Increased Ca\(^{2+}\) Sensitivity of the Ryanodine Receptor Mutant RyR2\(^{R4496C}\) Underlies Catecholaminergic Polymorphic Ventricular Tachycardia

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Abstract—Cardiac ryanodine receptor (RyR2) mutations are associated with autosomal dominant catecholaminergic polymorphic ventricular tachycardia, suggesting that alterations in Ca\(^{2+}\) handling underlie this disease. Here we analyze the underlying Ca\(^{2+}\) release defect that leads to arrhythmia in cardiomyocytes isolated from heterozygous knock-in mice carrying the RyR2\(^{R4496C}\) mutation. RyR2\(^{R4496C}\) littermates (wild type) were used as controls. [Ca\(^{2+}\)]\(_i\), transients were obtained by field stimulation in fluo-3–loaded cardiomyocytes and viewed using confocal microscopy. In our basal recording conditions (2-Hz stimulation rate), [Ca\(^{2+}\)]\(_i\), transients and sarcoplasmic reticulum Ca\(^{2+}\) load were similar in wild-type and RyR2\(^{R4496C}\) cells. However, paced RyR2\(^{R4496C}\) ventricular myocytes presented abnormal Ca\(^{2+}\) release during the diastolic period, viewed as Ca\(^{2+}\) waves, consistent with the occurrence of delayed afterdepolarizations. The occurrence of this abnormal Ca\(^{2+}\) release was enhanced at faster stimulation rates and by \(\beta\)-adrenergic stimulation, which also induced triggered activity. Spontaneous Ca\(^{2+}\) sparks were more frequent in RyR2\(^{R4496C}\) myocytes, indicating increased RyR2\(^{R4496C}\) activity. When permeabilized cells were exposed to different cytosolic [Ca\(^{2+}\)]\(_i\), RyR2\(^{R4496C}\) showed a dramatic increase in Ca\(^{2+}\) sensitivity. Isoproterenol increased [Ca\(^{2+}\)]\(_i\), transient amplitude and Ca\(^{2+}\) spark frequency to the same extent in wild-type and RyR2\(^{R4496C}\) cells, indicating that the \(\beta\)-adrenergic sensitivity of RyR2\(^{R4496C}\) cells remained unaltered. This effect was independent of protein expression variations because no difference was found in the total or phosphorylated RyR2 expression levels. In conclusion, the arrhythmic potential of the RyR2\(^{R4496C}\) mutation is attributable to the increased Ca\(^{2+}\) sensitivity of RyR2\(^{R4496C}\), which induces diastolic Ca\(^{2+}\) release and lowers the threshold for triggered activity. (Circ Res. 2009;104:201-209.)

Key Words: Ca\(^{2+}\) sparks \(\rightarrow\) [Ca\(^{2+}\)]\(_i\), transients \(\rightarrow\) ryanodine receptor \(\rightarrow\) excitation–contraction coupling \(\rightarrow\) CPVT

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease characterized by stress-induced, adrenergically mediated bidirectional or polymorphic ventricular tachycardia occurring in structurally normal hearts.\(^1\) During exercise or acute emotions, CPVT patients develop life-threatening ventricular arrhythmias, leading to syncope or sudden death. The first cardiac ryanodine receptor (RyR2) mutation identified in a CPVT family was R4497C.\(^2\) Today, more than 70 RyR2 mutations have been reported (http://www.fsm.it/cardmoc), and they comprise the most common genetic subtype of CPVT.\(^3\)–\(^7\) Although mutations in the calsequestrin gene can also cause CPVT.\(^8\)\(^9\)

Diverging results and conclusions have been generated from expression studies of RyR2\(^{R4496C}\) in heterologous systems. Jiang et al showed that RyR2\(^{R4496C}\) (the mouse equivalent of the human RyR2\(^{R4497C}\) mutation), when expressed in human embryonic kidney (HEK) cells, exhibits increased basal activity and increased sensitivity to luminal Ca\(^{2+}\).\(^10\) However, other authors found no difference in the basal activity of RyR2\(^{R4497C}\) but, instead, showed increased activity and gating frequency after protein kinase A phosphorylation\(^11\) or sarcoplasmic reticulum (SR) Ca\(^{2+}\) overload.\(^12\) The expression studies were carried out in a variety of models, which may explain the inhomogeneous findings. Furthermore, heterologous systems lack cardiac intracellular environment with all the RyR2 accessory proteins\(^11\) and most Ca\(^{2+}\)- handling proteins, so analysis in native cardiac myocytes is now critical to elucidate the mechanisms by which the mutation leads to cardiac arrhythmia.

Recently, a knock-in mouse model carrier of the RyR2\(^{R4496C}\) mutation was developed.\(^14\) Their phenotype presents extraordinary similarity with the clinical manifestations of patients carrying the RyR2\(^{R4497C}\) mutation, including the...
Abnormal Ca$^{2+}$ Release in RyR2$^{R4496C}$ Myocytes

Electrophysiological experiments in RyR2$^{R4496C}$ myocytes have evidenced DADs and triggered action potentials in the presence of adrenergic stimulation after stopping electric stimulation. Figure 1 shows Ca$^{2+}$ images in ventricular myocytes paced at 4 Hz in the presence of 1 μmol/L isoproterenol. The WT cardiomyocyte showed no spontaneous Ca$^{2+}$ release after electric pacing interruption (Figure 1A). On the contrary, the RyR2$^{R4496C}$ cell showed Ca$^{2+}$ waves evoking 2 [Ca$^{2+}$], transients, just after stimulation stopped, which was followed by several Ca$^{2+}$ waves, consistent with triggered activity and DADs (Figure 1B). These data suggest that abnormal Ca$^{2+}$ release underlies the electric abnormalities in RyR2$^{R4496C}$ cardiomyocytes. Because DADs are initiated by Ca$^{2+}$ waves that are in turn initiated by Ca$^{2+}$ sparks, we analyzed Ca$^{2+}$ sparks in RyR2$^{R4496C}$ cardiomyocytes. [Ca$^{2+}$], transients and waves characteristics are presented below.

