruments), and appropriate test simulations were run as described below. RyR2 gating is represented by a classical four-state equilibrium as in Keizer and Levine (1996), which includes two closed states, resting state (C1) and closed-adapted state (C2), and two open states, fully open state (O1) and open-adapted state (O2). At rest, the channel resides primarily in the closed state C1. Upon an increase in junctional Ca2+, the channel switches briefly to the open state O1, allowing Ca2+ to move through the channel before it reaches the closed-adapted state (transition to C2). Upon additional increases in Ca2+, the channel reopens by its transition to open-adapted state O2, displaying adaptive behavior in agreement with single-channel experiments (Györke and Fill, 1993). All of the rate constants are increased over the original Keizer and Levine (1996) values to adapt the model to the in vivo condition: RyR is located in the junctional space, where it is exposed to [Ca2+]jct exceeding 10.0 mM (Santana et al., 1996). In addition, changes of junctional [Ca2+] levels are more rapid than [Ca2+]i. Thus, the rate transition rates was added to the model as in Iyer et al. (1998). Moreover, modulation by luminal Ca2+ of state transition rates was added to the model as in Iyer et al. (2007). RyR2 transitions with the corresponding rate constants are depicted in the following scheme:

\[
\frac{dO2}{dt} = k^+_b [Ca^{2+}]_{jct} \cdot O1 - k^-_b \cdot O2
\]

\[
\frac{dC2}{dt} = k^+_c / k_{lumen\_deact} \cdot O1 - k^-_c \cdot k_{lumen\_act} \cdot C2
\]

\[
\frac{dO1}{dt} = C1 \cdot \left( k_c^+ \cdot k_{lumen\_act} \cdot [Ca^{2+}]_{jct} - k^-_c / k_{lumen\_deact} \right) + O2 \cdot k^+_c + C2 \cdot \left( k^-_c \cdot k_{lumen\_act} \right)
\]

We used the same RyR2 rate constants as Iyer et al. (2007), as indicated in Table S1, adjusted from the original Keizer and Levine (1996) work to compensate for the higher junctional [Ca2+].

Of note, the apparent rate constants for RyR2-state transitions (k^+_c \cdot k_{lumen\_act} for C1O1 state transition, k^-_c \cdot k_{lumen\_act} for C2O1, and k^+_c \cdot k_{lumen\_deact} for O1C2) are scaled by Hill functions of [Ca2+]SR, k_{lumen\_act} and k_{lumen\_deact} providing a graded modulation of RyR2 gating by [Ca2+]SR, as in Iyer et al. (2007). It was recently shown that RyR2 mutations can differently alter initiation and termination of Ca2+ release by differently affecting the Ca2+ sensitivity of the channel on the luminal side (Liu et al., 2015); therefore, we include distinct modulation of RyR2 activation and deactivation by [Ca2+]SR by using two different Hill parameters, k_{lumen\_act} for activating transitions and k_{lumen\_deact} for deactivating transition O1C2.

\[
k_{lumen\_act} = \frac{H_{max\_act} - H_{min\_act}}{1 + \left( \frac{[Ca^{2+}]_{SR}}{H_{50\_act}} \right)^{HN}}
\]

\[
k_{lumen\_deact} = \frac{H_{max\_deact} - H_{min\_deact}}{1 + \left( \frac{[Ca^{2+}]_{SR}}{H_{50\_deact}} \right)^{HN}}
\]

Mutation-driven changes of RyR2 gating are simulated in the model through appropriate adjustments of Hill constants (H_{max\_act}, H_{50\_act}, H_{max\_deact}, H_{50\_deact}) for the two [Ca2+]SR-dependent Hill parameters (k_{lumen\_act} and k_{lumen\_deact}). Initial values for Hill constants of WT and R4496C mutant RyR2 were initially chosen to fit previous data of single-channel currents recorded from RyR2s in lipid bilayers reporting the average P0 and opening times of WT and R4496C channels at different Ca2+ concentrations on the luminal side (Jiang et al., 2004, 2005). Our simple gating model was used to calculate changes in channel P0 in response to changes of [Ca2+]SR-dependent Hill parameters. Jiang et al. (2004).
report a single-channel overall average $P_0$ for mutant channels of 0.022 for WT channels and 0.077 for R4496C channels at 300 µmol/L $[Ca^{2+}]_{SR}$ and diastolic $[Ca^{2+}]_{jct}$ (<0.1 µmol/L). Because only luminal dependency of channel activation was investigated by Jiang et al. (2004) using a technique using fixed $Ca^{2+}$ concentrations and no data are available on channel deactivation, only $k_{lumen\_act}$ parameters could be derived from this work. However, as described below and in the main paper, changes of $k_{lumen\_act}$ parameters only do not reproduce all of our experimental results and additional changes of $k_{lumen\_deact}$ need to be introduced. Final values for $k_{lumen\_act}$ and $k_{lumen\_deact}$ constants were adjusted according to the results obtained with our cardiomyocyte model (see below) to fit our experimental values.

