In Situ Confocal Imaging in Intact Heart Reveals Stress-Induced Ca\textsuperscript{2+} Release Variability in a Murine Catecholaminergic Polymorphic Ventricular Tachycardia Model of Type 2 Ryanodine Receptor\textsuperscript{R4496C/+} Mutation

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Background—Catecholaminergic polymorphic ventricular tachycardia is directly linked to mutations in proteins (eg, type 2 ryanodine receptor [RyR2]\textsuperscript{R4496C} responsible for intracellular Ca\textsuperscript{2+} homeostasis in the heart. However, the mechanism of Ca\textsuperscript{2+} release dysfunction underlying catecholaminergic polymorphic ventricular tachycardia has only been investigated in isolated cells but not in the in situ undisrupted myocardium.

Methods and Results—We investigated in situ myocyte Ca\textsuperscript{2+} dynamics in intact Langendorff-perfused hearts (ex vivo) from wild-type and RyR2\textsuperscript{R4496C/+} mice using laser scanning confocal microscopy. We found that myocytes from both wild-type and RyR2\textsuperscript{R4496C/+} hearts displayed uniform, synchronized Ca\textsuperscript{2+} transients. Ca\textsuperscript{2+} transients from beat to beat were comparable in amplitude with identical activation and decay kinetics in wild-type and RyR2\textsuperscript{R4496C/+} hearts, suggesting that excitation-contraction coupling between the sarcolemmal Ca\textsuperscript{2+} channels and mutated RyR2\textsuperscript{R4496C/+} channels remains intact under baseline resting conditions. On adrenergic stimulation, RyR2\textsuperscript{R4496C/+} hearts exhibited a high degree of Ca\textsuperscript{2+} release variability. The varied pattern of Ca\textsuperscript{2+} release was absent in single isolated myocytes, independent of cell cycle length, synchronized among neighboring myocytes, and correlated with catecholaminergic polymorphic ventricular tachycardia. 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 an in situ confocal imaging approach suggest that mutated RyR2s are functionally normal at rest but display a high degree of Ca\textsuperscript{2+} release variability on intense adrenergic stimulation. Ca\textsuperscript{2+} release variability is a Ca\textsuperscript{2+} release abnormality, resulting from electric defects rather than the failure of the Ca\textsuperscript{2+} release response to action potentials in mutated ventricular myocytes. Our data provide important insights into Ca\textsuperscript{2+} release and electric dysfunction in an established model of catecholaminergic polymorphic ventricular tachycardia. (Circ Arrhythm Electrophysiol. 2012;5:841-849.)

Key Words: arrhythmia (mechanisms) ■ calcium ■ catecholaminergic polymorphic ventricular tachycardia ■ sarcoplasmic reticulum ■ ryanodine receptors

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. The genetic foundation of CPVT is linked to mutations within 2 important Ca\textsuperscript{2+} handling proteins: autosomal dominant mutations of intracellular Ca\textsuperscript{2+} release channels or type 2 ryanodine receptors (RyR2s)\textsuperscript{2,3} and autosomal recessive mutations of calsequestrin, a Ca\textsuperscript{2+}-binding protein in the sarcoplasmic reticulum (SR).\textsuperscript{4} Patients with CPVT mutations are typically healthy at rest but develop arrhythmias under emotional or physical stress. ECGs of CPVT patients reveal bidirectional ventricular tachycardia and polymorphic ventricular tachycardia.\textsuperscript{2}

