Shortened \( \text{Ca}^{2+} \) Signaling Refractoriness Underlies Cellular Arrhythmogenesis in a Postinfarction Model of Sudden Cardiac Death

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**Rationale:** Diastolic spontaneous \( \text{Ca}^{2+} \) waves (DCWs) are recognized as important contributors to triggered arrhythmias. DCWs are thought to arise when \([\text{Ca}^{2+}]_\text{SR}\) reaches a certain threshold level, which might be reduced in cardiac disease as a consequence of sensitization of ryanodine receptors (RyR2s) to luminal \( \text{Ca}^{2+} \).

**Objective:** We investigated the mechanisms of DCW generation in myocytes from normal and diseased hearts, using a canine model of post–myocardial infarction ventricular fibrillation (VF).

**Methods and Results:** The frequency of DCWs, recorded during periodic pacing in the presence of a \( \beta \)-adrenergic receptor agonist isoproterenol, was significantly higher in VF myocytes than in normal controls. Rather than occurring immediately on reaching a final \([\text{Ca}^{2+}]_\text{SR}\), DCWs arose with a distinct time delay after attaining steady \([\text{Ca}^{2+}]_\text{SR}\). Although the rate of \([\text{Ca}^{2+}]_\text{SR}\) recovery after the SR \( \text{Ca}^{2+} \) release was similar between the groups, in VF myocytes the latency to DCWs was shorter, and the \([\text{Ca}^{2+}]_\text{SR}\) at DCW initiation was lower. The restitution of depolarization-induced \( \text{Ca}^{2+} \) transients, assessed by a two-pulse protocol, was significantly faster in VF myocytes than in controls. The VF-related alterations in myocyte \( \text{Ca}^{2+} \) cycling were mimicked by the RyR2 agonist, caffeine. The reducing agent, mercaptopropionylglycine, or the CaMII inhibitor, KN93, decreased DCW frequency and normalized restitution of \( \text{Ca}^{2+} \) release in VF myocytes.

**Conclusions:** The attainment of a certain threshold \([\text{Ca}^{2+}]_\text{SR}\) is not sufficient for the generation of DCWs. Postrelease \( \text{Ca}^{2+} \) signaling refractoriness critically influences the occurrence of DCWs. Shortened \( \text{Ca}^{2+} \) signaling refractoriness due to RyR2 phosphorylation and oxidation is responsible for the increased rate of DCWs observed in VF myocytes and could provide a substrate for synchronization of arrhythmogenic events at the tissue level in hearts prone to VF. *(Circ Res. 2012;110:569-577.)*

**Key Words:** excitation-contraction coupling \( \bullet \) ryanodine receptor \( \bullet \) \( \text{Ca}^{2+} \) waves \( \bullet \) arrhythmia \( \bullet \) refractoriness

Sudden cardiac death resulting from ventricular tachyarrhythmias (ventricular tachycardia or ventricular fibrillation [VF]) remains the leading cause of mortality accounting for between 250,000 and 300,000 deaths in the United States each year. It has been established that a large proportion of sudden cardiac death occurs after myocardial infarction (MI). Abnormal \( \text{Ca}^{2+} \) handling has been implicated in a broad range of cardiac arrhythmias, including post-MI malignant ventricular arrhythmias. However, the specific mechanisms contributing to these arrhythmias have not been fully elucidated.

In the beating heart, most of the \( \text{Ca}^{2+} \) required for cardiac contractile activation is released from the sarcoplasmic reticulum (SR) via ryanodine receptor (RyR2) channels in response to \( \text{Ca}^{2+} \) entry during the systolic action potential (AP). After activation, SR \( \text{Ca}^{2+} \) release is terminated, due at least in part to an inhibitory effect of reduced SR \([\text{Ca}^{2+}]_\text{SR}\) on the RyR2 channels, that is, store-dependent deactivation. This mechanism also maintains refractoriness of \( \text{Ca}^{2+} \) signaling during the diastolic period, allowing the \( \text{Ca}^{2+} \) released into the cytosol to be effectively resequestered by the SR to be liberated again during the next cardiac cycle.