Final adjusted Hill constants for luminal calcium dependency parameters of WT and RyR2 mutants can be found in Table S2 and were used for all the simulations described, unless otherwise specified.

As expected by previous observations on single RYR2 channels (Jiang et al., 2002), modeled WT RyR2 peak $P_0$ is minimal at diastolic $[Ca^{2+}]_{jct}$ and rises sigmoidally with $[Ca^{2+}]_{jct}$ increase (Fig. S1 A). Luminal $[Ca^{2+}]_{i}$ actively modulates the RyR2 sensitivity to junctional $Ca^{2+}$: at higher $[Ca^{2+}]_{SR}$, the relationship between RyR2 $P_0$ and junctional $Ca^{2+}$ is left-shifted (Fig. S1 A), meaning that for any given $[Ca^{2+}]_{jct}$, even in the diastolic range (<150 nM), $P_0$ is higher at higher $[Ca^{2+}]_{SR}$ (Fig. S1 B). Changes of $k_{lumen\_act}$ Hill constants increase the sensitivity of modeled R4496C RyR2 to $[Ca^{2+}]_{jct}$ for channel activation: the mutant channel’s $P_0$–$[Ca^{2+}]_{jct}$ relationship is left-shifted in a range of diastolic luminal $Ca^{2+}$ concentrations (Fig. S1 C). Changes introduced by sim-

### Table S1

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### Table S2

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ulated mutation increased RyR2 $P_o$ at diastolic cytosolic $[\text{Ca}^{2+}]$ from <1% to nearly 5% at normal diastolic $[\text{Ca}^{2+}]_{\text{SR}}$; a slight increase of $[\text{Ca}^{2+}]_{\text{SR}}$ is sufficient to significantly increase diastolic $P_o$ above 10%, making the probability of massive propagated calcium release events in the cell relatively high. These effects are in line with the experimental observation of a two- to fourfold increase of the rate of Ca$^{2+}$ sparks in unstimulated cardiomyocytes from R4496C mutant mice when compared with WT (Fernández-Velasco et al., 2009). In WT RyR2, the same $[\text{Ca}^{2+}]_{\text{SR}}$ increase only slightly raises $P_o$ (to 2%; Fig. S1 D). According to our model of R4496C mutation, RyR2 channels have a higher probability of residing in an open state during diastole, especially when $[\text{Ca}^{2+}]_{\text{SR}}$ is high, increasing the rate of spontaneous diastolic Ca$^{2+}$ releases. Furthermore, Terentyev et al. (2003) showed that alterations of the luminal Ca$^{2+}$sensing capability of the RyR2 channels caused by reduced calsequestrin function (as in CPVT-associated CSQ mutations) led to faster RyR2 recovery from inactivation. Notably, $k_{\text{lumen, act}}$ also influences the reactivation transition (C2→O1), and changes of $k_{\text{lumen, act}}$ may directly explain the faster restitution properties in the presence of the R4496C mutation (see below). Moreover, myocytes carrying different CPVT-associated RyR mutations display elemental Ca$^{2+}$ sparks with higher amplitude and prolonged duration (Fernández-Velasco et al., 2009; Jung et al., 2012), consistent with delayed termination of release in the mutant compared with WT myocytes. Delayed termination of release was therefore incorporated in the mutant model, by decreasing maximal $k_{\text{lumen}}$ for the O1→C2 adaptation transition ($K_{\text{lumen, deact}}$). Notably, CSQ mutations or CSQ underexpression led to opposite effects on Ca release termination (i.e., faster release termination; Terentyev et al., 2003). As specified below, the inclusion of changes in $K_{\text{lumen, deact}}$ was essential to predict the abnormalities seen in R4496C myocytes.

