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In Situ Confocal Imaging in Intact Heart Reveals Stress-Induced Ca^{2+} Release Variability in a Murine CPVT Model of $RyR2^{R4496C+/-}$ Mutation

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Abstract

Background—Catecholaminergic polymorphic ventricular tachycardia (CPVT) is directly linked to mutations in proteins (e.g., $RyR2^{R4496C}$) responsible for intracellular Ca^{2+} homeostasis in the heart. However, the mechanism of Ca^{2+} release dysfunction underlying CPVT has only been investigated in isolated cells but not in the *in situ* undisrupted myocardium.

Methods and Results—We investigated *in situ* myocyte Ca^{2+} dynamics in intact Langendorff perfused hearts (*ex vivo*) from wildtype (WT) and $RyR2^{R4496C+/-}$ mice using laser scanning confocal microscopy. We found that myocytes from both WT and $RyR2^{R4496C+/-}$ hearts displayed uniform, synchronized Ca^{2+} transients. Ca^{2+} transients from beat to beat were comparable in amplitude with identical activation and decay kinetics in WT and $RyR2^{R4496C+/-}$ hearts, suggesting that excitation-contraction (EC) coupling between the sarcolemmal Ca^{2+} channels and mutated $RyR2^{R4496C+/-}$ channels remains intact under baseline resting conditions. Upon adrenergic stimulation, $RyR2^{R4496C+/-}$ hearts exhibited a high degree of Ca^{2+} release variability (CRV). The varied pattern of Ca^{2+} release was absent in single isolated myocytes, independent of cell cycle length, synchronized among neighboring myocytes, and correlated with CPVT. A similar pattern of action potential variability, which was synchronized among neighboring myocytes, was also revealed under adrenergic stress in intact hearts but not in isolated myocytes.

Conclusions—Our studies using *in situ* confocal imaging approach suggest that mutated RyR2s are functionally normal at rest but display a high degree of CRV upon intense adrenergic stimulation. CRV is a Ca^{2+} release abnormality resulting from electrical defects rather than the failure of the Ca^{2+} release response to action potentials in mutated ventricular myocytes. Our data provide important insights into Ca^{2+} release and electrical dysfunction in an established model of CPVT.

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Keywords

arrhythmia (mechanisms); calcium; catecholaminergic polymorphic ventricular tachycardia; sarcoplasmic reticulum; ryanodine receptors

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a lethal genetic disease characterized by exercise/stress-induced malignant ventricular arrhythmias and sudden cardiac death in young individuals with structurally normal hearts.[1] The genetic foundation of CPVT is linked to mutations within two important Ca^{2+} handling proteins: autosomal dominant mutations of intracellular Ca^{2+} release channels or type 2 ryanodine receptors (RyR2),[2, 3] and autosomal recessive mutations of calsequestrin (CASQ), a Ca^{2+} binding protein in the sarcoplasmic reticulum (SR).[4] Patients with CPVT mutations are typically healthy at rest but develop arrhythmias under emotional or physical stress. Electrocardiograms of CPVT patients reveal bidirectional ventricular tachycardia and polymorphic ventricular tachycardia.[2]

Both RyR2 and CASQ play important roles in controlling intracellular Ca^{2+} handling and homeostasis.[5, 6] Ca^{2+} release dysfunction is believed to be the underlying mechanism for the above mentioned ventricular arrhythmias in human patients due to the critical importance of the two key proteins in Ca^{2+} regulation of cardiomyocytes.[3, 7, 8] Indeed, many studies have identified Ca^{2+} handling defects using mutant channels in isolated lipid bilayers,[9–11] cultured myocytes, HEK 293 cells stably transfected with these mutants,[12–15] or in ventricular myocytes isolated from mouse models that carry mutant RyR2.[16–19] Notably, a recent study by Fernandez-Velasco, *et al.*, showed that, compared to wild type (WT) myocytes, untreated $RyR2^{R4496C+/-}$ myocytes have an enhancement of Ca^{2+} sensitivity and an increase in spontaneous Ca^{2+} release in diastole during electrical pacing, which is augmented by isoproterenol and increasing the pacing frequency.[20] Their results were further supported by Kang, *et al.*, who demonstrated spontaneous Ca^{2+} release events in both $RyR2^{R4496C+/-}$ ventricular and Purkinje cells.[21] However, these *in vitro* studies are controversial because it is unclear if these mutant proteins behave abnormally under resting conditions (i.e., in the absence of catecholamine stimulation).[22, 23] One unresolved issue, however, is that the resting defect in Ca^{2+} dynamics does not correspond to the clinical manifestations of CPVT patients with the same mutation, whose hearts are structurally normal and free of arrhythmias unless under stress.

A second question is related to the pathophysiology of CPVT RyR2 mutations, in particular how RyR2 mutations cause CPVT. Several previous studies demonstrated that ventricular myocytes harboring the RyR2 R4496C mutation are prone to spontaneous Ca^{2+} release and delayed after depolarizations (DADs).[13, 16, 20, 21] One of the leading hypotheses is that the mutation induces SR calcium leak in ventricular myocytes during diastole, thereby generating DADs that, in turn, trigger fatal cardiac arrhythmias.[24] Those previous results were obtained from isolated myocytes, and it remains unclear whether mutated RyR2s in physiologically coupled myocytes in intact heart behave differently from isolated myocytes during steady state beating. It is therefore important to study the nature of Ca^{2+} handling under physiological conditions in RyR2-mutated hearts.

In the present study, we aimed to identify the *in situ* feature of Ca^{2+} mis-handling in CPVT hearts and to enhance our understanding of Ca^{2+}-dependent arrhythmogenesis using a $RyR2^{R4496C+/-}$ mouse model. We performed *in situ*, *ex vivo* Ca^{2+} / action potential (AP) imaging in Langendorff-perfused intact hearts under near physiological conditions, using

laser scanning confocal microscopy at baseline and following catecholamine stimulation. We also mapped Ca^{2+} dynamics to simultaneously recorded ECGs. Our data demonstrate that myocyte EC coupling between the sarcolemmal Ca^{2+} channels and mutated $RyR2^{R4496C+/-}$ channels remains intact under baseline resting conditions. However, under intense adrenergic stress, we identified a previously unappreciated pattern of Ca^{2+} handling dysfunction in physiologically-coupled ventricular myocytes with the RyR2 R4496C mutation. Interestingly, highly variable SR Ca^{2+} release in $RyR2^{R4496C+/-}$ hearts was synchronized among neighboring myocytes and correlated with CPVT occurrence as measured by ECG. Similarly, with *in situ* AP imaging, we detected stress-induced beat-to-beat variability in AP, which was also synchronized among neighboring mutated myocytes.

