Increased Ca2+ sensitivity of the ryanodine receptor mutant RyR2R4496C underlies catecholaminergic polymorphic ventricular tachycardia

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Abstract

Cardiac ryanodine receptor (RyR2) mutations are associated with autosomal dominant catecholaminergic polymorphic ventricular tachycardia (CPVT), 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, WT) 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 (SR) Ca^{2+} load were similar in WT 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 after-depolarizations. The occurrence of this abnormal Ca^{2+} release was enhanced at faster stimulation rates and by β-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 WT and $RyR2^{R4496C}$ cells, indicating that the β-adrenergic sensitivity of $RyR2^{R4496C}$ cells remained unaltered. This effect was independent of protein expression variations since no difference was found in the total or phosphorylated RyR2 expression levels. In conclusion, the arrhythmogenic potential of the $RyR2^{R4496C}$ mutation is due to the increased Ca^{2+} sensitivity of $RyR2^{R4496C}$, which induces diastolic Ca^{2+} release and lowers the threshold for triggered activity.

MESH Keywords Adrenergic beta-Agonists ; pharmacology ; Animals ; Caffeine ; pharmacology ; Calcium Signaling ; drug effects ; Cardiac Pacing, Artificial ; Catecholamines ; metabolism ; Female ; Isoproterenol ; pharmacology ; Male ; Membrane Potentials ; Mice ; Mice, Transgenic ; Microscopy, Confocal ; Mutation ; Myocardial Contraction ; drug effects ; Myocytes, Cardiac ; drug effects ; metabolism ; Phosphorylation ; Ryanodine Receptor Calcium Release Channel ; genetics ; metabolism ; Sarcoplasmic Reticulum ; metabolism ; Tachycardia, Ventricular ; genetics ; metabolism ; physiopathology ; Time Factors

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 hearts1. During exercise or acute emotions, CPVT patients develop life-threatening ventricular arrhythmias leading to syncope or sudden death. The first RyR2 mutation identified in a CPVT family was R4497C2. Today, more than 70 RyR2 mutations have been reported (http://www.fsm.it/cardmoc/), and they comprise the most common genetic subtype of CPVT3–7, although mutations in the calsequestrin gene can also cause CPVT8,9.

Diverging results and conclusions have been generated from expression studies of $RyR2^{R4496C}$ in heterologous systems. Jiang and collaborators 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 (PKA) phosphorylation11 or sarcoplasmic reticulum (SR) Ca^{2+} overload12. 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 proteins13 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 developed14. Their phenotype presents extraordinary similarity with the clinical manifestations of patients carrying the $RyR2^{R4497C}$ mutation, including the development of bidirectional ventricular tachycardia. When exposed to adrenaline and caffeine, the $RyR2^{R4496C}$ cardiomyocytes develop delayed after-depolarizations (DADs)15, suggesting that triggered arrhythmias are elicited by adrenergic activation16. Here we demonstrate that untreated $RyR2^{R4496C}$

myocytes have increased spontaneous Ca^{2+} release in diastole during electrical pacing, due to the enhanced Ca^{2+} sensitivity of mutant RyR2; this abnormality is further augmented by exposure to isoproterenol and increasing pacing rates.

MATERIALS AND METHODS

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 digestion17. $[Ca^{2+}]_i$ transients and Ca^{2+}-sparks were viewed in isolated myocytes by confocal microscopy and analyzed using homemade routines.

A complete Methods section can be found in the Online Supplemental Material.

RESULTS

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 electrical stimulation15. 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 electrical pacing interruption (Fig. 1A). On the contrary, the $RyR2^{R4496C}$ cell showed Ca^{2+}-waves evoking two $[Ca^{2+}]_i$ transients, just after stimulation stopped, which was followed by several Ca^{2+}-waves, consistent with triggered activity and DADs (Fig. 1B). These data suggest that abnormal Ca^{2+} release underlies the electrical abnormalities in $RyR2^{R4496C}$ cardiomyocytes. Since DADs are initiated by Ca^{2+}-waves that are in turn initiated by Ca^{2+}-sparks18, we analyzed Ca^{2+}-sparks in $RyR2^{R4496C}$ cardiomyocytes. $[Ca^{2+}]_i$ transients and waves characteristics are presented below.

Ca^{2+}-sparks in $RyR2^{R4496C}$ myocytes

Figure 2A shows representative images of Ca^{2+}-sparks19. Ca^{2+} spark frequency was double in $RyR2^{R4496C}$ cells compared with WT cells ($p<0.001$, Figure 2B). This could be due to an increase in (i) the SR Ca^{2+} load, (ii) the level of RyR2 expression and/or phosphorylation, (iii) the diastolic $[Ca^{2+}]_i$ or (iv) changes in the intrinsic channel properties.

We estimated SR Ca^{2+} load in quiescent ventricular myocytes. $RyR2^{R4496C}$ showed reduced SR Ca^{2+} content (F/F_0: 7.0±0.5, n=11 in $RyR2^{R4496C}$ vs. 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 (Online Table I).