In disease settings, SR \( \text{Ca}^{2+} \) release can occur spontaneously rather than being triggered by the systolic action potential. Spontaneous \( \text{Ca}^{2+} \) releases, which occur in the form of diastolic self-propagating \( \text{Ca}^{2+} \) waves (DCWs), contribute to cardiac arrhythmogenesis by eliciting phase 4 oscillations of membrane potential known as delayed after-
depolarizations (DADs) and extrasystolic action potentials leading to triggered activity. Changes in the SR Ca\(^{2+}\) content and/or sensitivity of RyR2s to luminal Ca\(^{2+}\) have been recognized as important factors in the generation of DCWs.\(^\text{10}\) It has been suggested that spontaneous Ca\(^{2+}\) release arises whenever \([\text{Ca}^{2+}]_{\text{SR}}\) reaches a certain threshold level\(^\text{10}\)\(^-\text{12}\) and that this threshold is reduced in cardiac disease as a consequence of either genetic mutations in the RyR2 channel complex \(\text{ie, cation-chlorineotropic polymorphic ventricular tachycardia} (\text{CPVT})\) caused by mutations in RyR2 or calsequestrin \((\text{CSQ2})\) or acquired modifications in RyR2, leading to enhanced activation of RyR2s by luminal Ca\(^{2+}\)\(^\text{6,13}\). However, nearly all data supporting this view have been obtained either by steady-state recordings from RyR2 channels or in resting \(\text{ie, nonstimulated}\) cells, and therefore provide little information about spontaneous Ca\(^{2+}\) release during physiologically relevant Ca\(^{2+}\) cycling.

The goal of the present study was to investigate the molecular mechanisms of cellular arrhythmogenesis during physiologically relevant pacing using a well-established canine model of post-MI VF and sudden cardiac death.\(^\text{14}\) To this end, we performed simultaneous measurements of cytosolic and luminal Ca\(^{2+}\) changes during pacing-induced Ca\(^{2+}\) cycling in myocytes isolated from normal and diseased hearts. Additionally, we investigated the role of modifications of RyR2s by phosphorylation and oxidation in arrhythmogenesis in myocytes from heart in which VF was inducible. We found that spontaneous Ca\(^{2+}\) release was indeed influenced by levels of intra-SR Ca\(^{2+}\). However, rather than occurring once a final \([\text{Ca}^{2+}]_{\text{SR}}\) level had been reached, DCWs arose with a distinct time delay (latency) that was significantly shortened in cells from diseased hearts when compared with myocytes from control hearts. The shortened latency in VF myocytes is attributable to the modification of RyR2s by CaMKII-phosphorylation and oxidation.

## Methods

### Model Description

The principles governing the care and use of animals, as expressed in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health \((\text{NIH} \text{ publication No. 85–323, revised 1996})\) and as adopted by the American Physiological Society, were followed at all times during this study. In addition, the Ohio State University Institutional Animal Care and Use Committee approved all animal procedures used in this study.

The surgical procedures, and the arrhythmia risk stratification test \((\text{exercise plus ischemia})\) used to classify the dogs as to susceptibility to VF have been previously described.\(^\text{14,15}\) Briefly, heartworm-free mixed-breed dogs \((\text{male/female, 2–3 years of age})\) were anesthetized and a myocardial infarction was induced via 2-stage occlusion of the left anterior descending coronary artery. The ligation of this vessel produced an anterolateral infarction \((\sim 17\% \text{ of left ventricular mass})\),\(^\text{14}\) from the region between the papillary muscle and the apex. At the time of the surgery, a pulsed Doppler flow transducer and a vascular occluder were placed around the left circumflex coronary artery. After a 3- to 4-week recovery period, susceptibility to VF was assessed using a standardized exercise plus ischemia test.\(^\text{14,15}\) This exercise plus ischemia test has been shown to induce VF reproducibly \((\text{up to at least 5 months})\) in those animals exhibiting an initial positive test result \((\sim 60\% \text{ of the total post-MI animals})\).\(^\text{14}\) The animals with a positive test were promptly resuscitated and at least 1 week later, the animals were euthanized and the heart removed for preparation of ventricular myocytes \((\text{see below})\). Studies were only performed on the susceptible \(\text{ie, VF}^+\) \((n = 18)\) dogs. Myocytes were also obtained from control \(\text{ie, noninfarcted}\) dogs \((n = 14)\).