Ca$^{2+}$ Sparks in RyR2$^{R4496C}$ Myocytes

Figure 2A shows representative images of Ca$^{2+}$ sparks. [Ca$^{2+}$] spark frequency was double in RyR2$^{R4496C}$ cells compared with WT cells (P<0.001, Figure 2B). This could be attributable to an increase in (1) the SR Ca$^{2+}$ content, (2) the level of RyR2 expression and/or phosphorylation, (3) the diastolic [Ca$^{2+}$], or (4) changes in the intrinsic channel properties.

We estimated SR Ca$^{2+}$ load in quiescent ventricular myocytes. RyR2$^{R4496C}$ cells showed reduced SR Ca$^{2+}$ content (F/F$_0$: 7.0±0.5, n=11 in RyR2$^{R4496C}$ versus 8.5±0.5 in WT, n=10, P<0.05), ruling out SR Ca$^{2+}$ overload. No major alteration in Ca$^{2+}$ spark characteristics was observed (Table I in the online data supplement).

We performed Western blots of total and phosphorylated RyR2 in hearts in basal conditions and following isoproterenol perfusion. Neither the total RyR2 expression nor the level of phosphorylated RyR2 (P-Ser 2809) was different between WT and RyR2$^{R4496C}$ (supplemental Figure I). We also performed functional experiments challenging the cells with 1 μmol/L isoproterenol. This procedure increased Ca$^{2+}$ spark occurrence in both WT and RyR2$^{R4496C}$ myocytes (Figure 2A and 2B) by the same percentage (Figure 2C). Ca$^{2+}$ spark characteristics in the presence of isoproterenol are provided in supplemental Table I. Similar results were found using a lower isoproterenol concentration (100 nmol/L) (supplemental Figure II, A). Furthermore, treatment of RyR2$^{R4496C}$ myocytes with either a protein kinase A blocker (KT5720) or a Ca$^{2+}$/calmodulin-dependent protein kinase II blocker (KN93) failed to decrease Ca$^{2+}$ spark frequency (supplemental Figure III). These data rule out an increase in the total RyR2 expression or a higher level of basal phosphorylation as an explanation for the higher Ca$^{2+}$ spark occurrence in RyR2$^{R4496C}$ myocytes.

Resting cytoplasmic [Ca$^{2+}$], measured using Fura-2, was similar between WT and RyR2$^{R4496C}$ cells (ratios: 0.56±0.02 in 16 WT myocytes, 0.57±0.01 in 45 RyR2$^{R4496C}$ cells; P=0.05). Therefore, the increased Ca$^{2+}$ spark occurrence in quiescent RyR2$^{R4496C}$ cells was not caused by differences in the resting intracellular Ca$^{2+}$.

Ca$^{2+}$ sparks are produced by the opening of RyR2 clusters. The increase in total Ca$^{2+}$ spark frequency in RyR2$^{R4496C}$ could be attributable to a greater number of clusters firing Ca$^{2+}$ sparks or to the increased propensity of some clusters to fire repetitively, becoming “eager” clusters. We analyzed our data discriminating specific sites presenting multiple Ca$^{2+}$
sparks during the recording time (∼20 seconds). Firing sites were counted as the sites where we recorded at least 1 Ca2+ spark. Figure 2D shows that the RyR2R4496C myocytes presented more firing sites and that isoproterenol increased the number of sites in both WT and RyR2R4496C cells. This indicates that the RyR2R4496C cells presented more Ca2+ sparks attributable to the existence of more active RyR2 clusters (Figure 2E). We also measured the maximum number of Ca2+ sparks recorded at the same site in each group and found that this was also significantly increased in RyR2R4496C myocytes and further enhanced by β-adrenergic stimulation (Figure 2F). Taken together, these data suggest that RyR2R4496C cells present more Ca2+ sparks because of more active RyR2s clusters and a greater probability of repetitive openings of these clusters in the RyR2R4496C myocytes.

We next explored whether RyR2R4496C presents abnormal Ca2+ sensitivity. We analyzed Ca2+ sparks in permeabilized cells exposed to various cytoplasmic [Ca2+]i. Figure 3A illustrates enhanced Ca2+ sparks occurrence in a RyR2R4496C cell at 30 mmol/L [Ca2+]i. At all tested [Ca2+]i, Ca2+ sparks were much more frequent in RyR2R4496C than in WT myocytes, consistent with increased cytosolic Ca2+ sensitivity (Figure 3B). Analysis of the Ca2+ spark characteristics in permeabilized cells essentially confirmed the results obtained in intact cells (supplemental Table II). We estimated SR Ca2+ content in permeabilized cells and found that at all tested [Ca2+]i, the caffeine-evoked [Ca2+]i transient was significantly decreased in the RyR2R4496C cells (Figure 3C). Thus, the higher Ca2+ spark occurrence in RyR2R4496C myocytes was not attributable to either a higher level of Ca2+ stored in the SR or an alteration of calsequestrin expression evaluated by Western blots (data not shown). Figure 3D shows the luminal Ca2+ dependence of Ca2+ sparks, apparently consistent with increased luminal Ca2+ sensitivity. However, cytosolic and luminal Ca2+ vary concurrently. The high Ca2+ spark occurrence recorded in RyR2R4496C at very low intracellular Ca2+ might suggest that, rather than increasing RyR2R4496C Ca2+ sensitivity, this mutation renders the RyR2 intrinsically active. We repeated the experiments at 0 Ca2+ (0.5 mmol/L EGTA). In this condition, the occurrence of Ca2+ sparks was indistinguishable between WT and RyR2R4496C cells (Figure 3E and 3F, left), indicating that RyR2R4496C hyperactivity requires cytosolic Ca2+. To ensure that the SR was not depleted in our experimental conditions,
we applied caffeine. A robust caffeine-induced Ca\(^{2+}\)/H\(^{100}\) transient could be evoked (Figure 3E and 3F, right), proving that there was significant luminal Ca\(^{2+}\)/H\(^{100}\) to promote Ca\(^{2+}\)/H\(^{100}\) sparks. Altogether, our results show that the RyR2\(\text{R4496C}\) mutation increases the Ca\(^{2+}\)/H\(^{100}\) sensitivity of the channel. The RyR2 has 2 affinity Ca\(^{2+}\)/H\(^{100}\) binding sites on the cytosolic portion: one of high affinity that activates the channel and one of low affinity. Because ryanodine binds to open RyRs, we examined the Ca\(^{2+}\)/H\(^{100}\) dependence of [\(^{3}\text{H}\)]ryanodine binding in heart crude membrane preparations. Bell-shaped curves were obtained for both WT and RyR2\(\text{R4496C}\), but Ca\(^{2+}\)/H\(^{100}\)-induced maximal activation of RyR2\(\text{R4496C}\) was reached at 1 order of magnitude lower in RyR2\(\text{R4496C}\) (Figure 3G), indicating that the cytosolic Ca\(^{2+}\)/H\(^{100}\) sensitivity of RyR2 is greatly increased.
We normalized the \([3H]\)ryanodine binding and fitted data to the Hill equation\(^20\) to get the values for Ca\(^{2+}\) affinity to the RyR activation (\(K_a, 21.9\pm 8.3\) mmol/L versus 4.9\(\pm\)1.0 mmol/L, \(n=4; P<0.003;\) for WT and RyR2R4496C membranes, respectively) and inactivation (6.5\(\pm\)0.6 mmol/L versus 4.8\(\pm\)1.1 mmol/L; \(P>0.05;\) for WT and RyR2R4496C membranes) sites. These results show a 4.5-fold increase of cytosolic Ca\(^{2+}\) sensitivity for the RyR2R4496C.