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Both RyR2 and calsequestrin play important roles in controlling intracellular Ca\textsuperscript{2+} handling and homeostasis.\textsuperscript{5,6} Ca\textsuperscript{2+} release dysfunction is believed to be the underlying mechanism for the above-mentioned ventricular arrhythmias in human patients because of the critical importance of the 2 key proteins in Ca\textsuperscript{2+} regulation of cardiomyocytes.\textsuperscript{3,7,8}
Indeed, many studies have identified Ca\(^{2+}\) handling defects using mutant channels in isolated lipid bilayers,\(^8\)–\(^11\) cultured myocytes, human embryonic kidney 293 cells stably transfection with these mutants,\(^12\)–\(^15\) or in ventricular myocytes isolated from mouse models that carry mutant RyR2.\(^16\)–\(^19\) Notably, a recent study by Fernández-Velasco et al.\(^20\) showed that, compared with wild-type (WT) myocytes, untreated RyR2\(^{-4496C+/−}\) myocytes have enhancement of Ca\(^{2+}\) sensitivity and an increase in spontaneous Ca\(^{2+}\) release in diastole during electric pacing, which is augmented by isoproterenol and increasing the pacing frequency. Their results were further supported by Kang et al.\(^21\) who demonstrated spontaneous Ca\(^{2+}\) release events in both RyR2\(^{-4496C+/−}\) ventricular and Purkinje cells. However, these in vitro studies are controversial because it is unclear whether these mutant proteins behave abnormally under resting conditions (ie, 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 delay afterdepolarizations.\(^13\),\(^18\)–\(^20\),\(^21\) One of the leading hypotheses is that the mutation induces SR calcium leaks in ventricular myocytes during diastole, thereby generating delayed afterdepolarizations, which, 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\(^{2+}\) mishandling in CPVT hearts and to enhance our understanding of Ca\(^{2+}\)-dependent arrhythmogenesis using an RyR2\(^{-4496C+/−}\) 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 after catecholamine stimulation. We also mapped Ca\(^{2+}\) dynamics to simultaneously recorded ECGs. Our data demonstrate that myocyte excitation-contraction coupling between the sarcolemmal Ca\(^{2+}\) channels and mutated RyR2\(^{-4496C+/−}\) 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\(^{-4496C+/−}\) mutation. Interestingly, highly variable SR Ca\(^{2+}\) release in RyR2\(^{-4496C+/−}\) 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 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 ECG and in situ confocal AP imaging in intact hearts was adapted from published reports.\(^28\)–\(^30\) Ca\(^{2+}\) imaging in adult single isolated ventricular myocytes and in primary cultured neonatal myocytes was performed as previously described.\(^28\)–\(^29\) Confocal Ca\(^{2+}\)/AP images were analyzed offline with custom routines composed of Interactive Data Language image analysis software (ITK 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 box plots. Multiple regression analysis was performed to determine the correlation coefficient and significance. Student t tests were applied for pairwise comparison. Bonferroni procedure after a global test based on a linear mixed-effects model was performed for multiple group comparisons (NCSS, Kaysville, UT). A compound symmetry correlation structure was assumed for linear mixed-effects model tests. \(P<0.05\) was considered statistically significant. Expanded methods are available in the online-only Data Supplement.

**Results**

**Normal Excitation-Contraction Coupling in In Situ RyR2\(^{-4496C+/−}\) Myocytes at Rest**

RyR2\(^{-4496C+/−}\) 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.\(^25\)–\(^27\) We examined autonomous Ca\(^{2+}\) signals, initiated by sinus rhythm, in ventricular myocytes. In line scan mode, each myocyte from both WT and RyR2\(^{-4496C+/−}\) 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 excitation-contraction coupling between the sarcolemmal Ca\(^{2+}\) channels and mutated RyR2\(^{-4496C+/−}\) channels (eg, 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\(^{-4496C+/−}\) myocytes, indicating that mutated RyR2s are not leaky under resting condition.