### Ca\(^{2+}\) Imaging and Electrophysiology

Myocytes were isolated from the left lateral ventricular midmyocardium as previously described.\(^\text{16}\) Whole-cell patch clamp recordings of AP were performed with an Axopatch 200B amplifier \((\text{Molecular Devices, Sunnyvale, CA})\) using the external solution \((\text{mmol/L})\): 140 NaCl, 5.4 KCl, 2.0 CaCl\(_2\), 0.5 MgCl\(_2\), 10 HEPES, and 5.6 glucose \((\text{pH 7.4})\). Patch pipettes were filled with the following solution \((\text{mmol/L})\): 90 K-aspartate, 50 KCl, 5 MgATP, 5 NaCl, 1 MgCl\(_2\), 0.1 Tris GTP, 10 HEPES; \(\text{pH 7.2}\). Potassium salts of the following Ca\(^{2+}\) indicators were added to a pipette solution to monitor cytosolic Ca\(^{2+}\): Fluo-3 \((0.06 \text{ mmol/L})\) or Rhod-2 \((0.1 \text{ mmol/L})\) or Fluo-4FF \((0.2 \text{ mmol/L})\) \((\text{Life Technologies, Grand Island, NY})\).

Intracellular Ca\(^{2+}\) imaging was performed using an Olympus Fluoview 1000 and a Nikon A1R confocal microscopes. Free intra-SR Ca\(^{2+}\) levels were measured by loading myocytes with 10 \(\mu\text{mol/L}\) Fluo-5N AM \((\text{Life Technologies, Grand Island, NY})\) for 3 to 3.5 hours at 37°C. The SR Ca\(^{2+}\) levels and intra-SR Ca\(^{2+}\) dynamics can be significantly affected by alterations in the SR volume and/or changes in the level of CSQ2, a major intra-SR Ca\(^{2+}\) buffer. We assessed potential changes in the SR spatial organization by comparing fluorescence recovery after photobleaching \((\text{FRAP})\) of SR-entrapped flou-5N in control and VF myocytes.\(^\text{17}\) As shown in Online Figure I, flou-5N FRAP rate was not different in control and VF myocytes suggesting that no significant alterations in the SR spatial organization occurred in VF. We also measured the levels of CSQ2 in control and VF. As shown in Online Figure II, the level of CSQ2 was not significantly altered in VF. To monitor cytosolic Ca\(^{2+}\) in field-stimulation experiments, myocytes were loaded with 9 \(\mu\text{mol/L}\) Rhod-2 \(\text{AM}\) for 25 minutes at room temperature; 30–60 minutes was allowed for deesterification.

### Reagents

All reagents were from Sigma-Aldrich, Inc \((\text{St. Louis, MO})\), unless otherwise indicated.

### Analysis

Results are presented as mean±SEM. Statistical significance was evaluated either by the appropriate Student \(t\) test or by 1-way ANOVA with Tukey post hoc test where appropriate. A probability value of <0.05 was considered significant.

## Results

### Frequency of DCWs and DADs Are Increased in VF Myocytes

We examined the propensity toward proarrhythmic Ca\(^{2+}\) waves and DADs in a model of postinfarction sudden cardiac
death (VF). As we previously reported, VF hearts in this model are characterized by abnormally high RyR2 activity but normal SERCA and NCX function. First, we performed recordings of cytosolic Ca\(^{2+}\) and membrane potential in paced (at 0.5 Hz) VF and control myocytes stimulated at 0.5 Hz in the presence of 100 nmol/L isoproterenol, a β-adrenergic receptor agonist. Asterisks mark action potentials triggered by DADs. Average frequency of DADs recorded in VF myocytes (0.43±0.06 per cycle, n=13) was significantly higher when compared with controls (0.14±0.05 per cycle, n=10, P<0.05). Number of cells displaying DCWs and DADs is higher in VF than in control.