Methods

Animal experiments were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee at the University of Iowa. In situ confocal Ca^{2+} imaging in intact hearts with / without ex vivo electrocardiogram, in situ confocal Aaction Potential (AP) imaging in intact hearts were adapted from published reports.[25–27] Ca^{2+} imaging in adult single isolated ventricular myocytes and in primary cultured neonatal myocytes were performed as previously described.[28, 29] Confocal Ca^{2+} / AP images were analyzed offline with custom routines composed with IDL image analysis software (ITT VIS Inc., Boulder, CO).[30] Pseudo ECG data were processed offline with Clampfit 10. Data were expressed as mean ± SE, and median with interquartile range in boxplots. Multiple regression analysis was performed to determine the correlation coefficient and significance. Student's t-tests were applied for pair-wise comparison. Bonferroni procedure following a global test based on linear mixed effects model was performed for multiple group comparisons (NCSS, Kaysville, Utah). A compound symmetry correlation structure was assumed for linear mixed effects model tests. A p value of <0.05 was considered statistically significant. Expanded methods are available in Supplementary Material.

Results

Normal EC coupling in in situ $RyR2^{R4496C+/-}$ myocytes at rest

$RyR2^{R4496C+/-}$ mutant mice are susceptible to CPVT under catecholamine stimulation.[31] We performed *in situ* Ca^{2+} imaging of ventricular myocytes from intact hearts attached to an oxygenated Langendorff-perfusion system.[25] We examined autonomous Ca^{2+} signals, initiated by sinus rhythm, in ventricular myocytes. In linescan mode, each myocyte from both WT and $RyR2^{R4496C+/-}$ hearts displayed uniform, synchronized Ca^{2+} transients. Ca^{2+} transients from beat-to-beat were comparable in amplitude with identical activation and decay kinetics between these groups (Figure 1), suggesting that EC coupling between the sarcolemmal Ca^{2+} channels and mutated $RyR2^{R4496C+/-}$ channels (e.g. calcium-induced calcium release) remains intact under baseline resting conditions. In addition, spontaneous Ca^{2+} sparks or waves were rarely observed at diastolic phase during steady state beating in both WT and $RyR2^{R4496C+/-}$ myocytes, indicating mutated RyR2s are not leaky under resting condition.

High beat-to-beat variability of Ca^{2+} transients in $RyR2^{R4496C+/-}$ myocytes under adrenergic stress

CPVT occurs under physical or emotional stress in patients with the $RyR2^{R4496C+/-}$ mutation and are elicited exclusively by adrenergic stress.[2] $RyR2^{R4496C+/-}$ mice also display similar ECG abnormalities under high catecholamine and caffeine stimulation.[32] To further understand Ca^{2+} performance in $RyR2^{R4496C+/-}$ myocytes during high adrenergic stress, we next compared *in situ* Ca^{2+} dynamics of WT and $RyR2^{R4496C+/-}$ myocytes in intact hearts

under sinus rhythm with perfusion of epinephrine (1 μM) and caffeine (0.6 mM), a condition that induces CPVT in RyR2$^{R4496C+/-}$ mice.[16, 31, 32] In WT myocytes, epinephrine plus caffeine increased the amplitude of Ca^{2+} transients and accelerated both the activation and decay kinetics (Figure 2A, D–F). Epinephrine with caffeine also significantly accelerated the kinetics of Ca^{2+} transients (both activation and relaxation) in RyR2R$^{4496C+/-}$ myocytes (Figure 2D–F). Strikingly, RyR2$^{R4496C+/-}$ myocytes under high sympathetic stress exhibited high beat to beat Ca^{2+} release variability (CRV) in the amplitude of Ca^{2+} transients (Figure 2BC). While some Ca^{2+} transients with a higher amplitude were detected in mutant myocytes after adrenergic stimulation, many of the Ca^{2+} transients were markedly reduced, which resulted in a lower average amplitude as compared to WT myocytes (Figure 2D). It should be noted that these transients with reduced amplitude differed from asynchronous Ca^{2+} waves. Specifically, they were synchronized with fast rising kinetics (T_{peak}), similar to that of WT myocytes (Figure 2E). CRV activity often followed an irregular pattern of amplitude fluctuation of Ca^{2+} transients. In RyR2$^{R4496C+/-}$ myocytes, CRV was reversible and disappeared upon washout of epinephrine and caffeine, suggesting that this CRV defect is induced by adrenergic stress. Variance analysis from thousands of Ca^{2+} transient samples (5–7 hearts in each group) further supports our observation that Ca^{2+} transients are highly varied in amplitude from beat-to-beat under high sympathetic stress conditions, but not at baseline (Figure 3AB). Importantly, this CRV pattern was not observed in single isolated RyR2$^{R4496C+/-}$ myocytes under field stimulation with the same adrenergic stimulation (Supporting Information, Figure S1). Our data suggest that adrenergic stress-induced CRV requires physiological cellular coupling in RyR2$^{R4496C+/-}$ hearts.

High CRV is not due to beating interval variability

We postulated that the high CRV observed in mutated myocytes from intact hearts is due to variable beating intervals preceding each Ca^{2+} transient, which would cause varied SR Ca^{2+} loading under *ex vivo* conditions. To test this hypothesis, we measured the time intervals preceding each Ca^{2+} transient and correlated it with the amplitude of the corresponding transient from WT and RyR2 mutant hearts. As shown in Figure 3C–F, the range of amplitudes of Ca^{2+} transients from WT myocytes (after epinephrine + caffeine) was narrow, while the amplitudes of Ca^{2+} transients from mutated myocytes were dispersed over a much wider range. More importantly, there was no correlation between the beating intervals and Ca^{2+} transient amplitudes in either WT or mutated myocytes under stress. This analysis clearly indicates that the high level of CRV observed in RyR2$^{R4496C+/-}$ myocytes under *ex vivo* conditions and with high sympathetic stimulation is not caused by beating interval variability and is independent of stimulation frequency.