\([\text{Ca}^{2+}]\), Transients in RyR2R4496C Myocytes

To determine whether the alteration of diastolic Ca\(^{2+}\) spark frequency in RyR2R4496C cardiomyocytes has an impact during systole, we compared \([\text{Ca}^{2+}]\), transients and cell contraction at different pacing rates (2 Hz, 3 Hz, and 4 Hz) (supplemental Table III). At 2 Hz, the \([\text{Ca}^{2+}]\), transient amplitude, its time to peak, the \([\text{Ca}^{2+}]\), transient decay time, and cellular contraction were similar (\(P>0.05\)) in WT and RyR2R4496C cells, consistent with normal heart function in mice at rest. As stimulation rate increased, weaker \([\text{Ca}^{2+}]\), transients were evoked both in WT and RyR2R4496C cells (Figure 4A). However, the decrease in \([\text{Ca}^{2+}]\), transient amplitude was more pronounced (\(P<0.05\)) in RyR2R4496C cells. This reduction was associated with both weaker cellular contraction (Figure 4B) and slower decay time (Figure 4C), with no difference in the time to peak (Figure 4D).

Because \([\text{Ca}^{2+}]\), transient amplitude depends on SR Ca\(^{2+}\) load, we investigated the SR Ca\(^{2+}\) content. Images of caffeine-evoked \([\text{Ca}^{2+}]\), transients obtained in WT and RyR2R4496C (R4496C) cells obtained after field stimulation at 4 Hz are shown in Figure 4E. As shown in Figure 4F, caffeine-evoked \([\text{Ca}^{2+}]\), transients were significantly smaller (by 24.7\%) after pacing the cell at 4 Hz in RyR2R4496C compared to WT myocytes, whereas no significant difference was observed at lower frequencies. Plotting the peak \([\text{Ca}^{2+}]\), transient versus the SR Ca\(^{2+}\) load relationship in WT and RyR2R4496C myocytes at various pacing rates. Open circles and bars indicate WT; blue circles and bars, RyR2R4496C. N numbers from A and F. *\(P<0.05\).
tractility. We next analyzed \([\text{Ca}^{2+}]_{i}\) transient in each cell tested. We found no difference between WT and RyR2R4496C cells. For example, at 4 Hz, fractional transient amplitude increase induced by 1 \(\mu\)mol/L isoproterenol on caffeine-evoked \([\text{Ca}^{2+}]_{i}\) transient after stimulating the cell at 2 Hz (n=26 vs n=25), 3 Hz (n=4 vs n=12), and 4 Hz (n=3 vs n=12), WT vs RyR2R4496C cells. B. Percentage of increase induced by 1 \(\mu\)mol/L isoproterenol on caffeine-evoked \([\text{Ca}^{2+}]_{i}\) transient under stress conditions. Ca2+ sparks were observed during the diastolic period in 17% of the RyR2R4496C cells paced at 2 Hz (9 of 54 RyR2R4496C myocytes). The percentage of cells exhibiting these events dramatically increased to 67% (12 of 18 cells) when RyR2R4496C cells were paced at 4 Hz in the presence of isoproterenol. This behavior was almost absent in WT cells, both in basal conditions (2 Hz: 1 of 55 WT cells versus 9 of 54 RyR2R4496C cells; P<0.01) and at 4 Hz and isoproterenol (2 of 14 WT cells versus 12 of 18 RyR2R4496C cells; P<0.01). As shown in Figure 1B, 1 single Ca2+ wave was able to evoke triggered activity after electric stimulation stopped. However, this was never the case during constant stimulation. To trigger a full \([\text{Ca}^{2+}]_{i}\) transient, we measured that 7.1±0.6 Ca2+ waves/100 \(\mu\)m/sec had to overlap during diastole, which only occurred under stress conditions. Ca2+ waves spread at similar velocity in the absence (117.3±14.7 \(\mu\)m/sec, n=13) or in the presence of 1 \(\mu\)mol/L isoproterenol (113.8±7.2 \(\mu\)m/sec, n=22).