As described below, when integrated in a comprehensive ventricular cardiomyocyte model, the modified Keizer–Levine formulation that we use is able to predict the behavior of RyR channels during regularly paced stimulation. However, the deterministic formulation is unable to predict stochastic channel openings occurring during prolonged diastolic intervals. To compensate for this, an arbitrary function describing continuous Ca leakage from the SR has been added, as in Shannon et al. (2004). However, the presence of a fixed leakage equation, independent from the constants describing RyR2 gating, prevented a proper prediction of RyR2 abnormalities during prolonged diastolic periods in simulated R4496C myocytes (see below for details).

<table>
<thead>
<tr>
<th>Parameter Type</th>
<th>Experiment (Mean ± SD)</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$ transients (isolated myocytes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic $[\text{Ca}^{2+}]_i$</td>
<td>164 ± 31 nM</td>
<td>139 nM</td>
</tr>
<tr>
<td>Amplitude (peak-diast.$[\text{Ca}^{2+}]_i$)</td>
<td>599 ± 115 nM</td>
<td>551 nM</td>
</tr>
<tr>
<td>Time to peak</td>
<td>38.9 ± 1.5 ms</td>
<td>40 ms</td>
</tr>
<tr>
<td>Time to 50% decay</td>
<td>135.2 ± 7.2 ms</td>
<td>129 ms</td>
</tr>
<tr>
<td>Time to 90% decay</td>
<td>201 ± 13.4 ms</td>
<td>191 ms</td>
</tr>
<tr>
<td>Action potentials (isolated myocytes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest potential</td>
<td>$-78.56 ± 2.2$ mV</td>
<td>$-80$ mV</td>
</tr>
<tr>
<td>AP amplitude</td>
<td>113 ± 7.5 mV</td>
<td>120 mV</td>
</tr>
<tr>
<td>Time to 20% repolarization</td>
<td>7.1 ± 1.6 ms</td>
<td>7.6 ms</td>
</tr>
<tr>
<td>Time to 50% repolarization</td>
<td>37.4 ± 5.1 ms</td>
<td>36.3 ms</td>
</tr>
<tr>
<td>Time to 90% repolarization</td>
<td>79.2 ± 7.2 ms</td>
<td>76.1 ms</td>
</tr>
<tr>
<td>Force twitches (ventricular trabeculae)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>26.8 ± 4.5 mN/mm$^2$</td>
<td>29 mN/mm$^2$</td>
</tr>
<tr>
<td>Time to peak</td>
<td>64.9 ± 3.7 ms</td>
<td>70 ms</td>
</tr>
<tr>
<td>Time to 50% relaxation</td>
<td>46.1 ± 1.8 ms</td>
<td>51 ms</td>
</tr>
<tr>
<td>Time to 90% relaxation</td>
<td>141 ± 11.3 ms</td>
<td>151 ms</td>
</tr>
</tbody>
</table>
The mathematical Ca$^{2+}$-handling model used in this model is described in detail by Shannon et al. (2004). Although the original model by Shannon et al. was developed based on ventricular rabbit myocytes, we found that the equations describing Ca$^{2+}$ fluxes well predicted intracellular Ca$^{2+}$ handling in mouse cardiomyocytes, after proper adaptations in the action potential section (details below). Therefore, we adopted the original equations from Shannon et al. without changes, with the exception of those describing RyR2 function (see above). All equations and initial conditions can be found in the attached model file. In brief, structural features include four compartments (in series): a junctional cleft, a subsarcomemmal (SL) compartment, a bulk cytosolic compartment, and the SR space. Ca$^{2+}$ released into the junctional cleft diffuses to the SL space where it is extruded by the sarcolemal Ca-transport proteins, and to the bulk cytosol where SR Ca$^{2+}$ uptake occurs. We used the same equations and constants for calcium reuptake functions as in Shannon et al. (2004).