CRV is synchronized among neighboring myocytes with mutant RyR2

An advantage of *in situ* confocal imaging is that it allows examination of the Ca^{2+} dynamics of multiple, physiologically interconnected myocytes simultaneously. The data in Figure 2B&C revealed a coordinated pattern of CRV between two neighboring cells from RyR2$^{R4496C+/-}$ hearts. To further confirm this phenomenon, we evaluated the pattern of CRV among multiple myocytes from RyR2$^{R4496C+/-}$ hearts. Surprisingly, we found that CRV is indeed present in a coordinated pattern among many neighboring myocytes (Figure 4). This novel pattern of Ca^{2+} release dysfunction was consistently observed in RyR2$^{R4496C+/-}$ hearts during adrenergic stress but not in WT hearts. Interestingly, CRV was not observed in RyR2$^{R4496C+/-}$ cultured neonatal myocytes or among neighboring cells under both control and adrenergic stimulation conditions (Supporting Information, Figure S2). Collectively, these data strongly suggest that CRV is an integrated, tissue level response to adrenergic stress, and that the source of CRV is likely not ventricular myocytes themselves, but from an abnormality in the upstream electrical signal.

CRV is associated with CPVT

In order to test if increased CRV was related to CPVT, we performed simultaneous recordings of *in situ* confocal linescan Ca^{2+} images and real time *ex vivo* ECG measurements in Langendorff perfused intact hearts. WT hearts exhibited normal ECGs and regular, stable Ca^{2+} transients from beat-to-beat among different neighboring myocytes (Figure 5A) in both the absence and presence of epinephrine and caffeine. $RyR2^{R4496C+/-}$ hearts also displayed normal ECG and Ca^{2+} dynamics at baseline. However, under high sympathetic stimulation, CRV occurred in $RyR2^{R4496C+/-}$ hearts simultaneously with CPVT (Figure 5B). These data suggest that CRV, a tissue level measure of SR Ca^{2+} release abnormality in $RyR2^{R4496C+/-}$ hearts, is associated with electrical abnormalities, including CPVT.

Physiologically coupled $RyR2^{R4496C+/-}$ myocytes have abnormal APs

To investigate the underlying mechanism of CRV, we then measured the *in situ* APs under the same recording conditions as for *in situ* Ca^{2+} imaging,[27] except that a fast voltage-sensitive dye, ANNINE-6plus, was loaded into the intact hearts through Langendorff perfusion to track the dynamic changes of transmembrane potential. We applied this imaging technique to examine the *in situ* electrical properties of $RyR2^{R4496C+/-}$ myocytes in intact hearts under adrenergic stress. Surprisingly, we found that $RyR2^{R4496C+/-}$ myocytes also exhibited high heterogeneity in AP morphology under adrenergic stress in comparison to WT myocytes (Figure 6AB). On average, $RyR2^{R4496C+/-}$ myocytes had similar ratio of fluorescence change (indicative of AP amplitude) and shorter AP duration than those of WT myocytes (Figure 6CD). Variance analysis showed that mutant myocytes had a greater variability in both AP amplitude and duration (Figure 6EF). More importantly, AP variability was also synchronized among neighboring myocytes from the $RyR2^{R4496C+/-}$ hearts (Figure 6B & Supporting Information, Figure S3). By contrast, AP studies in single isolated myocytes using conventional current clamp recordings showed no difference in variance in these parameters between WT and $RyR2^{R4496C+/-}$ hearts under adrenergic stress (Supporting Information, Figure S4), suggesting that *in situ* electrical abnormalities in $RyR2^{R4496C+/-}$ hearts are non-ventricular in origin. Taken together, our data support the notion that electrical abnormalities of non-ventricular origin underlie the mechanism of CRV in physiologically coupled ventricular myocytes.

Discussion

Ca^{2+} handling abnormalities play an important role in the pathophysiology of heart disease, including heart failure, arrhythmias, and sudden cardiac death.[33] Patients with a specific mutation (R4496C) in the cardiac Ca^{2+} release channel, RyR2, suffer from exercise/stress induced CPVT and sudden cardiac death. The genetically modified mouse model bearing the same mutation provides an excellent model for studying the mechanisms of Ca^{2+}-dependent arrhythmogenesis and the pathophysiology of CPVT.[32] In this study, we applied the *in situ* confocal Ca^{2+} imaging techniques to study Ca^{2+} handling in intact hearts.[25] This approach allows investigation under near physiological conditions. To our surprise, we obtained distinctly different results as compared to results in ventricular myocytes isolated from these same mice.[20, 21, 34] We found that 1) mutated RyR2s are functionally normal during non-stressed, resting condition; 3) we identified a new pattern of Ca^{2+} release dysfunction, CRV, in $RyR2^{R4496C+/-}$ but not WT myocytes under adrenergic stress; 4) CRV is an integrated, tissue level response of mutated myocytes to adrenergic stress and is observed in intact hearts but not in single isolated myocytes; 5) CRV is synchronized among neighboring myocytes, independent of beating interval or stimulation frequency; 6) CRV is tightly associated with CPVT; and 7) AP variability in intact hearts (but not in single isolated myocytes) is the likely cause of CRV.