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 $RyR2^{R4496C}$ (Online Fig. 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 (Fig. 2B) by the same percentage (Fig. 2C). Ca^{2+}-spark characteristics in the presence of isoproterenol are provided in Online Table I. Similar results were found using a lower isoproterenol concentration (100 nmol/L) (Online Fig. IIA). Furthermore, treatment of $RyR2^{R4496C}$ myocytes with either a PKA blocker (KT5720) or a CaMKII blocker (KN93) failed to decrease Ca^{2+}-spark frequency (Online Fig. 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 $[Ca^{2+}]_i$, 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 due 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 (about 20 s). Firing sites were counted as the sites where we recorded at least one Ca^{2+}-spark. Fig. 2D shows that the $RyR2^{R4496C}$ myocytes presented more firing sites, and that isoproterenol increased the number of sites in both WT and $RyR2^{R4496C}$ cells. This indicates that the $RyR2^{R4496C}$ cells presented more Ca^{2+}-sparks due to the existence of more active RyR2 clusters (Fig. 2E). We also measured the maximum number of Ca^{2+}-sparks recorded at the same site in each group and found that this was also significantly increased in $RyR2^{R4496C}$ myocytes and further enhanced by β-adrenergic stimulation (Fig. 2F). Taken together, these data suggest that $RyR2^{R4496C}$ cells present more Ca^{2+}-sparks because of more active RyR2s clusters and a greater probability of repetitive openings of these clusters in the $RyR2^{R4496C}$ myocytes.

We next explored whether RyR2^{R4496C} presents abnormal Ca^{2+} sensitivity. We analyzed Ca^{2+}-sparks in permeabilized cells exposed to various cytoplasmic $[Ca^{2+}]_i$. Figure 3A illustrates enhanced Ca^{2+}-sparks occurrence in a RyR2^{R4496C} cell at 30 nmol/L $[Ca^{2+}]_i$. At all tested $[Ca^{2+}]_i$, Ca^{2+}-sparks were much more frequent in RyR2^{R4496C} than in WT myocytes, consistent with increased cytosolic Ca^{2+} sensitivity (Fig. 3B). Analysis of the Ca^{2+}-spark characteristics in permeabilized cells essentially confirmed the results obtained in intact cells (Online Table II). We estimated SR Ca^{2+} content in permeabilized cells and found that at all tested $[Ca^{2+}]_i$ the caffeine-evoked $[Ca^{2+}]_i$ transient was significantly decreased in the RyR2^{R4496C} cells (Fig. 3C). Thus the higher Ca^{2+} spark occurrence in RyR2^{R4496C} myocytes was not due to either a higher level of Ca^{2+} stored in the SR or an alteration of calsequestrin level evaluated by Western-blots (data not shown). Fig. 3D shows the luminal Ca^{2+} dependence of Ca^{2+}-sparks, apparently consistent with increased luminal Ca^{2+} sensitivity. However cytosolic and luminal Ca^{2+} vary concurrently. The high Ca^{2+}-spark occurrence recorded in RyR2^{R4496C} at very low intracellular Ca^{2+} might suggest that, rather than increasing RyR2^{R4496C} Ca^{2+} sensitivity, this mutation renders the RyR2 intrinsically active. We repeated the experiments at zero Ca^{2+} (0.5 mmol/L EGTA). In this condition, the occurrence of Ca^{2+}-sparks was indistinguishable between WT and RyR2^{R4496C} cells (Fig. 3.E&F, left), indicating that RyR2^{R4496C} hyperactivity requires cytosolic Ca^{2+}. To ensure that the SR was not depleted in our experimental conditions, we applied caffeine. A robust caffeine-induced Ca^{2+} transient could be evoked (Fig. 3E&F, right), proving that there was significant luminal Ca^{2+} to promote Ca^{2+}-sparks. Altogether, our results show that the RyR2^{R4496C} mutation increases the Ca^{2+} sensitivity of the channel. The RyR2 has 2 affinity Ca^{2+} binding sites on the cytosolic portion: one of high affinity that activates the channel and one of low affinity that inactivates it. Since ryanodine binds to open RyRs, we examined the Ca^{2+}-dependence of [^{3}H] ryanodine binding in heart crude membrane preparations. Bell-shape curves were obtained for both WT and RyR2^{R4496C} but Ca^{2+}-induced maximal activation of RyR2^{R4496C} was reached at one order of magnitude lower in RyR2^{R4496C} (Fig. 3G), indicating that the cytosolic Ca^{2+} sensitivity of RyR2 is greatly increased. We normalized the [^{3}H]ryanodine binding and fitted data to the Hill equation20 to get the values for Ca^{2+} affinity to the RyR activation (Ka, 21.9±8.3 μmol/L vs. 4.9±1.0μmol/L, n=4, $p<0.003$ for WT and RyR2^{R4496C} membranes, respectively) and inactivation (6.5±0.6 mmol/L vs. 4.8±1.1 mmol/L; $p>0.05$ for WT and RyR2^{R4496C} membranes) sites. Theses results show a 4.5 fold increase of cytosolic Ca^{2+} sensitivity for the RyR2^{R4496C}.

$[Ca^{2+}]_i$ transients in RyR2^{R4496C} myocytes

To determine whether the alteration of diastolic Ca^{2+} spark frequency in RyR2^{R4496C} cardiomyocytes has an impact during systole, we compared $[Ca^{2+}]_i$ transients and cell contraction at different pacing rates (2 Hz, 3 Hz, and 4 Hz) (Online table III). At 2 Hz, the $[Ca^{2+}]_i$ transient amplitude, its time to peak, the $[Ca^{2+}]_i$ transient decay time and cellular contraction, were similar ($p>0.05$) in WT and RyR2^{R4496C} cells, consistent with normal heart function in mice at rest. As stimulation rate increased, weaker $[Ca^{2+}]_i$ transients were evoked both in WT and RyR2^{R4496C} cells (Fig. 4A). However, the decrease in $[Ca^{2+}]_i$ transient amplitude was more pronounced ($p<0.05$) in RyR2^{R4496C} cells. This reduction was associated with both weaker cellular contraction (Fig. 4B) and slower decay time (Fig. 4C), with no difference in the time to peak (Fig. 4D).