**High Beat-to-Beat Variability of Ca\(^{2+}\) Transients in RyR2\(^{-4496C+/−}\) Myocytes Under Adrenergic Stress**

CPVT occurs under physical or emotional stress in patients with the RyR2\(^{-4496C+/−}\) mutation and are elicited exclusively by adrenergic stress.\(^2\) RyR2\(^{-4496C+/−}\) mice also display similar ECG abnormalities under high catecholamine and caffeine stimulation.\(^32\) To further understand Ca\(^{2+}\) performance in RyR2\(^{-4496C+/−}\) myocytes during high adrenergic stress, we next compared in situ Ca\(^{2+}\) dynamics of WT and RyR2\(^{-4496C+/−}\) myocytes in intact hearts under sinus rhythm with perfusion of epinephrine (1 \(\mu\)mol/L) and caffeine (0.6 mmol/L), a condition that induces CPVT in RyR2\(^{-4496C+/−}\) mice.\(^31\)\(^32\) In WT myocytes, epinephrine plus caffeine increased the amplitude of Ca\(^{2+}\) transients and accelerated both the activation and decay kinetics (Figure 2A, 2D–2F). Epinephrine with caffeine also significantly accelerated the kinetics of Ca\(^{2+}\) transients (both activation and relaxation) in RyR2\(^{-4496C+/−}\) myocytes (Figure 2D–2F). Strikingly, RyR2\(^{-4496C+/−}\) myocytes under high sympathetic stress exhibited high beat-to-beat Ca\(^{2+}\) release variability (CRV) in the amplitude of Ca\(^{2+}\) transients (Figure 2B and 2C).
Although 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 compared with 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$_{\text{peak}}$), similar to that of WT myocytes (Figure 2E). CRV activity often followed an irregular pattern of amplitude fluctuation of Ca$^{2+}$ transients. In RyR2R4496C+/− myocytes, CRV was reversible and disappeared...
on 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 3A and 3B). Importantly, this CRV pattern was not observed in single isolated RyR2R4496C+/− myocytes under field stimulation with the same adrenergic stimulation (online-only Data Supplement Figure SI). Our data suggest that adrenergic stress-induced CRV requires physiological cellular coupling in RyR2R4496C+/− hearts.

**High CRV Is Not Because of Beating Interval Variability**

We postulated that the high CRV observed in mutated myocytes from intact hearts is because of 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 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 to 3F, the range of amplitudes of Ca$^{2+}$ transients from WT myocytes (after epinephrine+caffeine) was narrow, whereas 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 RyR2R4496C+/− myocytes under ex vivo conditions and with high sympathetic stimulation is not caused by beating interval variability and is independent of stimulation frequency.

**Figure 3.** Ca$^{2+}$ release variability (CRV) in type 2 ryanodine receptor (RyR2)$^{R4496C+/−}$ myocytes from intact hearts is independent of the beating interval preceding each transient. A and 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/F₀ variance from wild-type (WT) and RyR2$^{R4496C+/−}$ myocytes under resting (control) and stress (epinephrine plus caffeine) conditions, respectively, are shown. B, Box plot of F/F₀ variance from each group. RyR2$^{R4496C+/−}$ myocytes with Epi + caff exhibited significant higher variance compared with other groups. Error bars inside the box denote means±SE. n=5 to 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 to F, No correlation between Ca$^{2+}$ transient amplitude (F/F₀) and the beating interval preceding each transient in WT (C) and mutant (D) hearts. C and 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 and F, Multiple regression analysis on data from 3 to 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 WT and mutant hearts. Epi indicates epinephrine; Caff, caffeine.
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 and 2C revealed a coordinated pattern of CRV between 2 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 (online-only Data Supplement Figure SII). Collectively, these data strongly suggest that CRV is an integrated, tissue-level response to adrenergic stress and that the source of CRV is not likely ventricular myocytes themselves but an abnormality in the upstream electric signal.