We found that mouse RyR2R4496C myocytes presented a higher incidence of Ca2+ waves not only in the presence of isoproterenol but also at higher pacing rates (Figure 6C). We then measured Ca2+ sparks in the diastolic period at different stimulation frequencies, when it was possible to discriminate them. During diastole, the maximum number of Ca2+ sparks×sec\(^{-1}\) in RyR2R4496C myocytes under \(\beta\)-adrenergic stimulation increased with pacing rates (4.7±1.7 when paced at 2 Hz, n=9; 15.0±4.4 when paced at 3 Hz, n=4, P<0.05 compared with 2 Hz; and 24.8±5.4 when paced at 4 Hz, n=5, P<0.001 compared with 2 Hz). This could be attributable to the higher diastolic \([\text{Ca}^{2+}]_{i}\), induced by increasing pacing rates. The diastolic Ca2+ fluorescence measured in the same cells increased progressively with pacing rate (39.5±2.7 at 2 Hz, 46.1±1.6 at 3 Hz, and 50.9±3.0 at 4 Hz; P<0.05 compared with 2 Hz).

### Discussion

We show for the first time that beating cardiomyocytes bearing the RyR2R4496C mutation, equivalent to that found in
several CPVT families, exhibited arrhythmogenic behavior related to a dramatically enhanced occurrence of Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves during diastole. This elevated spontaneous Ca\(^{2+}\) release was further enhanced by \(\beta\)-adrenergic stimulation and increasing pacing rates, mimicking human exercise-induced ventricular tachycardia. The high activity of RyR2R4496C was attributable to a dramatic increase in its Ca\(^{2+}\) sensitivity, which lowered the release threshold to produce spontaneous activity during the diastolic period.

Mice bearing the RyR2R4496C mutation, which is equivalent to the human RyR2R4497C mutation first identified in a CPVT family,\(^\text{14}\) present ventricular tachycardia in response to adrenergic stimulation and caffeine in vivo. Isolated cells were patch-clamped and action potentials recorded. Under these conditions, DADs and triggered activity could be recorded when electric pacing was interrupted.\(^\text{15}\) Here we found parallel evidence of spontaneous intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) waves in similar experimental conditions (Figure 1). However, in life, ventricular myocytes are continuously paced unless there is a problem with automatic or conducting cells. Moreover, CPVT arises under stress conditions with adrenergic stimulation, which, among other effects, increases heart rate.

This study is the first to show that isolated RyR2R4496C ventricular myocytes displayed arrhythmogenic activity related to spontaneous Ca\(^{2+}\) release while they are electrically stimulated, thus mimicking human CPVT and demonstrating that RyR2R4496C was at the origin of the arrhythmia. In isolated cardiomyocytes paced at 2 Hz, we observed multiple Ca\(^{2+}\) sparks capable of triggering localized Ca\(^{2+}\) waves in more than 16% of RyR2R4496C myocytes (Figure 6C). With pacing rate increased to 4 Hz and under \(\beta\)-adrenergic stimulation, the RyR2 R4496C myocytes were remarkably more prone to evoke Ca\(^{2+}\) waves (in up to 66.7% of cells). We thus found that RyR2 R4496C cells showed higher spontaneous Ca\(^{2+}\) release even in basal conditions, and this feature was further enhanced by \(\beta\)-adrenergic stimulation and pacing rate, reaching a threshold for triggered activity.

The higher diastolic Ca\(^{2+}\) release in RyR2R4496C cells is correlated by higher frequency of spontaneous Ca\(^{2+}\) sparks (Figure 2). This increased activity could depend on the expression or phosphorylation level of RyR2, the amount of
Ca^{2+} stored in the SR, and/or the sensitivity of RyR2 R4496C to luminal \(^{21}\) or cytosolic Ca\(^{2+}\). We found no difference between WT and RyR2 R4496C hearts in total RyR2 expression, FKBP12.6 association,\(^{15}\) or RyR2 phosphorylation level, even after \(\beta\)-adrenergic stimulation (supplemental Figure I). Moreover, although Ca\(^{2+}\) waves and high Ca\(^{2+}\) spark frequency usually reflect Ca\(^{2+}\) overload,\(^{18}\) RyR2 R4496C myocytes presented this behavior even at lower SR Ca\(^{2+}\) load.

Our data in permeabilized cardiomyocytes show that at all cytoplasmic \([\text{Ca}^{2+}]_{\text{i}}\), tested, the Ca\(^{2+}\) spark frequency was higher in RyR2 R4496C than in WT cells, showing that the RyR2 R4496C is hyperactive at any given \([\text{Ca}^{2+}]_{\text{i}}\), and indicating Ca\(^{2+}\) hypersensitivity. However, Ca\(^{2+}\) spark frequencies in WT and RyR2 R4496C cells were similar in absence of cytosolic Ca\(^{2+}\). Under these conditions, SR Ca\(^{2+}\) load was also similar in both experimental groups, suggesting that RyR2 R4496C sensitivity to luminal Ca\(^{2+}\) is maintained under these unphysiologic circumstances. Nevertheless, in the presence of cytosolic Ca\(^{2+}\), RyR2 R4496C behaves as hypersensitive to both luminal\(^{10}\) and cytosolic Ca\(^{2+}\) (Figure 3). It is not easy to unequivocally assign distinct roles for cytoplasmic versus luminal Ca\(^{2+}\) in situ because of the inherent interdependence of these Ca\(^{2+}\) compartments in living cells.