Because $[Ca^{2+}]_i$ transient amplitude depends on SR Ca^{2+} load, we investigated the SR Ca^{2+} content. Images of caffeine-evoked $[Ca^{2+}]_i$ transients evoked after electrical stimulation at 4 Hz are shown in Figure 4E. As shown in Figure 4F, caffeine-evoked $[Ca^{2+}]_i$ transients were significantly smaller (by 24.7%) after pacing the cell at 4 Hz in RyR2^{R4496C} compared to WT myocytes, while no significant difference was observed at lower frequencies. Plotting the peak $[Ca^{2+}]_i$ transient vs. the SR Ca^{2+} load for the three different pacing rates (Fig. 4G) provided similar correlations in WT and RyR2^{R4496C} myocytes. These results suggest that a decrease in SR Ca^{2+} load accounts for the reduction in systolic $[Ca^{2+}]_i$ transients and the associated lower contraction observed at the highest pacing rates in both cell groups. Interestingly, the decrease in SR load with increasing pacing rate was accentuated in RyR2^{R4496C} myocytes. To get an idea of how much Ca^{2+} is released at each twitch with respect to the total amount of Ca^{2+} stored, we evaluated the fractional release by normalizing the electrically-evoked $[Ca^{2+}]_i$ transient to the caffeine-evoked $[Ca^{2+}]_i$ transient in each cell tested. We found no difference between WT and RyR2^{R4496c} cells. For example at 4 Hz, fractional release was 0.80±0.04 in WT cells (n=8) and 0.82±0.06 in RyR2^{R4496C} myocytes (n=21). Altogether, these experiments unmask rate-dependent systolic Ca^{2+}-release defects in RyR2^{R4496C} cells in relation with impaired recovery of SR Ca^{2+} load, which might be secondary to diastolic Ca^{2+} leak.

However, the relevance of this systolic defect may be limited in vivo, since increased heart rate is usually associated with high sympathetic drive, thus increased contractility. We next analyzed $[Ca^{2+}]_i$ transients under β-adrenergic stimulation. Images of $[Ca^{2+}]_i$ transients evoked by field stimulation at 4 Hz are shown in Figure 5A, top. The increase in $[Ca^{2+}]_i$ transient amplitude induced by isoproterenol was similar in WT and RyR2^{R4496C} cells, (Fig. 5A, bottom, Online Fig. IIB, Online Table III). We also found a similar effect of isoproterenol on SR Ca^{2+} load in both experimental groups (Fig. 5B). Altogether, these data suggest that the systolic dysfunction at high

pacing rates persists during sympathetic stimulation ($p<0.01$), and that the incremental effect of β-adrenergic stimulation is identical in WT and $RyR2^{R4496C}$.

Nature of the arrhythmogenic activity of $RyR2^{R4496C}$ myocytes

The arrhythmic activity of the isolated cell presented in Fig. 1 does not reflect the reality of ventricular myocytes, which are under constant electrical stimulation. Moreover, CPVT is induced by stress, meaning β-adrenergic stimulation and elevated heart rhythm. During electrical stimulation and in the presence of isoproterenol, the $RyR2^{R4496C}$ myocytes developed spontaneous $[Ca^{2+}]_i$ transients and after-contractions, consistent with triggered activity, as opposed to the WT cells (Fig. 6A&B). During diastole, the $RyR2^{R4496C}$ myocytes showed spontaneous Ca^{2+} release as Ca^{2+}-sparks or small Ca^{2+}-waves. Occasionally (arrow in Fig. 6B), spontaneous Ca^{2+} releases reached the threshold to produce non-sustained triggered activity and after-contractions. Ca^{2+}-spark evoked Ca^{2+}-waves were observed during the diastolic period in 17% of the $RyR2^{R4496C}$ cells paced at 2 Hz (9 out of 54 $RyR2^{R4496C}$ myocytes). The percentage of cells exhibiting these events dramatically increased to 67% (12 out of 18 cells) when $RyR2^{R4496C}$ cells were paced at 4 Hz in the presence of isoproterenol. This behavior was almost absent in WT cells, both in basal conditions (2Hz: 1 out of 55 WT cells vs. 9 out of 54 $RyR2^{R4496c}$ cells $p<0.01$) and at 4 Hz and isoproterenol (2 out of 14 WT cells vs. 12 out of 18 $RyR2^{R4496C}$ cells, $p<0.01$). As shown in Fig. 1B, one single Ca^{2+}-wave was able to evoke triggered activity after electrical stimulation stopped. However, this was never the case during constant stimulation. In order to trigger a full $[Ca^{2+}]_i$ transient, we measured that 7.1±0.6 Ca^{2+}-waves*100μm^{-1} had to overlap during diastole, which only occurred under stress conditions. Ca^{2+}-waves spread at similar velocity in the absence (117.3±14.7 μm/s, n=13) or in the presence of 1μmol/L isoproterenol (113.8±7.2 μm/s, n=22).

We found that mouse $RyR2^{R4496C}$ myocytes presented a higher incidence of Ca^{2+}-waves not only in the presence of isoproterenol but also at higher pacing rates (Fig. 6C). We then measured Ca^{2+}-sparks in the diastolic period at different stimulation frequencies, when it was possible to discriminate them. During diastole, the maximum number of Ca^{2+}-sparks*s^{-1} in $RyR2^{R4496C}$ myocytes under β-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 due to the higher diastolic $[Ca^{2+}]_i$ induced by increasing pacing rates. The diastolic Ca^{2+} 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 $RyR2^{R4496C}$ 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 β-adrenergic stimulation and increasing pacing rates, mimicking human exercise-induced ventricular tachycardia. The high activity of $RyR2^{R4496C}$ was due 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 $RyR2^{R4496C}$ mutation, which is the equivalent to the human $RyR2^{R4497C}$ mutation first identified in a CPVT family14, 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 electrical pacing was interrupted15. Here we found parallel evidence of spontaneous intracellular Ca^{2+} release and Ca^{2+}-waves in similar experimental conditions (Fig. 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 report is the first to show that isolated $RyR2^{R4496C}$ ventricular myocytes displayed arrhythmogenic activity related to spontaneous Ca^{2+} release while they are electrically stimulated, thus mimicking human CPVT and demonstrating that $RyR2^{R4496C}$ was at the origin of the arrhythmia. In isolated cardiomyocytes paced at 2Hz, we observed multiple Ca^{2+}-sparks capable of triggering localized Ca^{2+}-waves in more than 16% of $RyR2^{R4496C}$ myocytes (Fig. 6C). With pacing rate increased to 4 Hz and under β-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 β-adrenergic stimulation and pacing rate, reaching threshold for triggered activity.