CRV Is Associated With CPVT

To test whether increased CRV was related to CPVT, we performed simultaneous recordings of in situ confocal line scan 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 electric 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, except that a fast voltage-sensitive dye, ANNINE-6plus, was loaded into the intact hearts through Langendorff perfusion to track dynamic changes of transmembrane potential. We applied this imaging technique to examine in situ electric 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 with WT myocytes (Figure 6A and 6B). On average, RyR2\(^{R4496C+/−}\) myocytes had a similar ratio of fluorescence change (indicative of AP amplitude) and shorter AP duration than those of WT myocytes (Figure 6C and 6D). Variance analysis showed that mutant myocytes had a greater variability in both AP amplitude and duration (Figure 6E and 6F). More importantly, AP variability was also synchronized among neighboring myocytes from the RyR2\(^{R4496C+/−}\) hearts (Figure 6B; online-only Data Supplement Figure SIII). In 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 (online-only Data Supplement Figure SIV), suggesting that in situ electric abnormalities in RyR2\(^{R4496C+/−}\) hearts are nonventricular in origin. Taken together, our data support the notion that electric abnormalities of nonventricular 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. 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<sup>2+</sup>-dependent arrhythmogenesis and the pathophysiology of CPVT. In the present study, we applied in situ confocal Ca<sup>2+</sup> imaging techniques to study Ca<sup>2+</sup> handling in intact hearts. This approach allows investigation under near-physiological conditions. To our surprise, we obtained distinctly different results compared with results by guest on December 11, 2017 http://circep.ahajournals.org/ Downloaded from
in ventricular myocytes isolated from these same mice.\textsuperscript{20,21,34} We found that (1) mutated RyR2s are functionally normal during a nonstressed, resting condition; (2) we identified a new pattern of Ca\textsuperscript{2+} release dysfunction, CRV, in RyR2\textsuperscript{R4496C+/−}, but not WT myocytes under adrenergic stress; (3) 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; (4) CRV is synchronized among neighboring myocytes, independent of beating interval or stimulation frequency; (5) CRV is tightly associated with CPVT; and (6) AP variability in intact hearts (but not in single isolated myocytes) is the likely cause of CRV.

### Normal Ca\textsuperscript{2+}-Induced Ca\textsuperscript{2+} Release Function in Ventricular Myocytes With RyR2\textsuperscript{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 whether the dysfunctional Ca\textsuperscript{2+} handling is constantly present and is exacerbated with stress or whether the mutated channels are normal at resting state and become dysfunctional only with adrenergic stimulation. The answer to this issue may provide important insights into 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 on high protein kinase A stimulation (10-fold increase in channel open probability).\textsuperscript{3,10} George and Lai\textsuperscript{18} also showed unaltered baseline Ca\textsuperscript{2+} activity in atrial myocyte cell line (HL-1) transfected with mutated RyR2s. However, these results were challenged by other groups who reported an increase in abnormal Ca\textsuperscript{2+} release and higher sensitivity of mutant RyR2s compared with WT at resting conditions. Jiang et al.\textsuperscript{11,13} reported that CPVT mutations enhance the sensitivity of the RyR2 channel to luminal Ca\textsuperscript{2+} activation and lower the threshold for spontaneous Ca\textsuperscript{2+} release in the setting of Ca\textsuperscript{2+} overload. Recently, Fernández-Velasco et al.\textsuperscript{26} showed an increased incidence of Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} waves at baseline in RyR2\textsuperscript{R4496C+/−} myocytes, which is further enhanced by either isoproterenol or high pacing rates. Liu et al.\textsuperscript{27} reported similar results of increased spark frequency at baseline and after isoproterenol challenge in isolated RyR2\textsuperscript{R4496C+/−} myocytes. However, when we examined Ca\textsuperscript{2+} signals from intact WT and mutant hearts using the in situ imaging method, we found that RyR2-mutated myocytes exhibit no abnormal Ca\textsuperscript{2+} release, eg, Ca\textsuperscript{2+} waves, at resting conditions (Figure 1). These discrepancies may be explained by differences in experimental conditions, which resulted in exposure of RyR2 to different intraluminal Ca\textsuperscript{2+} levels. Enzymatic and mechanical dissociation of heart tissue exerts a significant stress to the myocytes, causing myocyte Ca\textsuperscript{2+} overload and spontaneous Ca\textsuperscript{2+} release.\textsuperscript{14,16,37} Instead, under conditions that did not exist or cause SR Ca\textsuperscript{2+} overloading, such as in lipid bilayers,\textsuperscript{9,10} cultured HL-1 myocytes,\textsuperscript{15} 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\textsuperscript{2+} overload.\textsuperscript{13} In the absence of SR Ca\textsuperscript{2+} overload (ie, in situ under resting conditions or studies with lipid bilayers\textsuperscript{9,10} or cultured HL-1 atrial myocytes),\textsuperscript{35} the R4496C mutation-linked Ca\textsuperscript{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\textsuperscript{2+} signaling is predicted to lead to alterations in myocyte gene transcription, which could eventually promote cardiac pathophysiology (eg, cardiomyopathy).