Although unzipping of amino and central RyR2 domains has been reported to be involved in some forms of enhanced RyR2 activity,\(^{22,23}\) the R4496C mutation is far from those domains, making that mechanism unlikely. Differential FKBP12.6 association also cannot explain the increased RyR2 sensitivity reported here, because there is unaltered RyR2-FKBP12.6 association in this animal model.\(^{15}\) The increase in Ca\(^{2+}\) spark frequency of RyR2 R4496C is likely to reflect an enhancement of its open probability \((P_\text{o})\), consistent with data obtained by single channel analyses.\(^{10}\) Our data demonstrate that, in its normal environment (ie, in native cardiomyocytes), RyR2 R4496C has augmented Ca\(^{2+}\) sensitivity rather than increased \(P_\text{o}\) per se. Indeed, Ca\(^{2+}\) spark occurrence, measured in permeabilized cells exposed to different \([\text{Ca}^{2+}]_{\text{i}}\), concentrations, was significantly increased in RyR2 R4496C at all \([\text{Ca}^{2+}]_{\text{i}}\), tested except at 0 Ca\(^{2+}\), indicating that the channel needs Ca\(^{2+}\) to become hyperactive. The RyR2 R4496C mutation is located in the C-terminal portion of the channel (cytosolic side\(^{24,25}\)), close to the proposed molecular region involved in Ca\(^{2+}\)-dependent activation (residues 4485 to 4494).\(^{23}\) The RyR has highly reactive cysteines capable of forming disulfide bonds.\(^{26}\) It is thus plausible that the highly reactive cysteine introduced by the mutation, interacts with other cysteines of the channel, inducing a conformational change that renders the RyR hyper-sensitive to Ca\(^{2+}\). The conformational change might render more accessible to Ca\(^{2+}\) the E3987 residue, identified as important in Ca\(^{2+}\) sensitivity.\(^{27}\) However, the low affinity Ca\(^{2+}\) sensing of the RyR2 R4496C seems to be normal because the Ca\(^{2+}\) inhibition found in the [\(\text{H}\)]ryanodine binding experiments is similar to WT RyR2 (Figure 3G). Experiments in the RyR2 R4496C tertiary structure are needed to investigate whether this point mutation induces conformational changes favoring Ca\(^{2+}\) binding to the activating sites in the RyR2.

Even though RyR2 R4496C basal activity was dramatically higher than that of WT (Figure 2), \(\beta\)-adrenergic stimulation increased their activity to the same extent suggesting that: (1) the 2 mechanisms (increased Ca\(^{2+}\) sensitivity of the RyR2 R4496C mutant and the effect of \(\beta\)-adrenergic stimulation) are distinct and cumulative and (2) the \(\beta\)-adrenergic regulation of RyR2 R4496C is not modified. Nevertheless, \(\beta\)-adrenergic stimulation further increased the already elevated diastolic Ca\(^{2+}\) leak in RyR2 R4496C cells probably by increasing the SR Ca\(^{2+}\) load,\(^{28}\) further enhancing the RyR2 R4496C cell propensity to trigger DADs and allowing the occurrence of spontaneous activity (Figures 1 and 6).\(^{29,30}\)

At basal conditions (2 Hz in our experimental setting), the \([\text{Ca}^{2+}]_{\text{i}}\), transients in WT and RyR2 R4496C myocytes were similar. The \([\text{Ca}^{2+}]_{\text{i}}\), transient decay times were also similar, suggesting a normal function of SERCA (sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase). However, the rate-dependent decrease in \([\text{Ca}^{2+}]_{\text{i}}\), transients and contraction, which is normal in mice cardiomyocytes, was more pronounced in RyR2 R4496C (Figure 4). This reduction can be accounted for by a decrease in SR Ca\(^{2+}\) load (Figure 4). Therefore, the increase in diastolic Ca\(^{2+}\) leak becomes critical for the systolic function only at the highest pacing rates. Such a negative staircase, observed in normal mice, may depend on the interval between 2 consecutive twitches, while SERCA replenishes the SR with Ca\(^{2+}\). Enhancement of this phenomenon in RyR2 R4496C cells seems to indicate that, because RyR2 R4496C myocytes show more Ca\(^{2+}\) waves during diastole at high pacing rate, an imbalance between Ca\(^{2+}\) leak and Ca\(^{2+}\) uptake results in SR Ca\(^{2+}\) depletion, although a possible alteration in RyR2 R4496C refractoriness could account for this phenomenon. However, in humans, the staircase is positive, which further supports the lack of contractile impairment in CPVT patients.

The decrease in SR Ca\(^{2+}\) load with pacing rate can also reflect the higher Ca\(^{2+}\) leak at higher stimulation frequencies (Figure 6C) and could partly depend on a phenomenon known as Ca\(^{2+}\) current facilitation. By this phenomenon, the total amount of Ca\(^{2+}\) entry is enhanced when stimulation frequency is increased, mainly attributable to slowing of the Ca\(^{2+}\) current inactivation.\(^{31}\) This longer Ca\(^{2+}\) entry during the diastolic period can further activate RyR2 R4496C thereby evoking more Ca\(^{2+}\) waves and rhythmic disorders at higher pacing rates.

In conclusion, our study shows that beating RyR2 R4496C cardiomyocytes present high spontaneous \([\text{Ca}^{2+}]_{\text{i}}\) release during diastole, because of a dramatic increase in Ca\(^{2+}\) sensitivity of the RyR2 R4496C. This diastolic Ca\(^{2+}\) leak is responsible for both DADs and decreased SR Ca\(^{2+}\) load at high pacing rates. Our findings in cardiomyocytes provide a link between the data observed with the heterologous expression of RyR2 R4496C mutation and the macroscopic phenotype observed at the whole heart (ECG). By characterizing the function of mutant RyR2, we provide a detailed definition of how CPVT mutations cause DAD and triggered arrhythmias. Furthermore, the identification of abnormal Ca\(^{2+}\) sensitivity in RyR2 as the key factor for arrhythmogenesis supports the interest of the RyR2 in the development of novel therapeutic targets.
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Disclosures
None.

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Methods

Cell isolation

Ventricular cardiomyocytes from male and female RyR2^{R4496C} +/- mice (RyR2^{R4496C}) and their wild type RyR2^{R4496C}-/- (WT) littermates were isolated using a standard enzymatic digestion ¹. Only rod-shaped cells, quiescent when unstimulated and excitable were used for the experiments. Excitability of the cells was also tested before cell permeabilization for Ca^{2+} sparks measurement. This protocol also allowed reaching steady-state SR Ca^{2+} content, critical in the zero cytosolic Ca^{2+} measurements.

Imaging

[Ca^{2+}], transients and Ca^{2+} sparks were recorded in intact myocytes loaded with fluorescent Ca^{2+} dye (Fluo-3 AM)² and under control Tyrode perfusion (in mmol/L): 140 NaCl, 4 KCl, 1.1 MgCl₂, 10 HEPES, 10 glucose, 1.8 CaCl₂; pH=7.4, with NaOH). To record [Ca^{2+}], transients, cells were excited at 2, 3 or 4 Hz by field stimulation using two parallel Pt electrodes. Spontaneous Ca^{2+} sparks were obtained in quiescent cells after [Ca^{2+}], transients recordings. SR Ca^{2+} load was estimated by rapid caffeine (10 mmol/L) application, after 1 min of stimulation to reach the steady state.