The higher diastolic Ca^{2+} release in $RyR2^{R4496C}$ cells is correlated by higher frequency of spontaneous Ca^{2+}-sparks (Fig. 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 luminal21 or cytosolic Ca^{2+}. We found no difference between WT and $RyR2^{R4496C}$ hearts in total RyR2

expression, FKBP12.6 association14 or RyR2 phosphorylation level, even after β-adrenergic stimulation (Online Fig. I). Moreover, although Ca^{2+}-waves and high Ca^{2+}-spark frequency usually reflect Ca^{2+}overload18, $RyR2^{R4496C}$ myocytes presented this behavior even at lower SR Ca^{2+} load.

Our data in permeabilized cardiomyocytes show that at all cytoplasmic $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, 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 luminal10 and cytosolic Ca^{2+}(Fig. 3). It is not easy to unequivocally assign distinct roles for cytoplasmic vs. luminal Ca^{2+} in situ due to the inherent interdependence of these Ca^{2+} compartments in living cells.

While unzipping of amino and central RyR2 domains has been reported to be involved in some forms of enhanced RyR2 activity22,23, 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 model14. The increase in Ca^{2+}-spark frequency of $RyR2^{R4496C}$ is likely to reflect an enhancement of its open probability (P_o), consistent with data obtained by single channel analyses10. Our data demonstrate that, in its normal environment (i.e., in native cardiomyocytes) $RyR2^{R4496C}$ has augmented Ca^{2+} sensitivity rather than increased P_o per se. Indeed, Ca^{2+}-spark occurrence, measured in permeabilized cells exposed to different $[Ca^{2+}]_i$ concentrations, was significantly increased in $RyR2^{R4496C}$ at all $[Ca^{2+}]_i$ tested except at zero 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 side24, 25), close to the proposed molecular region involved in Ca^{2+}-dependent activation (residues 4485–4494)23. The RyR has highly reactive cysteines capable of forming disulfide bonds26. It is thus plausible that the highly reactive cysteine introduced by the mutation, interacts with other cysteines of the channel, inducing a conformation change that renders the RyR hypersensitive to Ca^{2+}. The conformational change might render more accessible to Ca^{2+} the E3987 residue, identified as important in Ca^{2+} sensitivity27. However, the low affinity Ca^{2+} sensing of the $RyR2^{R4496C}$ seems to be normal since the Ca^{2+} inhibition found in the 3[H^+]ryanodine binding experiments is similar to WT RyR2 (Fig. 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 (Fig. 2), β-adrenergic stimulation increased their activity to the same extent suggesting that: (i) the two mechanisms (increased Ca^{2+} sensitivity of the $RyR2^{R4496C}$ mutant and the effect of β-adrenergic stimulation) are distinct and cumulative and (ii) the β-adrenergic regulation of $RyR2^{R4496C}$ is not modified. Nevertheless, β-adrenergic stimulation further increased the already elevated diastolic Ca^{2+} leak in $RyR2^{R4496C}$ cells probably by increasing the SR Ca^{2+} load28, further enhancing the $RyR2^{R4496C}$ cell propensity to trigger DADs and allowing the occurrence of spontaneous activity (Figs. 1&6) 29,30.

At basal conditions (2Hz in our experimental setting), the $[Ca^{2+}]_i$ transients in WT and $RyR2^{R4496C}$ myocytes were similar. The $[Ca^{2+}]_i$ transient decay times were also similar, suggesting a normal function of sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA). However, the rate-dependent decrease in $[Ca^{2+}]_i$ transients and contraction, which is normal in mice cardiomyocytes, was more pronounced in $RyR2^{R4496C}$ (Fig. 4). This reduction can be accounted for by a decrease in SR Ca^{2+} load (Fig. 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 two 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+} re-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+} with pacing rate can also reflect the higher Ca^{2+} leak at higher stimulation frequencies (Fig. 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 due to slowing of the Ca^{2+} current inactivation31. 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 Ca^{2+} release during diastole, due to 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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Figure 1

Triggered activity observed in RyR2^{R4496C} myocytes

Line-scan images of ventricular myocytes isolated from (**A**) a WT mouse and (**B**) RyR2^{R4496C} mouse during electrical stimulation (4Hz). The corresponding fluorescence traces are shown below. Red lines indicate electrical stimulation. After pacing stopped, the WT cell remains silent while the RyR2^{R4496C} cell shows Ca^{2+}-waves that induce two full contractions followed by isolated Ca^{2+}-waves consistent with DADs.