Attainment of a Certain \([\text{Ca}^{2+}]_{\text{SR}}\) Is Insufficient for Diastolic Release

It is generally assumed that Ca\(^{2+}\) waves arise when \([\text{Ca}^{2+}]\) inside the SR attains a certain critical level or “threshold.” To test whether DCWs observed in our myocyte experiments were associated with luminal Ca\(^{2+}\) reaching a critical level, we recorded intra-SR Ca\(^{2+}\) levels during DCW generation using the low-affinity Ca\(^{2+}\) indicator fluo-5N entrapped in the SR in VF versus control myocytes. We standardized the conditions for examining DCW generation by using a Ca\(^{2+}\) loading protocol composed of a train of standard AP-clamp pulses. Using this protocol, DCWs occurred consistently after stimulation in both VF and control myocytes, although in VF myocytes DCWs arose with a significantly shorter time delay after termination of stimulation (Figure 2A and 2C). As indicated by the intra-SR fluo-5N fluorescence signal, myocytes indeed exhibited a certain critical \([\text{Ca}^{2+}]_{\text{SR}}\) level at which DCWs arose and this \([\text{Ca}^{2+}]_{\text{SR}}\) was significantly lower in VF myocytes than in control cells (Figure 2A and 2B). Notably, in neither control nor VF myocytes did DCWs occur as soon as \([\text{Ca}^{2+}]_{\text{SR}}\) recovered to its final baseline level. Instead there was a distinct delay or latency between \([\text{Ca}^{2+}]_{\text{SR}}\) reaching the baseline and the onset of DCW. After a Ca\(^{2+}\) wave, baseline \([\text{Ca}^{2+}]_{\text{SR}}\) declined as expected, because some of the Ca\(^{2+}\) that forms the wave is removed from the cell by NCX, thus depleting the SR.

The shortened time to DCW could be due to either a faster refilling of the SR Ca\(^{2+}\) store and/or to shortening of the latency period at an already steady-state \([\text{Ca}^{2+}]_{\text{SR}}\) in VF myocytes compared with controls. As shown in Figure 2, the average SR Ca\(^{2+}\) refilling time was not significantly different between control and VF myocytes (Figure 2D). In contrast, the latency period (at steady \([\text{Ca}^{2+}]_{\text{SR}}\)) was significantly shorter in VF myocytes with respect to control (Figure 2E).

The functional state of the Ca\(^{2+}\) release channels and the probability of spontaneous Ca\(^{2+}\) release are known to depend on the SR Ca\(^{2+}\) content. To assess the role of different baseline SR Ca\(^{2+}\) levels in determining the probability and timing of DCWs in VF vs. control myocytes, we varied \([\text{Ca}^{2+}]_{\text{SR}}\) in control myocytes by varying the Ca\(^{2+}\) loading protocol. As shown in Online Figure III, lowering SR Ca\(^{2+}\) in control myocytes towards the levels observed in VF cells led to an increase in latency and to a decrease in the incidence of DCWs. Therefore, the fact that the time delay to DCWs is shorter in VF myocytes despite the reduced diastolic SR Ca\(^{2+}\) level suggests impairment of the ability of depleted diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) to deactivate RyR2s in VF myocytes.

Collectively, these results show for the first time that spontaneous Ca\(^{2+}\) waves do not arise as a result of luminal Ca\(^{2+}\) activating RyR2 at a certain critical \([\text{Ca}^{2+}]_{\text{SR}}\) threshold as is commonly assumed. Rather these data suggest that DCWs are associated with the failure of RyR2s to remain activated or inactivated/refractory during diastole.

Caffeine Mimics the Impact of VF on Myocyte Ca\(^{2+}\) Handling

To assess further the potential role of altered RyR2 functional activity in the generation of DCWs, we examined the effects of the RyR agonist caffeine on DCW occurrence and on changes in both cytosolic and SR luminal Ca\(^{2+}\) in control myocytes. Experiments were performed in myocytes exposed to 0.4–1 mmol/L caffeine and 100 mmol/L isoproterenol using the same AP-clamp simulation protocols as in the experiments in control and VF myocytes (Figure 2). Under these conditions, caffeine significantly reduced the \([\text{Ca}^{2+}]_{\text{SR}}\) at which DCWs occurred and shortened both the overall time delay and latency (at a constant \([\text{Ca}^{2+}]_{\text{SR}}\)) to DCWs in control myocytes (Figure 2B and 2C) to values similar to the alterations observed in VF myocytes. Of note, in addition to shortening latency, caffeine also shortened the SR Ca\(^{2+}\) refilling rate by a small but significant degree (Figure 2D).
SR Ca\(^{2+}\) Release Refractoriness Is Shortened in VF Myocytes