In a set of experiments, we recorded Ca^{2+} sparks on permeabilized myocytes³. After field-stimulating the cells in normal Tyrode solution, cells were perfused for 90 s with saponin (0.01%) added to an internal solution containing (mmol/L): 120 K-aspartate, 10 HEPES, 3 MgATP, 0.5 EGTA, 10 Na phosphocreatine, 5 U/mL creatine phosphokinase, 0.75 MgCl₂ and 8% dextran, pH 7.2. After permeabilization, Ca^{2+} sparks were recorded in cells perfused with the internal solution in the absence of saponin and containing 50 µmol/L pentapotassium Fluo-3 and various concentrations of free Ca^{2+} calculated with Maxchelator
SR Ca\textsuperscript{2+} load was estimated by rapid caffeine (20 mmol/L) application.

Images were obtained with confocal microscopy (Meta Zeiss LSM 510, objective w.i. 63x, n.a. 1.2) by scanning the cell with an Argon laser every 1.54 ms; fluorescence was excited at 488 nm and emissions were collected at >505 nm. Image analyses were performed by homemade routines using IDL software (Research System Inc.). Images were corrected for the background fluorescence. The fluorescence values (F) were normalized by the basal fluorescence ($F_0$) in order to obtain the fluorescence ratio ($F/F_0$). As previously\textsuperscript{4}, Ca\textsuperscript{2+} sparks were detected using an automated detection system and a criterion that limited the detection of false events while detecting most Ca\textsuperscript{2+} sparks.

**Intracellular [Ca\textsuperscript{2+}], measurements**

Diastolic [Ca\textsuperscript{2+}], was measured in quiescent cells loaded with 2.5 µmol/L Fura-2/AM (from a stock solution in a mixture of DMSO/Pluronic F-127). Images of Fura-2 fluorescence were digitally captured by a cooled CCD camera (Photometrics ,USA) with a oil-inmersion x40 objective mounted on an inverted microscope (Axiovert, Zeiss ,Germany), and acquired at 510 nm emission after excitations at 340 nm and 380 nm using a lambda-DG4 excitation system (Sutter Instrument Company, USA). Ca\textsuperscript{2+} signals were then analyzed by using the Metafluor software (Universal Imaging Corporation, USA).

**Protein analysis**

The hearts extracted from WT and RyR2\textsuperscript{R4496C} mice were retrogradely perfused through the aorta with Tyrode’s solution at constant flow at 37°C for 1.5 min to wash blood, and they were then perfused with Tyrode’s solution or with Tyrode’s solution supplemented with 1 µmol/L isoproterenol for 5 min before rapid freezing. The Tyrode’s solutions were supplemented with 20 mmol/L NaF plus 0.005 mmol/L okadaic acid, to keep the phosphorylation status of the proteins.
Preparation of mouse whole heart homogenates and crude membrane fractions.

Mouse hearts were perfused for 5 min with Ca\(^{2+}\) free Tyrode solution (pH 7.4) complemented with 20 mmol/L NaF plus 0.005 mmol/L okadaic acid. Hearts were then frozen in liquid nitrogen and crushed into fine powder using a mortar and pestle. The powder was suspended in 1 ml ice-cold sucrose buffer (0.3 mol/L sucrose, 20 mmol/L HEPES, 20 mmol/L NaF, pH = 7.2 with KOH) plus protease inhibitors (12 mmol/L leupeptin, 100 mmol/L phenylmethylsulphonyl fluoride, 500 mmol/L benzamidine, and 1 mg/ml aprotinin) and homogenized on ice with a glass homogenizer at 1000 rpm, 3 times 30 s each with pauses of 1 min in between. The total homogenate was centrifuged at 40,000 g for 35 min at 4 °C. The 40,000 g pellet (crude membrane fraction) was resuspended in the same sucrose buffer described above, aliquoted, quickly frozen and stored at -80°C before use. Protein concentrations were determined by the Bradford method.

The hearts were then homogenized using a hand-held blender in lysis buffer containing (in mmol/L): 10 Na\(_2\)Pipes, 0.3 sucrose, pH 7.4, plus a complete protease inhibitors solution (Roche). The homogenate was spun at 8000 g for 10 min at 4°C and supernatants were frozen and stored at -80°C for western blotting analysis. Protein concentrations were determined by the Bradford assay (Bio-Rad). Immunoblot analysis was performed by NuPAGE MOPS Bis-Tris acrylamide gel 4-12% (Invitrogen).

The proteins were detected, respectively, with rabbit anti-Casq (ABR, PA1-913), mouse anti-RyR2 (ABR, MA3-916), rabbit anti-Phospho-RyR2-Ser2809 (Badrilla), rabbit PLB-Thr17 (Badrilla), rabbit PLB-Ser16 (Badrilla), mouse anti-PLB (MA3-922 ABR), and rabbit anti-GAPDH (Bethyl) as primary antibody and HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (ABR, respectively, SA1-100 and SAI-200) as secondary antibody. In addition to GAPDH immunostaining, ponceau staining was used in all blots to assure equal loading. Chemiluminescent detection was carried out with a specific reagent called Western Blotting Substrate (Pierce Biotechnology).