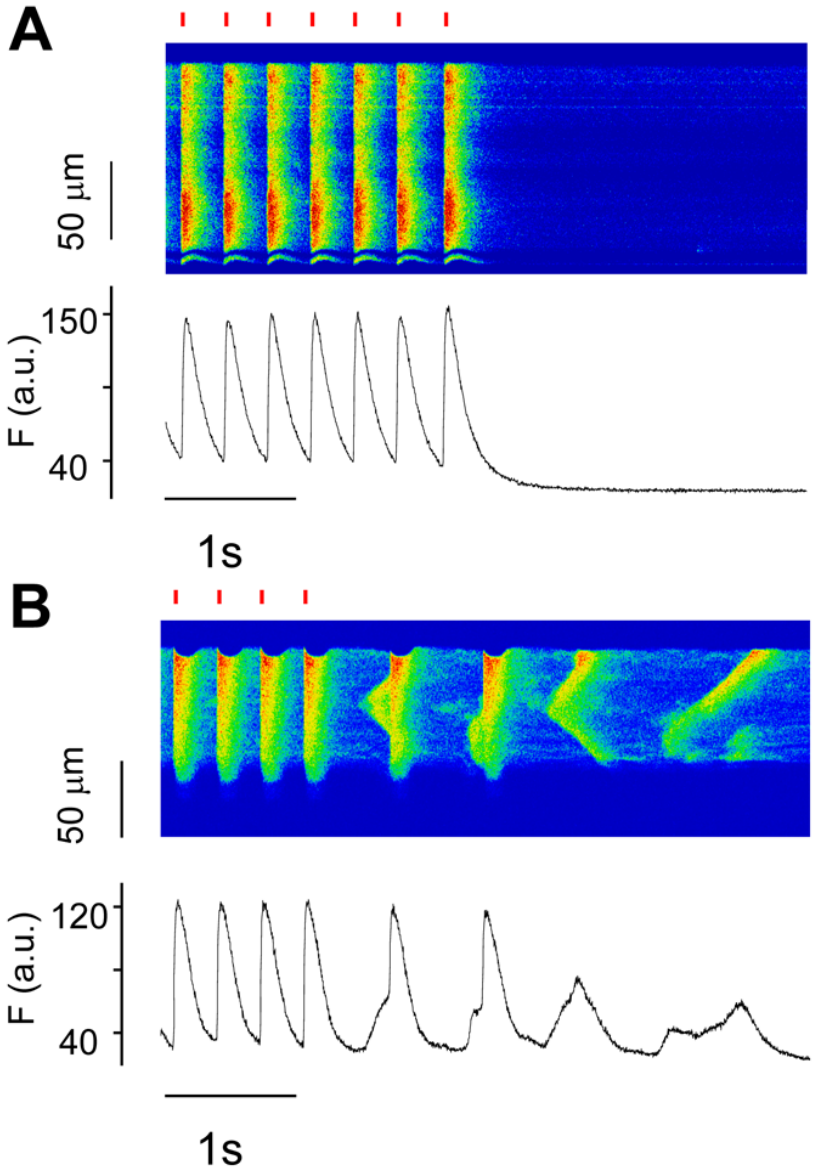

Figure 2

Ca^{2+}-spark in intact $RyR2^{R4496C}$ myocytes

A. Line-scan images obtained in a WT and a $RyR2^{R4496C}$ cell (R4496C) in the absence (top) or presence (bottom) of 1 μmol/L isoproterenol. **B**. Average of Ca^{2+}-spark occurrence in WT cells without (white bar, n=37) and with 1 μmol/L isoproterenol (hatched bar, n=10), and in $RyR2^{R4496C}$ cells without (blue bar, n=43) and with 1 μmol/L isoproterenol (hatched blue bar, n=17). **C**. Percentage of increase induced by 1 μmol/L isoproterenol on Ca^{2+}-spark frequency in 10 WT and 17 $RyR2^{R4496C}$ cells. **D**. Average of sites where Ca^{2+}-sparks are recorded within the same cell during the recording period (18 s). **E**. Probability in each cell to present sites that fire repetitively. **F**. Maximum number of Ca^{2+}-sparks recorded in the same site. For D, E & F: n= 37 WT cells, n=10 WT cells in the presence of 1 μmol/L isoproterenol; n=43 $RyR2^{R4496C}$ myocytes and n=17 $RyR2^{R4496C}$ myocytes under 1 μmol/L isoproterenol perfusion. *p<0.05, **p<0.01, ***p<0.001 vs. the same group in the absence of isoproterenol. †p<0.05, ††p<0.01, †††p<0.001 vs WT.