In brief, Ca$^{2+}$ is exported from the cell by the sarcolemmal (SL) Ca pump and the NCX, and SR Ca$^{2+}$ uptake occurs by virtue of the SR Ca$^{2+}$ pump (SERCA). SL transport proteins were considered to be evenly distributed across the membrane with 11% in the junctional cleft and 89% in the SL compartment. Both the sarcolemmal Ca pump and the NCX were dependent on the free Ca$^{2+}$ concentration just under the SL membrane in the SL compartment ([Ca$^{2+}$]$_{SL}$). The NCX included modulation of activity by external Na$^{+}$ and Ca$^{2+}$ and by [Ca$^{2+}$]$_{SL}$. The SERCA is found only in the cytosolic (i.e., nonjunctinal) portion of the SR membrane (90% of total membrane). SERCA was considered to be reversible with a forward and a reverse flux. Ca$^{2+}$ is only taken up from the bulk cytosolic compartment into the SR compartment where it is buffered by luminal proteins (calsequestrin).

Cytosolic calcium-buffering structures include: calmodulin, troponin, myosin, and SR surface buffers; $k_{on}$ and $k_{off}$ values for those buffers were left unchanged from the deriving models. Troponin binding includes a feedback from force development as described below.

Ca$^{2+}$ release from the SR (Jrel$_{SR}$) has been changed from the original Shannon model aiming to better describe the typical alterations found in CPVT-related mutations. We therefore used the calcium release simulation from Iyer et al. (2007), which is derived from previous work from Jafri et al. (1998) and is based on a model for RyR2 by Keizer and Levine (1996). The RyR2 kinetic model used here has been extensively described in the first section of this supplemental text.

In agreement with Shannon et al., in addition to the SR Ca$^{2+}$ release, a passive SR Ca$^{2+}$ leak flux (JLeak) was added. Experimental work on the R4496C mouse model showed that diastolic calcium release events (sparks) are more frequent in R4496C myocytes than in WT (Fernández-Velasco et al., 2009). However, in the absence of a clear quantification of the rate of Ca$^{2+}$ leakage from the SR, JLeak was left unchanged in the R4496C model (see below).

To test our model, we included a simulation of isoproterenol effect as in Shannon et al. (2004). According to the aforementioned work, isoproterenol potentiates ICa-L and increases SERCA function. In particular, when β-adrenergic stimulation is introduced in our model, SERCA pump rate is multiplied by a fixed factor of 2.3 and $I_{Ca,L}$ permeability by 3.75. Simulation of action potential in the original model in Shannon et al. was constructed from individual currents found in the normal rabbit ventricular myocyte. To adapt the model to mouse ventricular cardiomyocytes, equations and constants describing repolarizing membrane currents in the model were substituted with those coming from a mouse myocyte model from Bondarenko et al. (2004). In particular, equations describing seven potassium currents were inserted into our model unmodified from Bondarenko et al.: a slow transient outward K$^+$ current ($I_{K_{ss}}$), a time-independent K$^+$ current ($I_{K1}$), an ultra-rapidly activating delayed rectifier K$^+$ current ($I_{Kur}$), a non-inactivating steady-state K$^+$ current ($I_{ks}$), a rapid delayed rectifier K$^+$ current ($I_{Kd}$), and a slow delayed rectifier K$^+$ current ($I_{Ks}$). All equations describing the aforementioned currents were included in our model from Bondarenko et al. without changes and can be found in the supplemental model file. The equations describing fast sodium current were left unchanged from the original model (Shannon et al., 2004).

We checked whether the constants for calcium current required adaptation by emulating voltage-clamp experiments and comparing the model values with our experimental recordings. Adaptation of $I_{Ca,L}$ to mouse myocytes was found unnecessary. Therefore, the equations describing Ca$^{2+}$ current were adopted from the Shannon model without changes. As in Shannon et al. (2004), in our model 90% of the Ca$^{2+}$ channels are located in the junction and 10% in the nonjunctonal sarcolemna.