Chemiluminescent detection was carried out with a specific reagent called Western Blotting Substrate (Pierce Biotechnology).
**[^3H]ryanodine Binding Assay.**

[^3H]ryanodine binding experiments were performed with crude membrane fractions from individual hearts as described by El-Hayek and cols\(^5\). The incubation medium contained: 0.2 mol/L KCl, 20 mmol/L HEPES (pH 7.2 with KOH), 50 µg of cardiac membrane fraction, 10 nmol/L[^3H]ryanodine, 1 mmol/L EGTA, and CaCl\(_2\) necessary to set free Ca\(^{2+}\) in the range of 1 nmol/L to 10 mmol/L (total volume 100 µl). Ca\(^{2+}\)-EGTA ratios were calculated using the program MaxChelator (http://maxchelator.stanford.edu/). All incubations lasted 90 minutes at 36°C. Samples were run in duplicate, filtered onto glass fiber filters (Whatman GF/B), and washed three times with 5 ml of cold water using a Brandel M-24R cell harvester. The filters were placed in scintillation vials, 8 ml of liquid scintillation mixture was added, and the retained radioactivity was measured in a Beckman LS-6500 β-counter. The specific binding was defined as the difference between the binding in the absence (total binding) and presence (nonspecific binding) of 20 µmol/L unlabeled ryanodine. Fitting of data was accomplished with the computer program Origin (version 7.5, Microcal Inc., Northampton, MA).

**Statistical analysis**

Data are presented as means ± SEM. Statistical significance was evaluated by analysis of Student’s t-test or ANOVA followed by the Bonferroni multiple comparisons test or chi square whenever appropriate. Differences with values of p<0.05 were considered statistically significant.
REFERENCES


TABLES

Online Table I. Ca\(^{2+}\)-spark characteristics in intact cells.

<table>
<thead>
<tr>
<th></th>
<th>Peak (F/F(_{0}))</th>
<th>FWHM (µm)</th>
<th>FDHM (ms)</th>
<th>Ttp (ms)</th>
<th>N (sparks/cells/mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.77±0.02</td>
<td>1.20±0.02</td>
<td>15.99±0.44</td>
<td>9.42±0.33</td>
<td>609/38/7</td>
</tr>
<tr>
<td>WT +Iso</td>
<td>1.71±0.02</td>
<td>1.20±0.03</td>
<td>17.40±0.74</td>
<td>10.31±0.55</td>
<td>297/10/5</td>
</tr>
<tr>
<td>RyR2(^{R4496C})</td>
<td>1.70±0.01**</td>
<td>1.17±0.01</td>
<td>15.95±0.74</td>
<td>9.15±0.21</td>
<td>1284/43/6</td>
</tr>
<tr>
<td>RyR2(^{R4496C}+Iso)</td>
<td>1.69±0.01</td>
<td>1.15±0.01</td>
<td>16.53±0.38</td>
<td>9.56±0.27</td>
<td>886/17/6</td>
</tr>
</tbody>
</table>

WT: (Wild type: RyR2\(^{R4496C}-/-\) mice) RyR2\(^{R4496C}\): transgenic (RyR2\(^{R4496C}-/+\) mice). Iso: 1 µM isoproterenol. FWHM: Full width at half maximum; FDHM: Full duration at half maximum; Ttp: time to peak. ** p<0.01 WT, WT vs. RyR2\(^{R4496C}\) mice.
Online Table II. Ca\textsuperscript{2+}-spark characteristics in permeabilized cells.

<table>
<thead>
<tr>
<th>[Ca\textsuperscript{2+}]_i (nM)</th>
<th>Peak (F/F\textsubscript{0})</th>
<th>FWHM (µm)</th>
<th>FDHM (ms)</th>
<th>Ttp (ms)</th>
<th>n (sparks/cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 WT</td>
<td>1.51±0.06</td>
<td>1.03±0.07</td>
<td>12.24±1.12</td>
<td>16.52±1.38</td>
<td>55/6</td>
</tr>
<tr>
<td>RyR2\textsuperscript{R4496C}</td>
<td>1.53±0.06</td>
<td>0.99±0.04</td>
<td>12.05±1.06</td>
<td>14.22±1.11</td>
<td>53/6</td>
</tr>
<tr>
<td>7.5 WT</td>
<td>1.49±0.01</td>
<td>1.23±0.01</td>
<td>19.23±0.32</td>
<td>18.27±0.45</td>
<td>909/9</td>
</tr>
<tr>
<td>RyR2\textsuperscript{R4496C}</td>
<td>1.40±0.00***</td>
<td>1.17±0.06***</td>
<td>20.72±0.15***</td>
<td>14.82±0.20***</td>
<td>3584/7</td>
</tr>
<tr>
<td>15 WT</td>
<td>1.41±0.06</td>
<td>1.08±0.00</td>
<td>19.72±0.22</td>
<td>15.54±0.25</td>
<td>1992/10</td>
</tr>
<tr>
<td>RyR2\textsuperscript{R4496C}</td>
<td>1.20±0.02***</td>
<td>0.98±0.00***</td>
<td>19.14±0.15*</td>
<td>14.78±0.23*</td>
<td>4247/6</td>
</tr>
<tr>
<td>30 WT</td>
<td>1.79±0.00</td>
<td>1.50±0.00</td>
<td>18.85±0.12</td>
<td>12.93±0.11</td>
<td>6082/23</td>
</tr>
<tr>
<td>RyR2\textsuperscript{R4496C}</td>
<td>1.62±0.00***</td>
<td>1.41±0.00***</td>
<td>17.65±0.09***</td>
<td>11.82±0.10***</td>
<td>7971/19</td>
</tr>
<tr>
<td>50 WT</td>
<td>1.60±0.00</td>
<td>1.38±0.00</td>
<td>17.26±0.09</td>
<td>10.77±0.06</td>
<td>12341/37</td>
</tr>
<tr>
<td>RyR2\textsuperscript{R4496C}</td>
<td>1.60±0.00</td>
<td>1.40±0.00***</td>
<td>19.81±0.06***</td>
<td>10.18±0.04***</td>
<td>22797/44</td>
</tr>
</tbody>
</table>