Normal CICR function in ventricular myocytes with RyR2$^{R4496C+/-}$ mutation at rest

Whether mutant RyR2 channels are functionally normal at rest is a controversial yet fundamentally important question. Particularly for patients with this mutation, it is critical to determine if the dysfunctional Ca^{2+} handling is constantly present and is exacerbated with stress, or if the mutated channels are normal at resting state and become dysfunctional only with adrenergic stimulation. The answer to this issue may provide important insights in developing therapeutic strategies for CPVT patients. Marks and colleagues demonstrated using single channel recording in lipid-bilayers that RyRs harboring human mutations (including R4496C) show similar gating features as WT RyRs at baseline conditions but are much more leaky upon high protein kinase A stimulation (10-fold increase in channel open probability).[9, 10] George and Lai also showed unaltered baseline Ca^{2+} activity in HL-1 myocytes transfected with mutated RyR2s.[35] However, these results were challenged by other groups who reported an increase in abnormal Ca^{2+} release and higher sensitivity of mutant RyR2s compared to WT at resting conditions. Jiang *et al.* reported that CPVT mutations enhance the sensitivity of the RyR2 channel to luminal Ca^{2+} activation and lower the threshold for spontaneous Ca^{2+} release in the setting of Ca^{2+} overload.[11, 13] Recently, Fernandez-Velasco *et al.* showed an increased incidence of Ca^{2+} sparks and Ca^{2+} waves at baseline in RyR2$^{R4496C+/-}$ myocytes, which is further enhanced by either isoproterenol or high pacing rates.[20] Liu *et al.* reported similar results of increased spark frequency at baseline and after isoproterenol challenge in isolated RyR2$^{R4496C+/-}$ myocytes.[19] However, when we examined Ca^{2+} signals from intact WT and mutant hearts using the *in situ* imaging method, we found RyR2 mutated myocytes exhibit no abnormal Ca^{2+} release, e.g., Ca^{2+} waves, at resting conditions (Figure 1). These discrepancies may be explained by differences in experimental conditions that resulted in exposure of RyR2 to different intra-luminal Ca^{2+} levels. Enzymatic and mechanical dissociation of heart tissue exerts a significant stress to the myocytes, causing myocyte Ca^{2+} overload and spontaneous Ca^{2+} release.[34, 36, 37] Instead, under conditions which did not exist or cause SR Ca^{2+} overloading such as in lipid bilayers, [9, 10] cultured HL-1 myocytes [35] and intact hearts (of the present study), findings were surprisingly consistent, that is, RyR2s carrying R4496C mutation are not leaky at baseline. It has been shown that CPVT-linked RyR2 mutations, including R4496C, increase the sensitivity of the RyR2 channel to SR Ca^{2+} overload.[13] In the absence of SR Ca^{2+} overload (i.e., *in situ* under resting conditions or studies with lipid bilayers [9, 10] or cultured HL-1 myocytes [35]), the R4496C mutation-linked Ca^{2+} release defect would not be apparent. Importantly, our *in situ* findings are also consistent with the clinical characteristics of CPVT patients whose hearts are structurally normal throughout their lives but only develop fatal arrhythmias under emotional or physical stress. If mutated RyR2 channels expressed in myocytes are continuously leaky at resting conditions, this persistent abnormality of intracellular Ca^{2+} signaling is predicted to lead to alterations in myocyte gene transcription that could eventually promote cardiac pathophysiology (e.g., cardiomyopathy).

Ca^{2+} release variability and electrical abnormalities in physiologically coupled myocytes with RyR2$^{R4496C+/-}$ mutation

In this study, we identified a new pattern of Ca^{2+} release dysfunction - a high degree of variation in Ca^{2+} transient amplitude in RyR2$^{R4496C+/-}$ myocytes. This pattern of Ca^{2+} release abnormality was present in mutated myocytes under adrenergic stimulation; it was not observed in WT cells, in RyR2 mutated myocytes under baseline conditions, or in mutated neonatal myocytes in culture. This result was only detectable by *in situ* imaging methods. Ca^{2+} release variability is distinct in nature from rapid pacing-induced Ca^{2+} alternans,[25] in that 1) CRV is independent of the cycle length; 2) the change in Ca^{2+} transient amplitude is predominantly very irregular; 3) CRV is not inducible in isolated myocytes (either WT or mutated myocytes); and 4) rapid high-rate pacing-induced Ca^{2+} alternans may not be coordinated or synchronized among different myocytes from beat to

beat.[25] These features suggest that proarrhythmic defects in RyR2$^{R4496C+/-}$ hearts are not limited to ventricular myocytes. Recently, two elegant studies using different approaches (optical mapping and confocal imaging) both concluded that the RyR2^{R4496C} mutation associated CPVT originated from abnormalities in Purkinje cells. Cerrone *et al.* studied the mechanisms and origin of CPVT arrhythmias in Langendorff-perfused RyR2$^{R4496C+/-}$ mouse hearts using optical mapping.[38] Their data provide compelling evidence that CPVT arrhythmias originate from the specialized conduction system. Very recently, Fishman and colleagues examined the frequency and severity of spontaneous Ca^{2+} activities in Purkinje cells in comparison to ventricular myocytes with the aid of a novel fluorescent reporter in the cardiac conduction system (including the distal Purkinje fiber network). These studies revealed that Ca^{2+} handling defects in Purkinje cells are more pronounced and frequent than those of ventricular myocytes with the same mutation.[21] Our data are consistent with these studies by demonstrating that CRV occurs in intact hearts but not in single isolated myocytes with RyR2 mutation, and that CRV is synchronized among different neighboring myocytes.

Our data suggest that CRV may originate from an abnormality in electrical activity in other regions, such as Purkinje fibers or ectopic ventricular foci. Providing additional support for this conclusion, our *in situ* AP optical recordings and single cell AP studies indicate that this defect is in electrical conduction system rather than ventricular in origin. Therefore, it is postulated that under stress conditions, defective RyR2-mediated abnormal Ca^{2+} release in Purkinje cells leads to aberrant electrical activities, thereby triggering variability in Ca^{2+} release in ventricular myocytes. We speculate that the mechanism is as follows: Under stress condition, spontaneous Ca^{2+} releases in Purkinje fibers with RyR2$^{R4496C+/-}$ mutation cause DADs, which conduct and produce abnormal APs in ventricular myocytes, as we have observed using *in situ* AP imaging technique. These abnormal APs, specifically, high variability of AP amplitude and duration but synchronized among neighboring ventricular myocytes, may lead to a varied magnitude of Ca^{2+} influx, causing coordinated Ca^{2+} release variability among mutated ventricular myocytes. Also contributing to the mechanism, it is very possible that upon stress the Purkinje fibers initiate the arrhythmia, which is further sustained by ventricular myocytes carrying defective mutant of RyR2s.