### CRV and Electric Abnormalities in Physiologically Coupled Myocytes With RyR2\textsuperscript{R4496C+/−} Mutation

In the present study, we identified a new pattern of Ca\textsuperscript{2+} release dysfunction—a high degree of variation in Ca\textsuperscript{2+} transient amplitude in RyR2\textsuperscript{R4496C+/−} myocytes. This pattern of Ca\textsuperscript{2+} release abnormality was present in mutated myocytes under adrenergic stimulation; it was not observed in WT cells, RyR2-mutated myocytes under baseline conditions, or mutated neonatal myocytes in culture. This result was only detectable by in situ imaging methods. CRV is distinct in nature from rapid pacing-induced Ca\textsuperscript{2+} alternans\textsuperscript{25} in that (1) CRV is independent of the cycle length, (2) change in Ca\textsuperscript{2+} transient amplitude is predominantly irregular, (3) CRV is not inducible in isolated myocytes (either WT or mutated myocytes), and (4) rapid high-rate, pacing-induced Ca\textsuperscript{2+} alternans may not be coordinated or synchronized among different myocytes from beat to beat.\textsuperscript{25} These features suggest that proarrhythmic defects in RyR2\textsuperscript{R4496C+/−} hearts are not limited to ventricular myocytes. Recently, 2 elegant studies using different approaches (optical mapping and confocal imaging) concluded that the RyR2\textsuperscript{R4496C} mutation–associated CPVT originated from abnormalities in Purkinje cells. Cerrone et al.\textsuperscript{38} studied the mechanisms and origin of CPVT arrhythmias in Langendorff-perfused RyR2\textsuperscript{R4496C+/−} mouse hearts using optical mapping. Their data provide compelling evidence that CPVT arrhythmias originate from the specialized conduction system. Recently, Fishman et al.\textsuperscript{39} examined the frequency and severity of spontaneous Ca\textsuperscript{2+} activities in Purkinje cells in comparison with 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\textsuperscript{2+} handling defects in Purkinje cells are more pronounced and frequent than those of ventricular myocytes with the same mutation.\textsuperscript{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 electric activity in other regions, such as Purkinje fibers. Providing additional support for this conclusion, our in situ AP optical recordings and single-cell AP studies indicate that this defect is in the electric conduction system rather than ventricular in origin. Therefore, it is postulated that under stress conditions, defective RyR2-mediated abnormal Ca\textsuperscript{2+} release...
in Purkinje cells leads to aberrant electric activities, thereby triggering variability in Ca$^{2+}$ release in ventricular myocytes. We speculate that the mechanism is as follows: Under stress conditions, spontaneous Ca$^{2+}$ releases in Purkinje fibers with RyR2R4496C−/− mutation cause delayed afterdepolarizations, 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 CRV 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 RyR2R4496C−/− 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 seem to be related to basal catecholamine levels because β-adrenergic blockade failed to completely prevent sudden cardiac death in patients with CPVT mutations. Future studies toward understanding the mechanisms underlying this difference are warranted.