To test directly the hypothesis that the shortened latency to DCW in VF myocytes involves impaired diastolic refractoriness of RyR2s, we used a 2-pulse protocol as depicted in Figure 3 (upper panels). In these experiments, by varying the interpulse interval, we determined the rate of restitution of the cytosolic Ca\(^{2+}\) transient amplitude, while simultaneously measuring [Ca\(^{2+}\)]\(_{SR}\) recovery in VF versus control myocytes. Of note, recovery of the peak L-type Ca\(^{2+}\) current recorded using the 2-pulse protocol was not different between control and VF myocytes (Online Figure IV). Consistent with the diminished refractoriness hypothesis, the rate of restitution of cytosolic Ca\(^{2+}\) transients was significantly faster in VF myocytes than in control (Figure 3A through 3D). In addition, comparison of Ca\(^{2+}\) transient restitution rates with [Ca\(^{2+}\)]\(_{SR}\) recovery rates within the same myocyte groups revealed that while in control myocytes, Ca\(^{2+}\) transient recovery lagged substantially behind SR Ca\(^{2+}\) refilling, the rates of these 2 processes were similar in VF myocytes (Figure 3A–D). To gain further insight into the role of [Ca\(^{2+}\)]\(_{SR}\) in the different Ca\(^{2+}\) release restitution properties in VF and control cells we plotted the amplitude of Ca\(^{2+}\) transient as a function of [Ca\(^{2+}\)]\(_{SR}\) during recovery in the 2 myocyte groups (Figure 3E). The release-[Ca\(^{2+}\)]\(_{SR}\) dependency in VF myocyte is markedly shifted to lower [Ca\(^{2+}\)]\(_{SR}\) values with respect to control. This shows that while even relatively small Ca\(^{2+}\) store reductions are able to effectively restrain SR Ca\(^{2+}\) release in control myocytes, this control mechanism is impaired in VF myocytes, in which nearly normal SR Ca\(^{2+}\) release is preserved at much lower intra-SR Ca\(^{2+}\) levels. Collectively, these data suggest that the SR Ca\(^{2+}\) release refractoriness is significantly shortened in VF myocytes compared with controls. This decrease of diastolic refractoriness in VF myocytes is attributable to reduced ability of RyR2 to become deactivated on the decline of [Ca\(^{2+}\)]\(_{SR}\) that follows SR Ca\(^{2+}\) release.

Oxidation and Phosphorylation of RyR2s Contribute to Shortened Ca\(^{2+}\) Release Refractoriness

We previously demonstrated that cardiomyocytes in the VF model used here are characterized by increased reactive oxygen species, increased levels of RyR2 thiol oxidation and augmented redox-sensitive RyR-mediated SR Ca\(^{2+}\) leak.\(^{18}\) Therefore, we tested the effect of the reducing agent N-2-mercaptopropionyl glycine (MPG) on proarrhythmic alterations of Ca\(^{2+}\) handling. Figure 4 shows that treating VF myocytes with 0.75 mmol/L MPG significantly reduced the propensity for DCW generation and restored both the restitution of Ca\(^{2+}\) release and the SR Ca\(^{2+}\) content to control levels. These improvements of parameters of myocyte Ca\(^{2+}\) handling were associated with a significant increase in free thiol content and an improvement of functional activity of single RyR2 channels from VF hearts treated with MPG (Online Figure V).