Limitations

In human patients with this or other similar CPVT-linked mutations, intense emotional or physical stress may trigger the appearance of symptoms, such as ventricular arrhythmias, syncope, and sudden cardiac death. However, in RyR2$^{R4496C+/-}$ mice, the combination of caffeine and epinephrine, instead of epinephrine alone, is necessary to induce CPVT. This may suggest that humans with the same mutation are more susceptible to Ca^{2+} disorder-induced CPVT than mice. This difference does not appear to be related to basal catecholamine levels since β-adrenergic blockade failed to completely prevent sudden cardiac death in patients with CPVT mutations.[39] Future studies towards understanding the mechanisms underlying this difference are warranted.

Summary

Our study using the *in situ* confocal imaging approach provides compelling evidence that the RyR2$^{R4496C+/-}$ mutants are functionally normal *in situ* under resting conditions, but display high degree of CRV upon intense adrenergic stimulation. CRV is an integrated, tissue level response of mutated myocytes to adrenergic stress and is closely correlated with CPVT. Our data reveal that CRV results from electrical defects rather than the failure of Ca^{2+} release response to action potentials in mutated ventricular myocytes. This study provides important insights into Ca^{2+} release and electrical dysfunction in an established model of CPVT and has important implications in understanding the mechanism of CPVT in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Ca^{2+} handling abnormalities play important roles in the pathophysiology of heart failure, arrhythmias, and sudden cardiac death. Patients with a specific mutation (R4496C) in the cardiac Ca^{2+} release channel, RyR2, suffer from exercise/stress-induced catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden cardiac death, but are typically healthy at rest. The reason that the resting defect in Ca^{2+} dynamics *in vitro* in isolated myocytes does not correspond to the clinical manifestations of CPVT patients with the same mutation is unexplained. In a genetically modified mouse model of CPVT ($RyR2^{R4496C+/-}$), we applied *in situ* confocal Ca^{2+} imaging techniques to study Ca^{2+} handling in undisrupted myocardium of intact hearts. This approach allows investigation under near physiological conditions. The $RyR2^{R4496C+/-}$ mutants were found to be functionally normal *in situ* under resting conditions, but had a high degree of Ca^{2+} release variability upon intense adrenergic stimulation. This new pattern of Ca^{2+} handling abnormality is an integrated, tissue level response of mutated myocytes to adrenergic stress, closely correlated with CPVT. Our data reveal that Ca^{2+} release variability results from electrical defects, likely originating from Purkinje fibers, rather than the failure of Ca^{2+} release response to action potentials in mutated ventricular myocytes. This study provides insights into Ca^{2+} release and electrical dysfunction in CPVT.

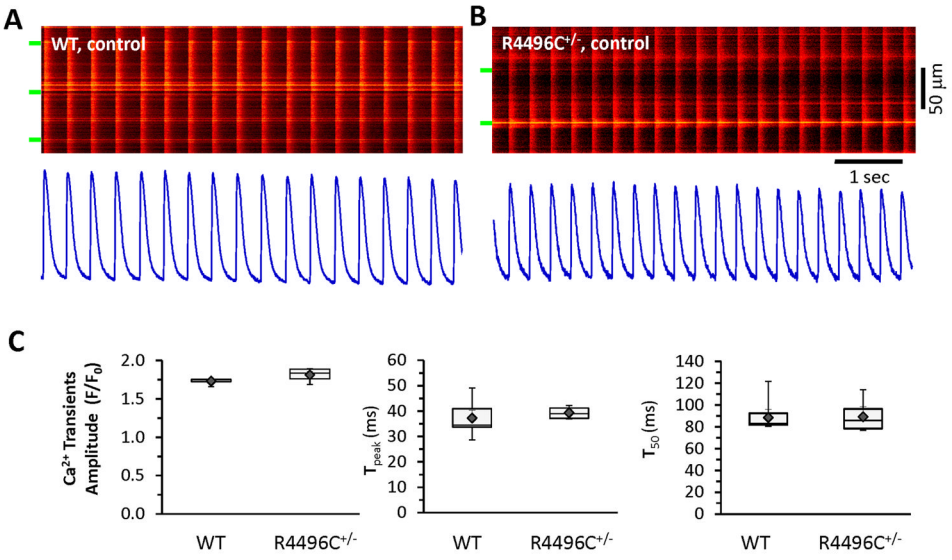

Figure 1.

In situ confocal Ca^{2+} imaging in WT and $RyR2^{R4496C+/-}$ hearts: normal Ca^{2+} transients under resting conditions. **A**–**B**, Autonomous, synchronized Ca^{2+} transients driven by sinus rhythm in (A) WT and (B) $RyR2^{R4496C+/-}$ hearts. Spontaneous Ca^{2+} waves or Ca^{2+} sparks were rarely detected during steady state beating under resting conditions. Bottom panels are spatial averages of Ca^{2+} signals from corresponding images. **C**, Boxplots of Ca^{2+} transients (amplitude, time to peak, and T_{50} relaxation), ◆ and error bars inside the box denotes mean ±SE. N=5–7 hearts, n=54 or 82 frames of transients (most of frames include multiple myocytes) for WT and $RyR2^{R4496C+/-}$, respectively. Green bars indicate edges of myocytes in this and subsequent figures.