WT: (Wild type: RyR2\textsuperscript{R4496C}/- mice) RyR2\textsuperscript{R4496C}: transgenic (RyR2\textsuperscript{R4496C}/+ mice). Iso: 1 µM isoproterenol. FWHM: Full width at half maximum; FDHM: Full duration at half maximum; Ttp: time to peak. * p<0.05, ***p<0.001 WT vs. RyR2\textsuperscript{R4496C} mice.
Online Table III. [Ca^{2+}] transient characteristics in WT and R4496C myocytes.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>F/F₀</th>
<th>τ (ms)</th>
<th>Contraction (%)</th>
<th>Time to peak (ms)</th>
<th>n</th>
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<tbody>
<tr>
<td>2Hz</td>
<td>WT</td>
<td>B</td>
<td>4.6±0.1</td>
<td>95.6±4.3</td>
<td>10.2±0.9</td>
<td>20.1±0.9</td>
</tr>
<tr>
<td></td>
<td>ISO</td>
<td></td>
<td>5.5±0.2***</td>
<td>79.9±3.4*</td>
<td>13.6±1.2*</td>
<td>25.7±5.6</td>
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<tr>
<td></td>
<td>R4496C</td>
<td>B</td>
<td>4.5±0.2</td>
<td>104.5±2.9</td>
<td>9.0±0.6</td>
<td>19.9±1.0</td>
</tr>
<tr>
<td></td>
<td>ISO</td>
<td></td>
<td>5.3±0.3*</td>
<td>84.5±3.3***</td>
<td>11.7±5.9*</td>
<td>23.1±3.1</td>
</tr>
<tr>
<td>3Hz</td>
<td>WT</td>
<td>B</td>
<td>3.8±0.5</td>
<td>100.7±10.3</td>
<td>9.2±1.6</td>
<td>28.1±4.2</td>
</tr>
<tr>
<td></td>
<td>ISO</td>
<td></td>
<td>5.1±0.4*</td>
<td>78.2±10.5*</td>
<td>11.0±1.3*</td>
<td>32.7±2.4</td>
</tr>
<tr>
<td></td>
<td>R4496C</td>
<td>B</td>
<td>3.3±0.2</td>
<td>112.0±6.7</td>
<td>8.9±1.6</td>
<td>29.0±4.5</td>
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<tr>
<td></td>
<td>ISO</td>
<td></td>
<td>3.7±0.3*</td>
<td>91.6±5.2*</td>
<td>14.8±0.9*</td>
<td>31.0±5.2</td>
</tr>
<tr>
<td>4Hz</td>
<td>WT</td>
<td>B</td>
<td>3.6±0.3</td>
<td>92.0±6.6</td>
<td>8.4±1.1</td>
<td>30.0±1.5</td>
</tr>
<tr>
<td></td>
<td>ISO</td>
<td></td>
<td>4.2±0.5**</td>
<td>84.4±6.0*</td>
<td>10.7±0.8*</td>
<td>31.7±3.1</td>
</tr>
<tr>
<td></td>
<td>R4496C</td>
<td>B</td>
<td>2.8±0.1†</td>
<td>113.6±6.9†</td>
<td>6.0±0.9†</td>
<td>28.5±1.0</td>
</tr>
<tr>
<td></td>
<td>ISO</td>
<td></td>
<td>3.1±0.2**</td>
<td>89.4±6.3**</td>
<td>10.0±0.8*</td>
<td>31.7±3.2</td>
</tr>
</tbody>
</table>

WT: (wild type, RyR2^{R4496C−/−} mice); R4496C: (RyR2^{R4496C+/+} mice). B= baseline, without Isoproterenol ISO= 1 μM Isoproterenol. F/F₀= [Ca^{2+}] transient amplitude, τ= [Ca^{2+}] transient decay time, * P<0.05, **p<0.01, *** P<0.001 B vs. ISO. †P<0.05 WT vs. R4496C.
Online Figure I. RyR2 and PLN protein levels were unchanged in RyR2\textsuperscript{R4496C} hearts.

The basal levels of RyR2, phospholamban, phospho-Ser-2809-RyR2, phospho-Thr-17-PLN and phospho-Ser-16-PLN were similar in WT and RyR2\textsuperscript{R4496C} hearts. Isoproterenol treatment significantly induced an increase in the level of phosphorylated proteins in both groups (n=2 hearts per genotype) * p <0.05 with respect to the same group in the absence of isoproterenol.

Online Figure II. 100 nmol/L isoproterenol induces similar response in WT and RyR2\textsuperscript{R4496C} myocytes.

A. Percentage of increase in Ca\textsuperscript{2+} spark frequency induced by 100 nmol/L isoproterenol in 6 WT and 7 RyR2\textsuperscript{R4496C} cells. B. Percentage of increase in [Ca\textsuperscript{2+}], transient amplitude induced by 100 nmol/L isoproterenol on caffeine evoked [Ca\textsuperscript{2+}], transients in WT (white bars) and RyR2\textsuperscript{R4496C} (hatched bars) myocytes after stimulating the cell at 2 Hz (n= 5 vs. n=6) and 4 Hz (n= 8 vs. n=13).

Online Figure III. PKA and CaMKII inhibitors failed to reduce the Ca\textsuperscript{2+} spark frequency in RyR2\textsuperscript{R4496C} myocytes.

A. Ca\textsuperscript{2+} spark frequency recorded in 8 RyR2\textsuperscript{R4496C} cells before (white bar) and during (gray bar) perfusion with the PKA inhibitor KT5720 (2 μmol/L). B. Ca\textsuperscript{2+} spark frequency recorded in 10 RyR2\textsuperscript{R4496C} cells before (white bar) and during (gray bar) perfusion with the CaMKII inhibitor KN93 (1 μmol/L).
Online Fig. I

1μmol/L ISO

kDa

P-Ser2809-RyR2
RyR2
P-Thr17-PLB
P-Ser16-PLB
PLB

10

260

% change vs. WT

RyR2
P-Ser2809-RyR2
P-Thr17-PLB
P-Ser16-PLB
PLB

WT
R4496C
WT + Iso
R4496C + Iso

WT
R4496C
WT + Iso
R4496C + Iso
Online Figure II

A

Iso effect on Ca\(^{2+}\) sparks occurrence (% of control)

Control  R4496C

B

Iso effect on \([Ca^{2+}]\) transient (% F/F\(_0\) of control)

Control  R4496C  Control  R4496C

2 Hz  4 Hz
Online Fig. III

A

Ca^{2+} Spark Frequency

R4496C  R4496C +KT5720

B

R4496C  R4496C +KN93