Increased phosphorylation of RyR2s by PKA or CaMKII has been implicated in increased susceptibility to cellular arrhythmias.\(^{6,21}\) Therefore, we also tested whether the phosphorylation of RyR2s was altered in VF myocytes. As
as a function of interpulse time. The recovery of the amplitude was fit to a monoexponential function with time constants of 287 ± 16 ms in control (n = 7), 197 ± 28 ms inVF untreated (n = 3–4), 342 ± 33 ms inVF myocytes treated with MPG (n = 4–5), and 380 ± 40 ms inVF treated with KN93 (n = 4–5). D. Representative recordings of cytosolic Ca2+ transients induced by 10 mmol/L caffeine imaged with Fluo-4FF in voltage-clamped control and VF myocytes. E. Average amplitude of caffeine-induced Ca2+ transients in control (n = 8) andVF myocytes, andVF myocytes treated with either 0.75 mmol/L MPG (n = 6) or 1 mmol/L KN-93 (n = 4). F. Representative line-scan images and temporal profiles of Rhod-2 fluorescence recorded in control andVF myocytes, and inVF myocytes treated with either 0.75 mmol/L MPG, a reducing agent, or 1 μmol/L KN-93, a CaMKII inhibitor. Cells were field-stimulated at 0.3 Hz. G. Frequency of DCWs (marked with arrows in F) was calculated for control (n = 29) andVF myocytes (n = 25) andVF myocytes treated with either MPG (n = 19) or KN-93 (n = 16). H. Proportion of cells displaying DCWs in each experimental group. All data presented in this figure were obtained in the presence of 100 mmol/L isoproterenol, a β-adrenergic receptor agonist. *P < 0.05 versus control; †P = 0.05 versusVF untreated.

Discussion

DCWs and DADs are recognized as important contributors to the pathogenesis of triggered arrhythmias, including CPVT and post-MI VF.3,24 Although alterations in RyR2 regulation have been implicated in arrhythmogenesis, the specific mechanisms responsible for Ca2+ wave generation and factors accounting for increased propensity toward DCWs in cardiac disease remain to be fully elucidated. In the present study, we used a canine model of post-MI tachyarrhythmia to show that susceptibility to DCWs is determined by alterations in the...
time- and Ca\(^{2+}\) store-dependent properties of RyR2s that contribute to the diastolic refractoriness of release required for normal myocyte Ca\(^{2+}\) cycling. The shortened RyR2 refractoriness is attributable to modifications of the channel protein by CaMKII phosphorylation and thiol oxidation. These findings provide new mechanistic insights into the control of SR Ca\(^{2+}\) release in the normal heart and illustrate how alterations in these control mechanisms lead to the arrhythmogenesis associated with acquired defects in the RyR2 channel.

**Role of Shortened Ca\(^{2+}\) Release Refractoriness in Arrhythmogenesis**

The current study examined the factors that account for the loss of dynamic stability of SR Ca\(^{2+}\) release in paced ventricular myocytes from hearts susceptible to malignant tachyarrhythmias. Our findings are schematically illustrated in Figure 6, showing the main components of the time period preceding DCWs after systolic Ca\(^{2+}\) release for control and VF myocytes. Based on the results of experiments with monitoring of [Ca\(^{2+}\)]\(_{SR}\) (Figure 2), the total time to DCW is comprised of a period of refilling of the SR Ca\(^{2+}\) store and a subsequent delay or latency period during which [Ca\(^{2+}\)]\(_{SR}\) remains constant. Whereas the SR Ca\(^{2+}\) refilling time was not significantly different between VF and control myocytes, the latency to DCWs at a constant [Ca\(^{2+}\)]\(_{SR}\) was significantly shortened in VF myocytes. The latency in the development of DCWs after reaching a steady-state [Ca\(^{2+}\)]\(_{SR}\) could result from RyR2s being refractory (RyR2s are completely or partially unresponsive) and/or may involve an idle period (during which RyR2 functional activity is completely recovered and Ca\(^{2+}\) waves can be initiated at any time by stochastic Ca\(^{2+}\) sparks). The duration of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) restitution measured in 2-pulse experiments (Figure 3) suggests that most of the latency to DCW in control myocytes can be attributed to RyR2 refractoriness. In VF myocytes, CICR restitution closely follows the SR Ca\(^{2+}\) refilling time. The relatively short idle periods after the functional recovery of RyR2s and before the onset of DCWs are similar between VF and control myocytes (Figure 6). The idle period is likely to reflect a delay associated with “spontaneous” activation of stochastic release sites recovered from refractoriness, ultimately leading to the generation of diastolic Ca\(^{2+}\) release.