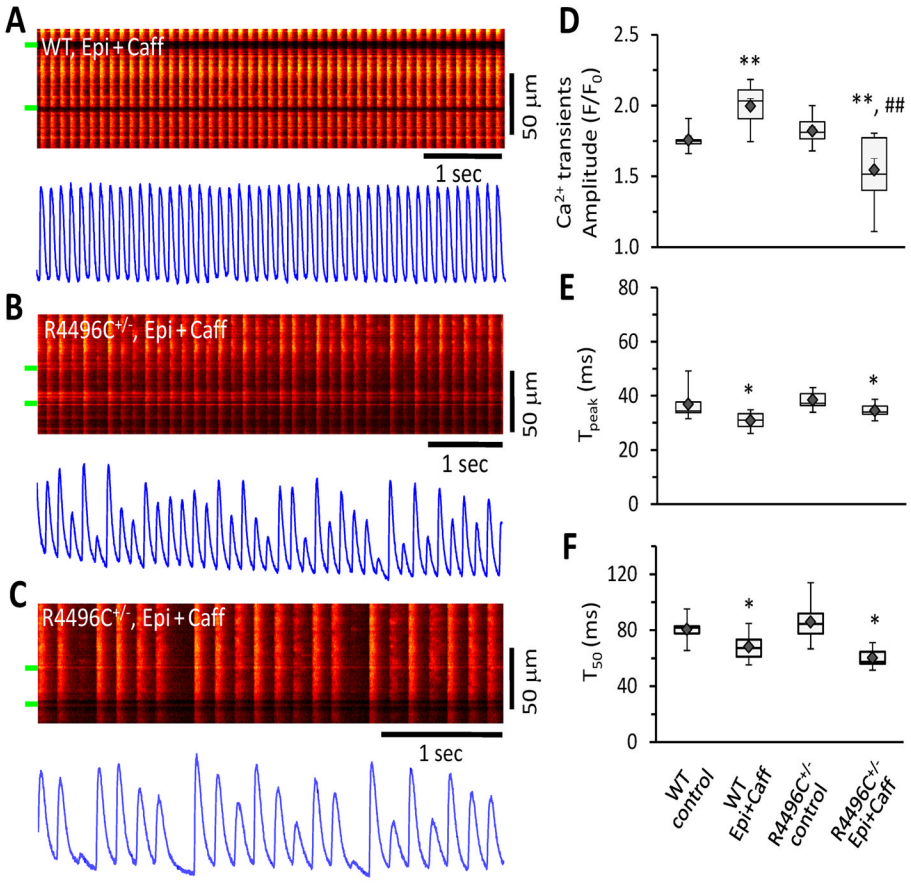

Figure 2.

High degree of Ca^{2+} release variability in $RyR2^{R4496C+/-}$ myocytes from intact heart as demonstrated by *in situ* confocal imaging after adrenergic stimulation. **A**, Autonomous Ca^{2+} transients in myocytes from WT hearts under epinephrine (1 μM) and caffeine (0.6 mM) perfusion. Beat-to-beat Ca^{2+} transients were stable in WT myocytes even under adrenergic stress challenge. **B**–**C**, Ca^{2+} transients from $RyR2^{R4496C+/-}$ myocytes of intact hearts, exhibiting high degree of variability in Ca^{2+} transient amplitude. These Ca^{2+} transients with varied amplitude were action potential-triggered events with a quick rising and decay phase, which is distinct from asynchronous propagated Ca^{2+} wave events. **D**–**F**, Boxplots of Ca^{2+} transients (amplitude, time to peak - T_{peak}, and decay rate T_{50}). ◆ and error bars inside the box denotes mean±SE. The averaged amplitude of Ca^{2+} transients of $RyR2^{R4496C+/-}$ myocytes under epinephrine plus caffeine perfusion was less than that of control. N=5–7 hearts for each group, n=107 or 74 frames of transients for WT and $RyR2^{R4496C+/-}$ myocytes, respectively. * $p<0.05$ and ** $p<0.01$ *vs* WT or $RyR2^{R4496C+/-}$ without epinephrine and caffeine (control), $^{\#\#}$ $p<0.01$ *vs* WT under the same condition (epinephrine and caffeine). Global test: p=0.00001, 0.005, 0.02 among the 4 groups, for D, E, F, respectively.