Summary
Our study using the in situ confocal imaging approach provides compelling evidence that the RyR2R4496C−/− mutants are functionally normal in situ under resting conditions but display a high degree of CRV on 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 electric defects rather than the failure of Ca$^{2+}$ release response to APs in mutated ventricular myocytes. The present study provides important insights into Ca$^{2+}$ release and electric dysfunction in an established model of CPVT and has important implications in understanding the mechanism of CPVT in patients.

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Disclosures
None.

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Ca²⁺ 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 Ca²⁺ release channel, type 2 ryanodine receptor, 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 Ca²⁺ 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 (type 2 ryanodine receptor 
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SUPPLEMENTAL MATERIAL

In situ confocal imaging in intact heart reveals stress-induced Ca$^{2+}$ release variability in a mouse CPVT model of RyR2$^{R4496C+/-}$ mutation

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Extended Methods

Animal model

Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No.85 to 23, revised 1985) and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. The generation and characterization of RyR2$^{R4496C+/-}$ heterozygous mice has been described recently (1) and is identical to the mice made by Priori group.(2) Both male and female RyR2$^{R4496C+/-}$ and wildtype littermates at ages of 2-4 months were used for studies.

In situ confocal Ca$^{2+}$ imaging in intact hearts with / without ex vivo electrocardiogram

Methods were adapted from published reports.(3, 4) Mice were heparinized (100 IU i.p.) and euthanized by pentobarbital (120 mg/kg, i.p.). Excised hearts were perfused with Rhod-2 AM (0.3 mM) containing Kreb- Henseleit’s solution (in mM: 120 NaCl, 24 NaHCO$_3$, 11.1 Glucose, 5.4 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 0.42 KH$_2$PO$_4$, oxygenated with 95% O$_2$ and 5% CO$_2$) at room temperature for 30 min via retrograde Langendorff perfusion system. Hearts were later transferred to another Langendorff apparatus (37 ºC) attached to the confocal microscope system after Rhod-2 loading was completed. The heart was placed onto a recording chamber for in situ confocal imaging (linescan) of Ca$^{2+}$ signals from epicardial myocytes under sinus rhythm. To avoid motion artifacts in Ca$^{2+}$ imaging, blebbistatin (10 µM, Sigma) and BDM (2,3-butanedione monoxime, 10 mM, Sigma) were added to the perfusion solution. Two ECG probes (silver wires) were placed on the apical subepicardium and right atrium, respectively. The pseudo ECG signal was continuously acquired by a Differential AC Amplifier (Model 1700, A& M Systems), digitized at 1K Hz online with Digidata 1440A A-D converter, stored with pClamp 10 software, and analyzed offline with Clampfit 10 (Molecular Devices, CA). The confocal linescan images were acquired at a rate of 3.07 ms or 1.93 ms per line. Unless otherwise specified, Ca$^{2+}$ transients were autonomously elicited by electrical signals from sinoatrial node.

In situ confocal AP imaging in intact heart
These studies were adapted from a published report. Similar to in situ Ca\(^{2+}\) imaging, a fast voltage-sensitive dye, ANNINE-6plus (3 µg/ml) was used to load the intact hearts through Langendorff-perfusion to track the dynamic changes of transmembrane potential. ANNINE-6plus fluorescence was recently shown to report changes in transmembrane voltage in cardiomyocytes with high fidelity (verified with direct microelectrode recordings of APs). We recorded ANNINE-6plus fluorescence using linescan confocal imaging at a scan rate of 1.54 ms per line from epicardial myocytes of intact hearts under sinus rhythm. Since changes in transmembrane potential are linearly correlated with ANNINE-6plus fluorescence changes, we used the absolute fluorescence ratio (ΔF/\(F_0\)) as an index of AP amplitude, and defined the time from peak to 50% decay as APD\(_{50}\).