Because this modeling project aimed to replicate mechanical measurements from multicellular preparations, we included a simulation of myofilament activation and force generation in isometric conditions. To better evaluate interval–force relations taking into account Ca$^{2+}$ dynamics, the computation model describes inter-
actions between force and Ca$^{2+}$ in detail. We use the model of contraction and cooperativity mechanisms of Ca$^{2+}$, troponin, tropomyosin, and cross-bridge formation by Rice et al. (1999), which also includes a feedback pathway from developed force to Ca$^{2+}$ handling and troponin binding. All equations were included in our model from Rice et al. without changes and can be found in the supplemental model file. Notably, the force generation model simulates a perfectly isometric condition where shortening does not occur. Therefore, a series compliance is not included. Our simplified cross-bridge kinetic model implies that isometric tension developed at every time point is directly proportional to the number of force-generating cross-bridges.

In turn, the number of force-generating cross-bridges depends directly on cytosolic calcium concentration; therefore, the kinetics and the amplitude of force twists directly reflect the features of intracellular calcium transients. In a subset of experiments, we measured sarcomere shortening at the peak of isometric twists (Ferrantini et al., 2014): in our preparations, sarcomere shortening was in the order of 5–10% of initial sarcomere length and was not different in WT and R4496C trabeculae, indicating that series elasticity is the same in CPVT and WT myocardium. It is, therefore, unlikely that the lack of series components affects the results.

The ability of our modified model to simulate the normal behavior of mouse myocardium in our experimental conditions was extensively tested by comparing experimental recordings from WT preparations with model simulations. A complete validation of the modified model was performed by comparing recorded and simulated amplitude and kinetics of action potentials, calcium transients, and force twists at steady state (>120 s stimulation) during 1-Hz pacing. The model provides a good quantitative approximation of WT mouse myocardial function, as shown in Table S3, where experimental data are compared with values obtained from the model.

3—Simulation of altered RyR2 gating in R4496C

The analysis of calcium handling and mechanics of mutant myocardium suggested that R4496C RyR2 mutation may affect properties of calcium release by altering the luminal Ca$^{2+}$-dependent regulation of the channel. Evidence coming from work on mutant channels reexpressed in bilayers (Jiang et al., 2002) or in expression vectors (Jiang et al., 2004) confirms an increased response of mutant channels to luminal calcium increments in terms of Po. In an in vivo setting, this behavior of mutant channels will likely give rise to increased spontaneous diastolic calcium releases, especially in the presence of SR calcium overload. The aforementioned changes of RyR2 gating were simulated in the mutant model through appropriate adjustments to the k_{lumen,act} and k_{lumen,deact}. k_{lumen,act} and k_{lumen,deact} are the factors that confer luminal Ca$^{2+}$ dependence to RyR2 opening and closing transitions (see above).

Initial values for Hill constants of R4496C mutant RyR2 were chosen to fit previous data of single RyR2 channel currents recorded in lipid bilayers that report the average P_o of WT and R4496C channels at different [Ca$^{2+}$]_SR (Jiang et al., 2002, 2004). These values were then adjusted to better fit our results by replicating in the model all experimental protocols that were performed in isolated cells and intact trabeculae.