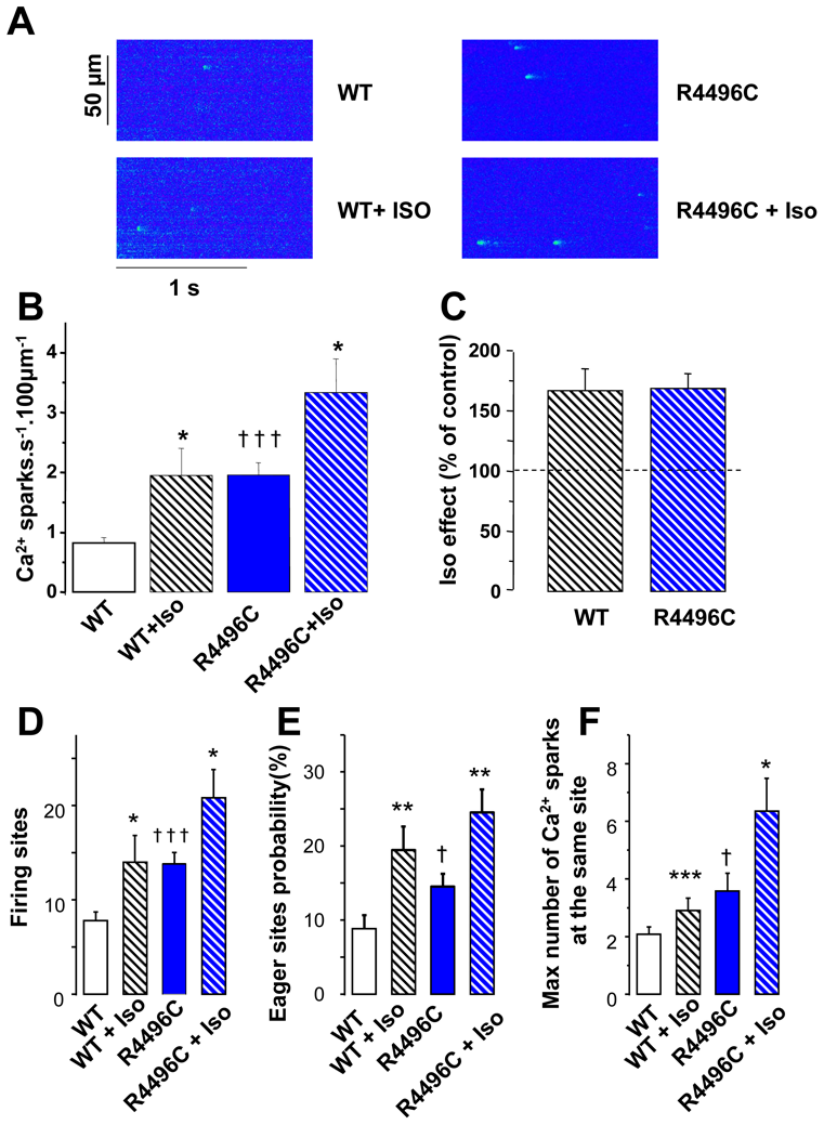

Figure 3

The increase in Ca^{2+}-spark frequency in $RyR2^{R4496C}$ cells depends on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

A. Line-scan images obtained at 30 nmol/L $[Ca^{2+}]_i$ in WT and $RyR2^{R4496C}$ (R4496C) permeabilized cells. **B**. Average of Ca^{2+}-spark frequencies obtained in permeabilized myocytes exposed at 7.5 nmol/L (n= 9 vs. n=7), 15 nmol/L (n=10 vs. n=6), 30 nmol/L (n= 23 vs. n=19), 50 nmol/L (n= 37 vs. n=44) and 100 nmol/L (n=8 vs. n=8) $[Ca^{2+}]_i$. WT cells vs. $RyR2^{R4496C}$ cells. Lines are Boltzmann fitting of the data. **C**. Caffeine-evoked (20 mmol/L) $[Ca^{2+}]_i$ transient in permeabilized myocytes at 7.5 nmol/L (n= 13 vs. n=11), 15 nmol/L (n=15 vs. n=10), 30 nmol/L (n=19 vs. n=20), 50 nmol/L (n=21 vs. n=33) and 100 nmol/L (n=9 vs. n=9) $[Ca^{2+}]_i$, WT cells vs. $RyR2^{R4496C}$ cells, expressed as % of the caffeine-evoked transient at 100 nmol/L in WT cells. The x axis labels indicate cytosolic $[Ca^{2+}]_i$ in nmol/L. **D**. Ca^{2+}-sparks frequencies plotted as a function of caffeine-evoked $[Ca^{2+}]_i$ transient. Numbers indicate cytosolic $[Ca^{2+}]_i$. **E**. Images of Ca^{2+}-sparks obtained at 0 nmol/L $[Ca^{2+}]_i$ in WT (top, left) and $RyR2^{R4496C}$ (R4496C, bottom, left) cells and during (right) 20 mmol/L caffeine application (arrow). **F**. Left. Ca^{2+}-spark frequency obtained in WT myocytes (n=6) and in $RyR2^{R4496C}$ myocytes (n=6). Right. Caffeine-evoked $[Ca^{2+}]_i$ transient amplitude (in F/F_0) measured at zero $[Ca^{2+}]_i$ in 15 WT cells and 16 $RyR2^{R4496C}$ cells. **G**. Specific Ca^{2+}-dependent [^{3}H]ryanodine binding curves to crude membrane fractions of WT (n=4) and R4496C+/+ (n=4) heart tissues. Values have been normalized and fitted to the equation $y=Bmax*([Ca^{2+}]^{na}/([Ca^{2+}]^{na}+K_a^{na}))(1-([Ca^{2+}]^{ni}/([Ca^{2+}]^{ni}+K_i^{ni})))+C$. Open symbols, WT; blue symbols, $RyR2^{R4496C}$. Solid line for WT and dotted line for $RyR2^{R4496C}$ cells or homogenates. *p<0.05, **p<0.01, ***p<0.001.