Although DCWs are considered to be the basis of triggered arrhythmias, the mechanism responsible for synchronization of Ca\(^{2+}\) waves and the ensuing membrane potential oscillations, between individual cardiac cells as required for the generation of ectopic action potentials remain to be elucidated. Our study demonstrated that DCWs in VF myocytes occur with a high degree of probability, reaching on average 0.5 per cycle (Figure 1), with the timing after systolic depletion tightly clustered around a median value of 1.5 seconds (standard deviation 0.3 seconds; Figure 2). Given their high probability and uniform timing, DCW rather than being spontaneous or random events seem to occur in a manner predetermined by refilling of the SR Ca\(^{2+}\) store and accelerated restitution of SR Ca\(^{2+}\) release. Thus, reduced refractoriness of Ca\(^{2+}\) signaling combined with uniform refilling of the SR after systolic SR Ca\(^{2+}\) release could provide a mechanism for temporal alignment of DCWs in multiple myocytes. We suggest that this synchronicity of abnormal Ca\(^{2+}\) release in multiple myocytes may be necessary for triggered activity and resulting tachyarrhythmias in VF hearts. This interpretation is consistent with recent studies.
by Wasserstrom et al. that showed that refilling of the SR Ca\(^{2+}\) store contributes to synchronization of Ca\(^{2+}\) waves and DADs in cardiac muscle during triggered activity induced by Ca\(^{2+}\) overload.

**Mechanism of Ca\(^{2+}\) Release Refractoriness and Its Impairment in VF Myocytes**

In addition to demonstrating the importance of refractoriness in preventing arrhythmogenic DCWs, our study provides insights into the mechanism of diminished refractoriness in VF myocytes that underlies the increased arrhythmogenic potential of myocytes from VF hearts. Previous studies from our and other laboratories have suggested that SR Ca\(^{2+}\) release is followed by a period of decreased store responsiveness (ie, refractoriness) due to lowered [Ca\(^{2+}\)]\(_{SR}\) that in turn, reduces RyR2 sensitivity to cytosolic Ca\(^{2+}\) (ie, store-mediated deactivation). In the present study, we showed that the luminal Ca\(^{2+}\)-dependence of CICR during restitution is markedly shifted toward lower luminal Ca\(^{2+}\) concentrations, suggesting that the ability of reduced luminal Ca\(^{2+}\) to inhibit SR Ca\(^{2+}\) release is impaired in VF myocytes (Figure 3E). Furthermore, sensitization of RyR2s to cytosolic Ca\(^{2+}\), caused by low doses of caffeine, significantly reduced the latency to DCWs in normal cells in a manner similar to that observed in VF myocytes (Figure 2). These results support the notion that Ca\(^{2+}\) release refractoriness involves SR Ca\(^{2+}\) store-dependent changes in RyR2 responsiveness to cytosolic Ca\(^{2+}\) and, moreover, that this mechanism is altered in VF.

**Impaired Ca\(^{2+}\) Release Refractoriness Versus Direct Activation by Luminal Ca\(^{2+}\) as Mechanisms for DCWs**

It has been previously proposed that diastolic Ca\(^{2+}\) release is a result of luminal Ca\(^{2+}\) activating RyR2 directly at luminal accessible sites, a mechanism referred to as store-overload-induced SR Ca\(^{2+}\) release. One important premise of this mechanism is that diastolic Ca\(^{2+}\) release arises as soon as the SR Ca\(^{2+}\) content reaches a critical level, that is, "threshold." In contrast to this expectation, our present study with direct monitoring of [Ca\(^{2+}\)]\(_{SR}\) in paced myocytes from VF and control hearts showed that DCWs do not arise immediately on [Ca\(^{2+}\)]\(_{SR}\) reaching a final "threshold" level but instead occur with a substantial time delay after the attainment of a baseline [Ca\(^{2+}\)]\(_{SR}\) (Figure 2). This delay or latency was observed in VF myocytes (Figure 2). These results support the notion that Ca\(^{2+}\) release refractoriness involves SR Ca\(^{2+}\) store-dependent changes in RyR2 responsiveness to cytosolic Ca\(^{2+}\) and, moreover, that this mechanism is altered in VF.