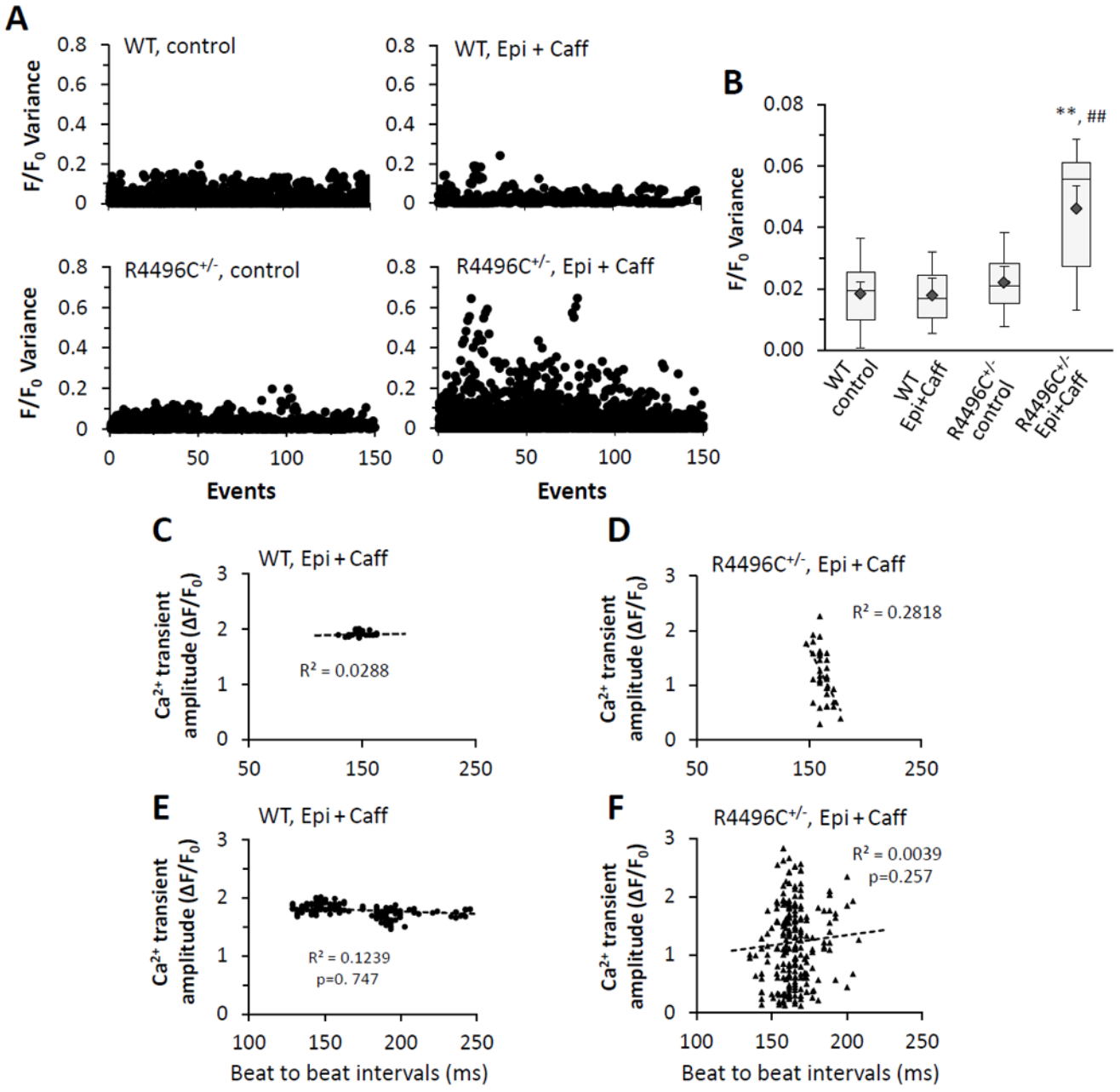

Figure 3.

Ca^{2+} release variability in $RyR2^{R4496C+/-}$ myocytes from intact hearts is independent of the beating interval preceding each transient. **A**–**B**, Variance analysis of CRV. **A**, Variance was calculated as the square of variation (sample value minus mean value) of each transient amplitude. Scatter plots of F/F_0 variance from WT and $RyR2^{R4496C+/-}$ myocytes under resting (control) and stress (epinephrine plus caffeine) conditions, respectively, are shown. **B**, Boxplot of F/F_0 variance from each group. $RyR2^{R4496C+/-}$ myocytes exhibited significant higher variance comparing to other groups, ◆ and error bars inside the box denotes mean ±SE. n=5–7 hearts, n=1947, 1502, 2405, or 2449, respectively, for each group. **p<0.01 *vs* $RyR2^{R4496C+/-}$ under control condition, ## p<0.01 vs WT under the same condition (epinephrine and caffeine). p=0.008 among the 4 groups (global test). **C**–**F,** No Correlation

between Ca^{2+} transient amplitude (F/F_0) and the beating interval preceding each transient in WT (C) and mutant (D) hearts. **C**–**D**, Examples displaying Ca^{2+} transient amplitude – time interval correlation from a representative Ca^{2+} image of WT (**C**) and $RyR2^{R4496C+/-}$ (**D**) heart, respectively, under epinephrine and caffeine stimulation. **E**–**F**, Multiple regression analysis on data from 3–4 hearts of WT and $RyR2^{R4496C+/-}$(n=163, 227 events, respectively), indicating no correlation between Ca^{2+} transient amplitude and time interval preceding each transient in both WTs and mutant hearts.

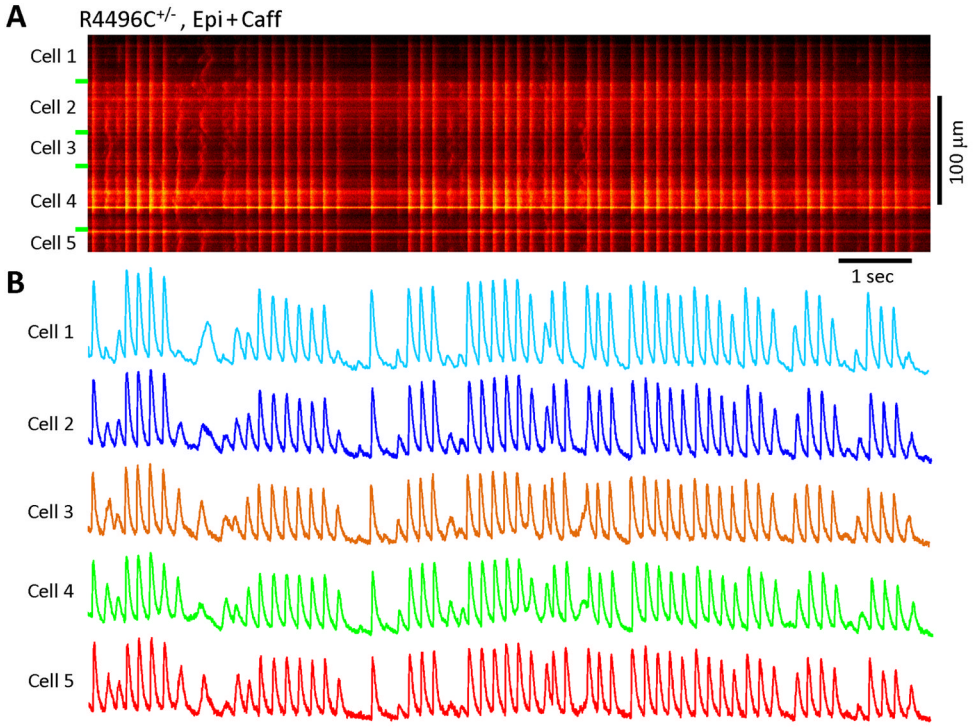

Figure 4.

Ca^{2+} release variability in $RyR2^{R4496C+/-}$ myocytes under sympathetic stress: synchrony among neighboring myocytes. **A**, A typical Ca^{2+} image of 5 myocytes from a $RyR2^{R4496C+/-}$ heart. **B**, Spatial profile of Ca^{2+} signals from each myocyte showing that stress-induced Ca^{2+} release variability was coordinated among many neighboring mutated myocytes.