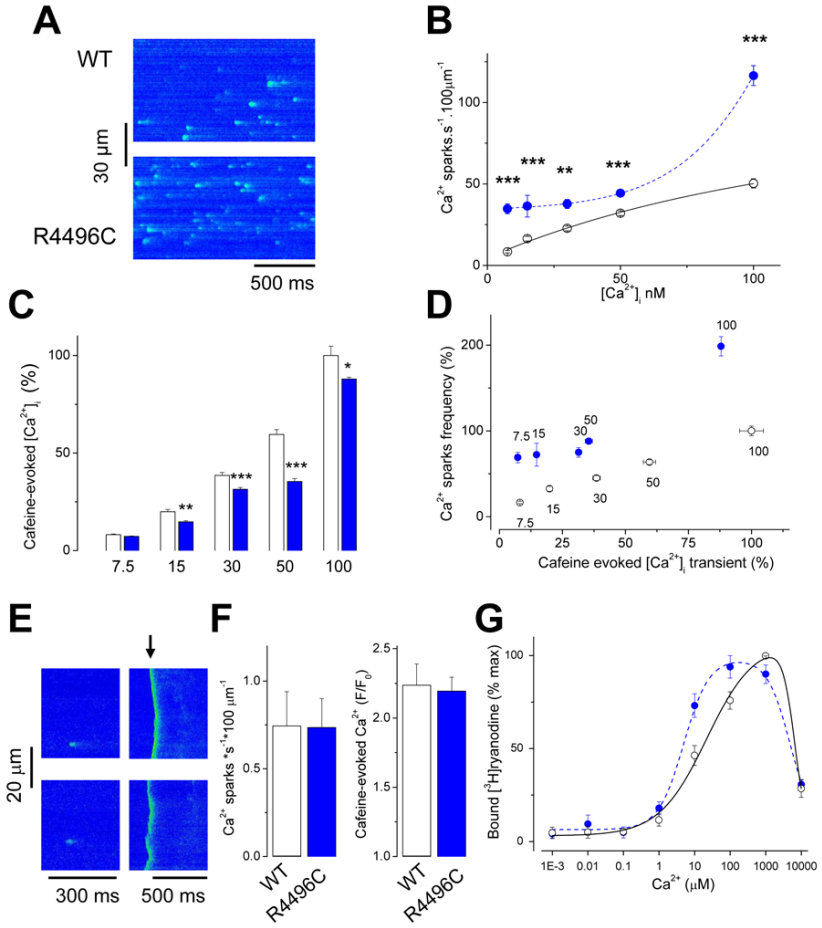

Figure 4

RyR2^{R4496C} myocytes show a rate-dependent decrease in $[Ca^{2+}]_i$ transients and SR Ca^{2+} load

A. Average of $[Ca^{2+}]_i$ transients amplitude (expressed as F/F_0, where F is the peak fluorescence signal and F_0 the diastolic fluorescence) of WT) vs. RyR2^{R4496C} myocytes obtained by field stimulation at 2 Hz (n= 50 vs. n=52), 3 Hz (n=16 vs. n=21) and 4 Hz (n=14 vs. n=21). **B. C**& **D**. Bar graphs comparing the average cell shortening (**B**), $[Ca^{2+}]_i$ transient decay time (**C**), or time to peak (**D**) at 4 Hz in WT (n=13) vs. RyR2 ^{R4496C} myocytes (n=19). **E**. Line-scan images of caffeine-evoked $[Ca^{2+}]_i$ transients obtained in WT and RyR2^{R4496C} (R4496C) cells obtained after field stimulation at 4 Hz. **F**. Average caffeine-evoked $[Ca^{2+}]_i$ transients (expressed as peak F/F_0, as in panel A) in WT vs. RyR2^{R4496C}) myocytes obtained following field stimulation at 2 Hz (n=33 vs. n=36), 3 Hz (n=7 vs. n=22) and 4 Hz (n= 8 vs. n=25). **G**. $[Ca^{2+}]_i$ transient-SR Ca^{2+} load relationship in WT and RyR2^{R4496C} myocytes at various pacing rates. Open circles and bars, WT; blue circles and bars, RyR2^{R4496C}. N numbers from panels A&F.*p<0.05.