**Ca\(^{2+}\) imaging in adult single isolated ventricular myocytes and in primary cultured neonatal myocytes**

Adult ventricular myocyte dissociation and confocal Ca\(^{2+}\) imaging were performed as previously described. Briefly, isolated myocytes loaded with Rhod-2 AM were subjected to external field stimulation (3 Hz / 5 Hz), and Ca\(^{2+}\) transients were recorded using linescan confocal imaging during steady state conditions at baseline and at least 3 minutes after epinephrine (Epi)+ caffeine (Caff) perfusion. This avoids the transient effects of caffeine on myocytes.

Neonatal myocytes were prepared as follows: primary cultures of neonatal ventricular myocytes were prepared from 1–2 day old WT or RyR2\(^{R496C/+}\) neonates. Neonatal mice were sacrificed by cervical dislocation. The ventricles were washed with cold PBS and minced finely in 1.5 ml of 0.05% trypsin, 0.2 mM Na-EDTA, and 0.5 mg/ml collagenase II, and then digested for 15 minutes. Ventricles were then resuspended several times in 1-ml Eppendorf tubes and incubated for another 15-30 minutes until the dissociation of cells was complete. Complete media (10% fetal bovine serum (FBS) in Dulbecco’s modified eagle medium (DMEM)) was added to terminate digestion. After centrifugation, cells were resuspended in complete media and pre-plated on a cell culture dish for 2 hours, thereby eliminating non-myocyte cells that adhere to the dish. Un-attached myocytes were plated onto laminin-coated glass surfaces and cultured under 5% CO\(_2\) in complete media for 12 hours. Next, the plating media was replaced with fresh complete media to remove dead cells. Myocytes were used after 48 hours in culture. Confocal Ca\(^{2+}\) imaging was performed as for adult ventricular myocytes, except that these neonatal cells exhibit spontaneous beating, thus no external field stimulation was required.

**Action potential (AP) recordings in single isolated myocytes**

Action potentials of single ventricular myocytes were evoked with 3 / 5 Hz stimulation and were recorded by perforated patch at 36±1°C. The bath solution contained (in mM) 134 NaCl, 10 HEPES, 11 glucose, 4 KCl, 1.8 CaCl\(_2\), and 1.2 MgCl\(_2\), with pH adjusted to 7.4 with NaOH. The pipette was filled with (in mM) 130 potassium aspartate, 10 NaCl, 10 HEPES, 0.04 CaCl\(_2\), 2.0 MgATP, 7.0 phosphocreatine, 0.1 NaGTP, and 240 µg/ml amphotericin B, with pH adjusted to 7.2 with KOH. Action
potential duration (APD) was assessed as the time from the AP upstroke to 50% repolarization to baseline (APD$_{50}$).

**Data analysis and statistics**

Confocal Ca$^{2+}$ / AP images were analyzed offline with custom routines composed with IDL image analysis software (ITT VIS Inc., Boulder, CO). Pseudo ECG data were processed offline with Clampfit 10. Data were expressed as mean ± SE (♦, mean plus/minus error bars) 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 comparisons. Bonferroni procedure following a global test based on linear mixed effects model was performed for multiple group comparisons (NCSS, LLC, Kaysville, Utah). The hearts were treated as random effects and the conditions (e.g., WT control, WT Epi + Caff, R4496C+/- Control and R4496C+/- Epi + Caff) as a fixed effect. A compound symmetry correlation structure was assumed for linear mixed effects model tests. A p value of <0.05 was considered statistically significant.