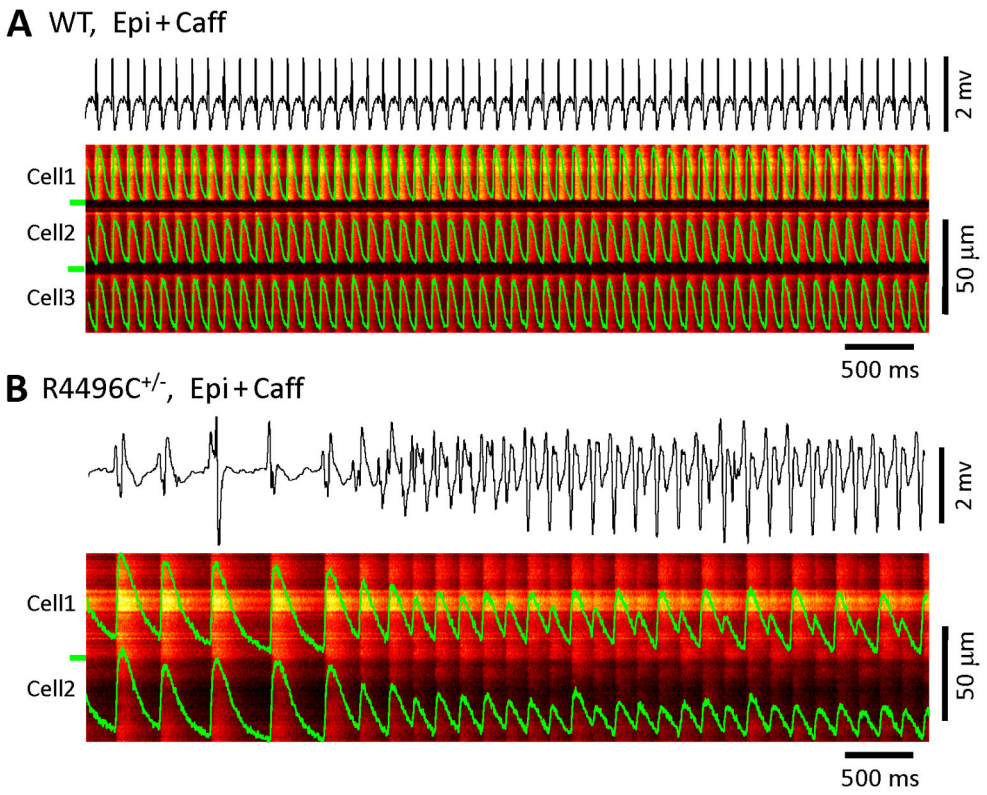

Figure 5.

Ca^{2+} release variability and CPVT in $RyR2^{R4496C+/-}$ hearts. Confocal Ca^{2+} imaging was performed with simultaneous recordings of *ex vivo* ECG in hearts with continuous Langendorff perfusion. **A**, A WT heart exhibited normal *ex vivo* ECG and stable Ca^{2+} transients from beat-to-beat under stress (Note: p wave is visible but has very small magnitude). **B**, $RyR2^{R4496C+/-}$ hearts concurrently displayed CRV and CPVT.

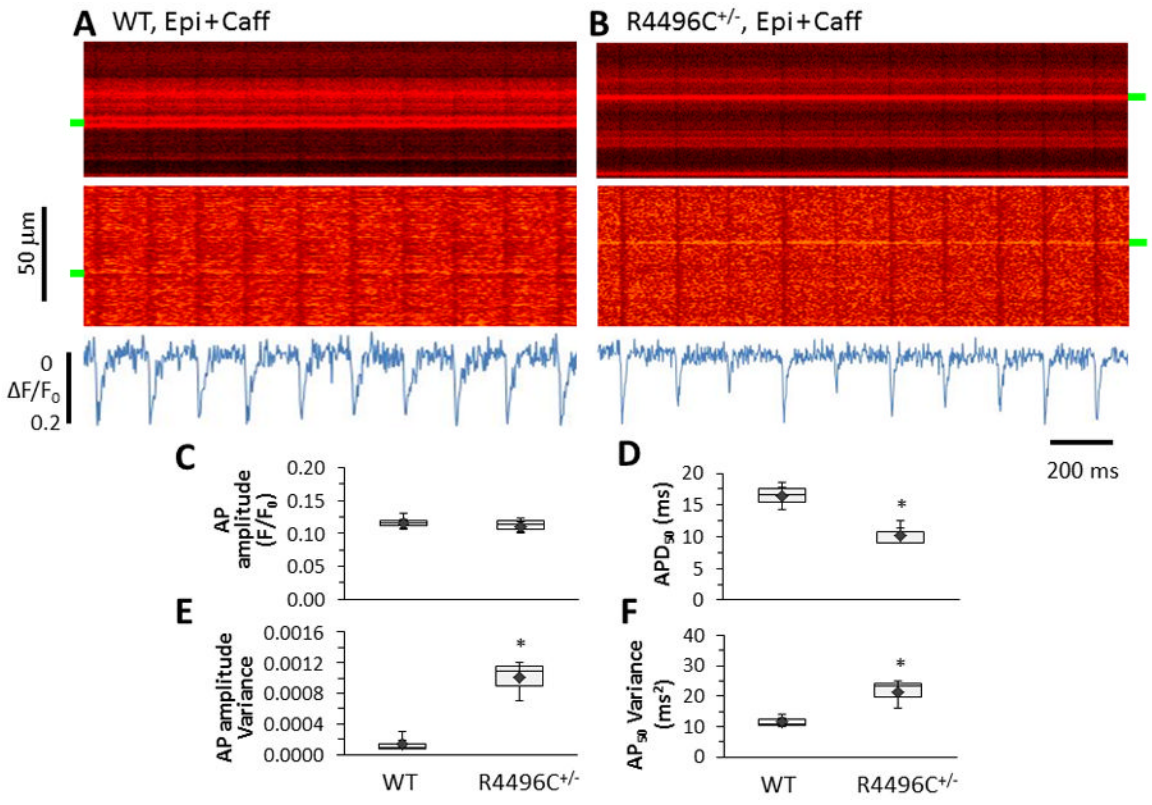

Figure 6.

In situ imaging of myocyte APs in intact hearts. **A**–**B**, Representative examples of ANNINE-6plus fluorescence from WT and $RyR2^{R4496C+/-}$ hearts perfused with epinephrine plus caffeine. Top to bottom: raw images, images after normalization, and spatial average of ANNINE-6plus fluorescence. $RyR2^{R4496C+/-}$ myocytes displayed beat-to-beat variation in AP morphology. **C**–**D**, Summary of AP amplitude ($\Delta F/F_0$) and APD_{50} in boxplots. **E**–**F**, Boxplots of variance of AP amplitude and APD_{50}. * $p<0.05$, n=4 and 5 for WT and $RyR2^{R4496C+/-}$ hearts, and 326 or 213 AP events for WT or $RyR2^{R4496C+/-}$ hearts, respectively.