To dissect the separate roles of altered RyR2 activation and deactivation in the mutant, we first introduced a higher [Ca$^{2+}$]_SR sensitivity only for RyR2 closed-to-open (activation) transitions (altered Hill constants of k_{lumen,act}). Initial values of H50_{act} and Hmax_{act} parameters of k_{lumen,act} for R4496C mutant were, respectively, 0.4 and 0.9, calculated from single-channel data according to Jiang et al. (2004). In agreement with the experiments, force–frequency and restitution protocols were run in the cardiomyocyte model several times while changing the k_{lumen,act} parameters: the best approximation of results from mutants was obtained when Hmax_{act} was increased from 0.5 to 1 and H50_{act} was decreased from 1 to 0.3 with respect to WT. This change leads to blunted simulated inotropic responses to high steady-state stimulation frequencies, as well as to a faster mechanical restitution of the mutant compared with WT myocardium. However, in direct contrast with our experimental data, increased [Ca$^{2+}$]_SR sensitivity of only the activating transitions of the RyR2 (k_{lumen,act}) predicts a markedly reduced amplitude of Ca$^{2+}$ transients and twitch force at 1 Hz (Fig. S2). Based on the observation that twitch force and systolic Ca$^{2+}$ transient in R4496C myocytes are not decreased at 1 Hz, an additional modification was introduced in the model by decreasing [Ca$^{2+}$]_SR-dependent R4496C RyR2 deactivation, thus slowing termination of calcium release. We therefore changed the parameters of the Hill function k_{lumen,deact} that is inversely related to the apparent rate of the deactivation transition O1C2 in the scheme shown above. Because changes in luminal-dependent deactivation could not be derived from single-channel data, we had to estimate the changes in the parameters for the mutant from our results. We tested simulated steady-state twitches amplitude at 1 Hz of mutant versus WT myocytes and compared them with experimental data. The best approximation of results was obtained when Hmax_{deact} was increased from 0.5 to 2 and H50_{deact} was decreased from 1 to 0.15. This change led to delayed channel deactivation and termination of Ca$^{2+}$ release at lower [Ca$^{2+}$]_SR. Under basal conditions, RyR2 deactivation at lower [Ca$^{2+}$]_SR restored amplitude, and kinetics of Ca$^{2+}$ transients and twitches (Fig. S2) further speeded up restitution while maintaining blunted inotropic responses to high stimulation frequencies. Delayed Ca$^{2+}$ release termination mitigated the effects
Myocardial contractility in CPVT of SR Ca\(^{2+}\) content depletion, approximating more closely the experimental results. Only by introducing altered [Ca\(^{2+}\)]\(_{SR}\) sensitivity of both RyR2 activation and deactivation is the model capable of quantitatively simulating most of the experimental results obtained with R4496C cardiomyocytes and trabeculae (Fig. S3). Notably, although the model well predicts the abnormal behavior of mutant RyR2 channels at steady-state stimulation and during short diastolic intervals, it is unable to predict the increase of spontaneous channel openings in mutant cardiomyocytes during prolonged diastolic periods. Indeed, the kinetic RyR2 model we used is deterministic and cannot predict stochastic openings when junctional calcium is very low (i.e., during stimulation pauses). Consequently, the model was unable to predict the reduction of post-rest potentiation of force and Ca transients in R4496C versus WT cardiomyocytes. Reduced coupled gating and altered intersubunit interaction in RyR2 mutants have been modeled, as described recently, as a reduction in cooperativity of activation of RyR2 (Wehrens et al., 2003). This phenomenon is generally associated with reduced RyR binding to calstabin; however, in R4496C mutation, there is no evidence of an active role of an altered calstabin binding in the determination of abnormalities (Liu et al., 2006). We therefore did not modify the cooperativity parameter of RyR2 in our simulation of mutants to better adapt it to our specific mutation. Because of the intrinsic limitations of our deterministic model, the parameters that were chosen for the R4496C mutant RyR2 model cannot represent the actual behavior of the channel. However, we think that the main conclusions that can be drawn from this modeling study, namely that increased sensitivity of RyR2 to luminal calcium for opening, recovery, and release termination, may be paralleled by comparable changes in the mutant channel behavior.

4—Limitations of the model

RyR2 gating schemes derived from single-channel data, such as the Keizer and Levine formulation, have several limitations. The Keizer and Levine scheme gives a reasonable description of RyR2 activation and adaptation as seen in single-channel studies (Györke and Fill, 1993). However, this model has a cytosolic calcium-dependent transition between two open states (O1→O2): once the channel reaches state O1, it will be exposed to the microdomain of high [Ca\(^{2+}\)] in the junctional space. This will induce a transition to O2, precluding any opportunity for cytosolic Ca\(^{2+}\)-dependent inactivation or adaptation (C2 state), if a single dyad is taken into account (Stern et al., 1999). However, when a Keizer-