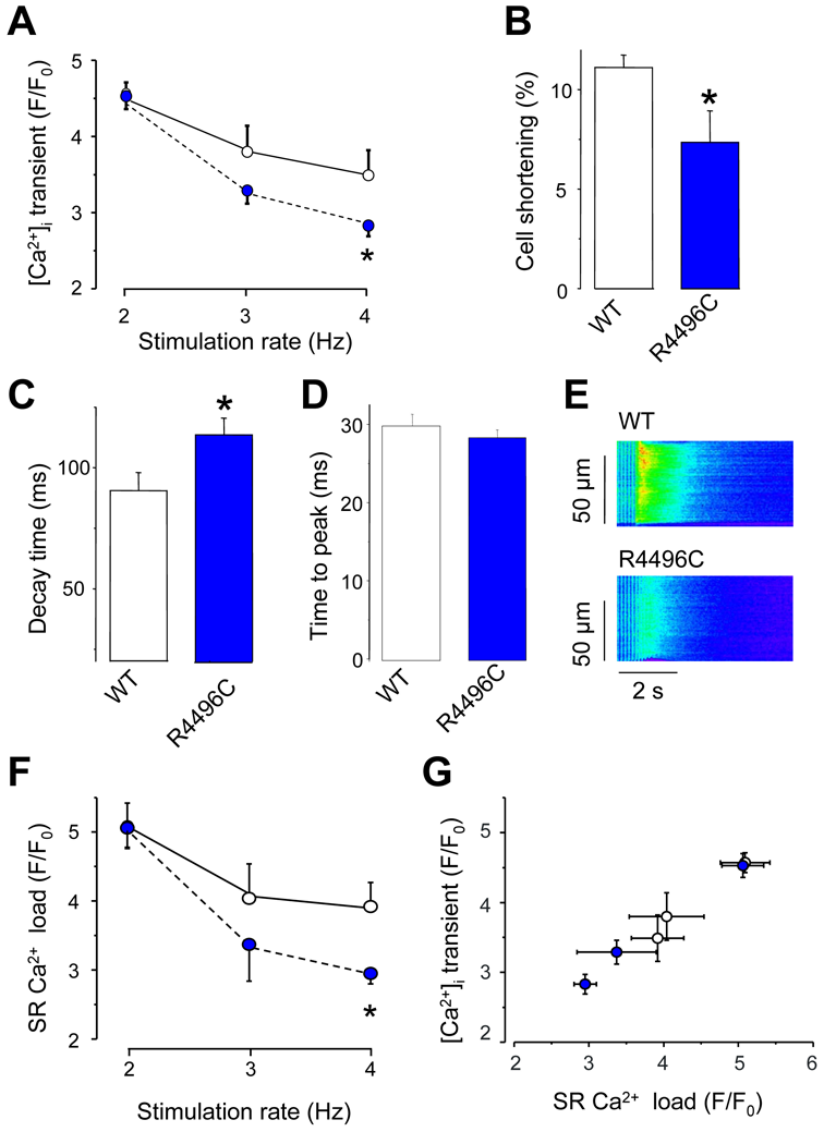

Figure 5

Conserved β-adrenergic responsiveness in RyR2^{R4496C} myocytes

A. Top. Line-scan images of $[Ca^{2+}]_i$ transients obtained in WT and RyR2^{R4496C} (R4496C) cells evoked by field stimulation at 4 Hz in the absence and the presence (ISO) of 1 μmol/L isoproterenol. **Bottom.** Percentage of $[Ca^{2+}]_i$ transient amplitude increase induced by 1 μmol/L isoproterenol at 2 (n=26 vs. n=25), 3 (n=4 vs. n=12) and 4 (n=3 vs. n=12) Hz, WT vs. RyR2^{R4496C} cells. **B**. Percentage of increase induced by 1 μmol/L isoproterenol on caffeine evoked $[Ca^{2+}]_i$ transients 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 (white hatched bars), RyR2^{R4496C} (blue hatched bars)

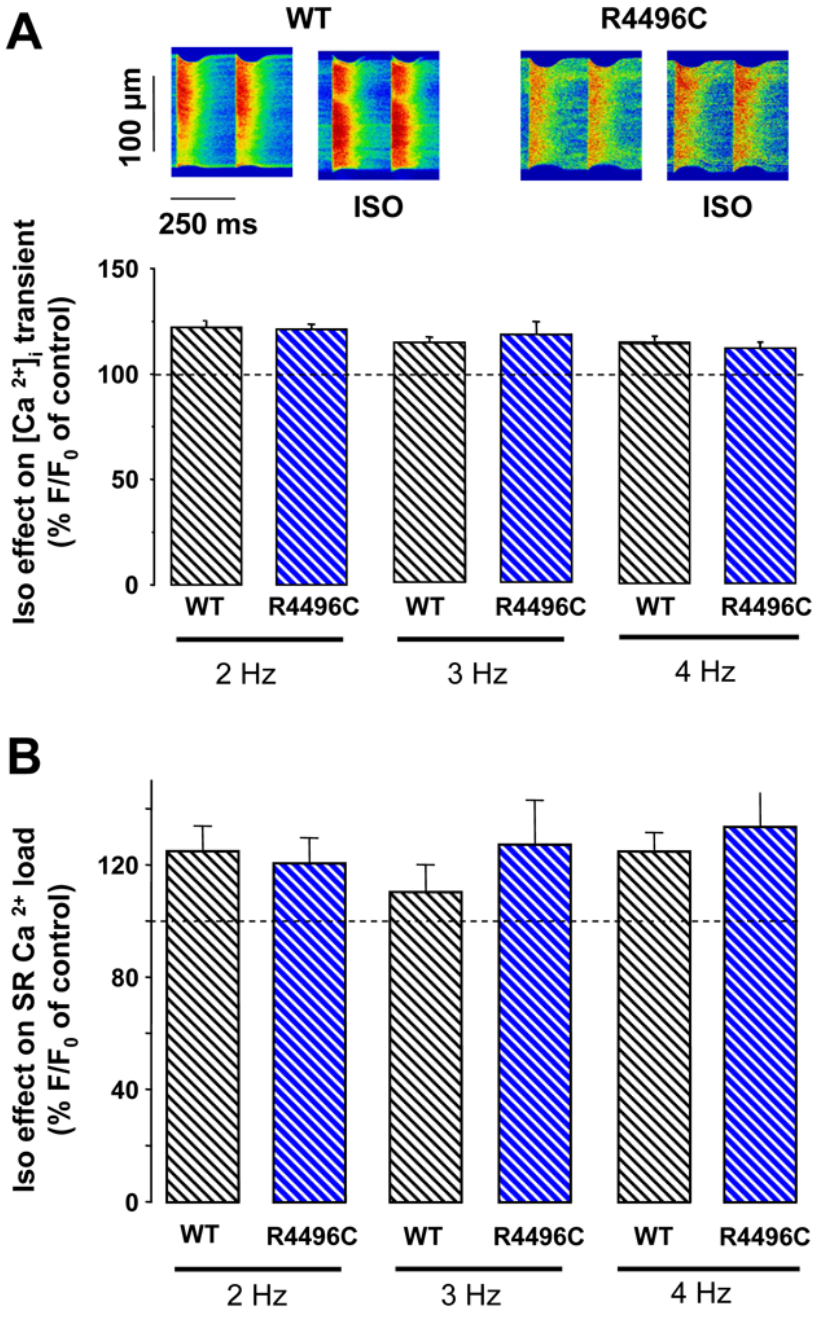

Figure 6

Arrhythmic activity depends on β-adrenergic stimulation and pacing rate

A. Line-scan images obtained in a WT cell paced at 2 Hz and superfused with 1 μmol/L isoproterenol. The corresponding fluorescence $[Ca^{2+}]_i$ transients and cell shortening profiles appear below. **B**. The same for a $RyR2^{R4496C}$ cell. The image shows multiple Ca^{2+}-sparks and/or Ca^{2+}-waves during diastole. The red arrow indicates triggered activity, maintained in panel **c** and spontaneously terminated in panel **d**. Red lines indicate electrical stimuli. **C**. Occurrence of abnormal diastolic Ca^{2+} release at 2, 3 and 4 Hz, in myocytes in absence (solid bars) or in presence (hatched bars) of 1 μmol/L of isoproterenol; WT, white bars; $RyR2^{R4496C}$, blue bars.*p<0.05, **p<0.01 with respect to WT. †p<0.05 with respect to $RyR2^{R4496C}$. ‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001 with respect to WT in the presence of isoproterenol. #p<0.05, ###p<0.001 with respect to $RyR2^{R4496C}$ at 2 Hz.