**Supplemental Figure legends**

**Figure S1. Variance of Ca$^{2+}$ transient amplitude in single isolated myocytes.** Isolated myocytes loaded with Rhod-2 AM were subjected to external field stimulation at 3Hz, and Ca$^{2+}$ transients were acquired at steady state. A-B, representative examples of confocal images from WT and RyR2$^{R4496C+/−}$ myocytes under control and at least 3 minutes after Epi+Caff treatment. Note the frequent spontaneous Ca$^{2+}$ waves during the diastolic phase in RyR2$^{R4496C+/−}$ myocyte under Epi+Caff stimulation. C. Variance analysis of Ca$^{2+}$ transient amplitude. Action potential-triggered Ca$^{2+}$ transients, but not spontaneous propagating Ca$^{2+}$ waves, were included in this analysis. n= 911, 758, 500 and 306 events, respectively. D. a boxplot of means of variance, n= 51, 58, 47 and 30 cells from 4 animals, respectively. No difference in variance in Ca$^{2+}$ transient amplitude was detected between WT and RyR2$^{R4496C+/−}$ myocytes perfused with Epi+Caff, suggesting CRV observed in intact RyR2$^{R4496C+/−}$ hearts was not a Ca$^{2+}$ release defect due to the mutation in ventricular myocytes themselves. E-F, Variance analysis of Ca$^{2+}$ transient amplitude under 5-Hz field stimulation in myocytes treated with Epi+Caff. No difference in variance in Ca$^{2+}$ transient amplitude was detected between WT and RyR2$^{R4496C+/−}$ myocytes perfused with Epi+Caff at 5 Hz stimulation too.

**Figure S2. No apparent CRV was observed in 2-day cultured neonatal myocytes with the R4496C mutation.** Ca$^{2+}$ transients were recorded in multi-connected neonatal myocytes under conditions of spontaneous beating. A, Representative images of Ca$^{2+}$ transients from WT and RyR2$^{R4496C+/−}$ neonatal myocytes treated with Epi+Caff. Green bars indicate the boundary of neighboring cardiomyocytes. B. Variance analysis of the amplitude of Ca$^{2+}$ transients, n=3711, 4450, 1752, 7521 events for WT, WT with
Epi+Caff, R4496C+/− and R4496C+/− with Epi+Caff, respectively. C. A boxplot of means of variance, n=22, 11, 11, 14, respectively. No difference was found between WT and RyR2R4496C+/− myocytes under either control or Epi+Caff treatment.

Figure S3. AP variation was synchronized between neighboring cells in RyR2R4496C+/− hearts. A, Raw and normalized images from a RyR2R4496C+/− heart under Epi+Caff perfusion. B, Spatial average of ANNINE-6plus fluorescence from Cell 1 and Cell 2 as indicated in Panel A, suggesting a synchronized pattern in AP alteration in physiologically-coupled RyR2R4496C+/− myocytes. Of note, the synchronized AP alterations were not observed in WT hearts under the same condition.

Figure S4. AP studies in single isolated myocytes using conventional current clamp recordings. A-B, Superimposed AP traces (100) from WT and RyR2R4496C+/− myocytes under control conditions and with adrenergic stress (Epi+Caff) at 3Hz stimulation. C-D, Boxplots of average data on APA and APD50 (3 Hz). E-F, Boxplots of variance of APA and APD50 under different conditions (3 Hz). G-J, Boxplots of average data at 5Hz stimulation. No differences were detected in AP amplitude (APA), duration (APD50), or in variance of these parameters between WT and RyR2R4496C+/− hearts under both control and adrenergic stress.

Figure S5. The influences of 2,3-butanedione monoxime (BDM) and blebbistatin (BB) on field stimulation Ca2+ transient amplitude and kinetics of single myocytes. A. Representative examples of steady state Ca2+ transients from WT and RYR2R4496C+/− myocytes under 3-Hz field stimulation, in control and BDM and BB perfusion. B. Average data on Ca2+ transient parameters (amplitude, time to peak and decay time), n=30, 20, 30, 20 cells from 3-4 hearts.

References


Figure S5

A

WT

R4496C^{+/−}

Control

BDM + BB

Scale: 50 μm

1 Hz

B

Ca transient amplitude (F/F₀)

WT control WT Epi+Caff R4496C^{+/−} control R4496C^{+/−} Epi+Caff

T_{peak} (ms)

WT control WT Epi+Caff R4496C^{+/−} control R4496C^{+/−} Epi+Caff

T_{50} (ms)

WT control WT Epi+Caff R4496C^{+/−} control R4496C^{+/−} Epi+Caff