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**Figure S2.** Altering the kinetics parameters of different RyR2 transitions leads to different effects. Increasing SR Ca\(^{2+}\) sensitivity of modeled mutant RyR2 for opening transition (changed \(k_{lumen\_act}\) only; middle column) leads to increased \(P_o\) of mutant channel versus WT at all levels of physiological diastolic [Ca\(^{2+}\)]\(_{SR}\). This leads to SR depletion, which in turn lowers the amount of Ca\(^{2+}\) released during a Ca\(^{2+}\) transient and thus reduces twitch force at 1 Hz, in direct contrast with experiments. Moreover, the kinetics of Ca transients is predicted to be faster in mutants, whereas no differences were found experimentally. By adding an appropriate change in the mutant [Ca\(^{2+}\)]\(_{SR}\)-dependent RyR2 deactivation (changed \(k_{lumen\_act}\) and \(k_{lumen\_deact}\); right column), SR content at 1 Hz is still depleted but Ca release termination occurs at lower SR content, thus increasing fractional release and the net amount of Ca released at each cycle. Basal Ca\(^{2+}\)-transient amplitude and kinetics are therefore restored, in agreement with experimental results.
Levine formulation is inserted in an integrative model of cardiomyocyte, as in Jafri et al. (1998), and the rate constants are adapted to the real cellular environment, RyR2 exhibits a proper adaptive behavior, leading to a stable simulation of excitation–contraction coupling in the whole myocyte. The RyR2 kinetic model used in this work cannot simulate cytosolic Ca^{2+}-dependent inactivation of the RyR2. However, the most relevant mechanism for Ca^{2+} release termination is luminal-dependent inactivation, i.e., RyR2 inactivation occurring when local SR Ca^{2+} concentration falls below a certain level (Sobie et al., 2002). The addition of an SR Ca^{2+}-sensing capability in our model, as in Iyer et al. (2007), led to a very stable simulation of Ca^{2+} cycling at steady state. Four-state Markovian models of RyR2, such as that proposed by Stern et al. (1999) and modified by Shannon et al. (2004) may be an improvement over the Keizer–Levine formulation, in that they include cytosolic Ca^{2+} inactivation and can predict transient channel responses. However, such additional features are likely to be of small additional value for the purposes of our work.

A second limitation of this model is that Ca^{2+} leak through RyR2 is not produced by a change in the diastolic P_o of the channel. Indeed, the ideal RyR2 gating model should be able to mimic the stochastic single-channel opening events that mediate Ca^{2+} sparks, and the increased rate of such events in the presence of the mutation. However, the model we use is deterministic and is unable to mimic stochastic channel-opening events. An overall different approach to RyR2 modeling would be required to mimic such events, with a strong stochastic parameter and a simulation of each single release unit. All RyR2 deterministic models, including the Keizer–Levine formulation and the four-state Markovian model by Stern et al. (1999) (originally included in the Shannon model) require a J_{leak} parameter to achieve Ca^{2+} balance. This is because none of these models can mimic stochastic RyR2 openings during diastole (e.g., at low cytosolic [Ca^{2+}]), which are physiologically present in both healthy and diseased cardiomyocytes. In order for a deterministic RyR2 model to have a significantly high diastolic P_o, one would need to remove all cooperativity parameters and all features of incremental opening, preventing the possibility of simulating steady-state periodic activations. Despite the observed increased rate of diastolic Ca^{2+} sparks in R4496C versus WT cells, the J_{leak} parameter was left unchanged in the mutant R4496C model. This way, our model well simulated the kinetics of RyR activa-

Figure S3. Predictive ability of the mathematical model. (A) Force–frequency relationship in WT and R4496C model myocytes: tension and SR Ca traces from the two modeled cells are shown superimposed (gray, WT; black, R4496C). The model accurately predicts the shape of the force–frequency curves and the blunted force response to low and high frequencies of stimulation in R4496C myocardium with respect to WT. (B) Model predictions and mechanical restitution curves in mutant (black continuous) versus WT (gray continuous). Notably, experimental average curves from RyR^{R4496C/+} (closed squares) and WT (open squares) ventricular trabeculae are shown superimposed to allow a direct comparison of model results with experimental data. The model accurately predicts the effects of the mutation.
tion and inactivation during systole but could not emulate the behavior of RyR2 channels during prolonged diastolic periods. Consequently, the model was unable to predict the reduction of post-rest potentiation of force and Ca transients in R4496C versus WT cardiomyocytes, as well as the abnormal behavior of mutants at low pacing rates (<0.5 Hz). In a subset of simulations, we demonstrated that a two-time increase of $J_{\text{leak}}$ correctly predicted the abnormal behavior during prolonged diastolic periods. Notably, the rate of calcium sparks during diastole in cells from R4496C mice is double than that of WT cells (Fernández-Velasco et al., 2009).

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S8 Myocardial contractility in CPVT