**Roles of Phosphorylation and Redox Modification of RyR2s**

Previous reports attributed proarrhythmic alterations in RyR2 function to either hyperphosphorylation by CAMKII or redox modifications of the channel protein in various disease settings. Our present study demonstrates that both of these mechanisms are involved in post-MI VF through affecting the refractory state of RyR2s. The vulnerability of RyR2 to oxidation is not surprising given the fact that it contains about 90 cysteines. Interestingly, targeting these mechanisms individually, by either an antioxidant treatment or CamKII inhibition, led to nearly complete normalization of Ca\(^{2+}\) handling parameters including RyR2 refractoriness. Moreover the effect of combined antioxidant and CamKII inhibition treatments in VF myocytes was not significantly different from that observed with either treatment alone (data not shown). Thus there was no additive effect of these interventions. One possible explanation for this result is that both types of modifications are required to induce an energetically unfavorable RyR2 conformation, such as "domain unzipping," associated with the increased RyR2 functional activity in VF. Altered RyR2 interdomain interactions caused by phosphorylation and redox modification of the channel protein have been previously reported to contribute to the hyperactive RyR2 phenotype associated with cardiac disease.

**Relationship to Arrhythmogenesis in CPVT and Heart Failure**

The mechanism of arrhythmogenesis in VF myocytes appears to be analogous to that described for CPVT linked to mutations in RyR2 and CASQ2. Indeed, in both disease settings, arrhythmogenesis is associated with altered luminal Ca\(^{2+}\) regulation of RyR2 and shortened refractoriness and is facilitated by β-adrenergic receptor stimulation. The proarrhythmic effects of isoproterenol in both CPVT and VF myocytes could be attributed to a combination of (1) phosphorylation of RyR2s by CamKII that would further contribute to altered RyR2 function, rendering them more sensitized and less refractory; and (2), an enhanced SERCA-mediated SR Ca\(^{2+}\) uptake that leads also to facilitated restitution from store-dependent deactivation. Thus, genetic and acquired defects in the RyR2 complex appear to be involved in a range of arrhythmia disorders, which are associated with dysregulated SR Ca\(^{2+}\) release. The basic pathophysiology of these arrhythmias hinges on the loss of Ca\(^{2+}\) signaling stability, due to the failure of RyR2 channels to deactivate and maintain appropriate refractoriness.
Our present results are consistent with accelerated Ca\(^{2+}\) release refractoriness observed in a canine model of chronic heart failure,\(^{31}\) although Ca\(^{2+}\) transient restitution was slowed in a rat model of heart failure.\(^{31}\) One possible explanation for these different results is a better preserved SR Ca\(^{2+}\) content and more extensive RyR2 phosphorylation in the canine as opposed to the rat studies performed with and without isoproterenol, respectively.

Conclusions

In summary, our study provides a unifying mechanistic framework for understanding Ca\(^{2+}\) handling in both normal physiology and disease settings. In normal myocytes, store-mediated deactivation of release is required for the stabilization of CICR, a process that is intrinsically prone to self-regeneration. Impairment of this stabilizing mechanism results in decreased Ca\(^{2+}\) signaling refractoriness, and increased propensity of myocytes to arrhythmogenic diastolic Ca\(^{2+}\) release and DADs in myocytes from VF hearts. RyR2 luminal Ca\(^{2+}\) regulation appears to be a site of integration for various physiological and pathophysiological influences on RyR2s, including cytosolic Ca\(^{2+}\), phosphorylation, redox modification, and genetic mutations, and may present a common target for treatment of different forms of cardiac diseases associated with altered RyR2 function.

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Disclosures

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**Novelty and Significance**

**What Is Known?**

- In multiple pathologies associated with both genetic and acquired defects in the cardiac ryanodine receptor (RyR2) channel, arrhythmias result from aberrant Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) in the form of spontaneous diastolic Ca\textsuperscript{2+} waves.
- Normal control of SR Ca\textsuperscript{2+} release involves Ca\textsuperscript{2+}-dependent activation of RyR2s followed by their store-dependent deactivation rendering the RyR2s refractory during diastole.
- Spontaneous diastolic Ca\textsuperscript{2+} waves are thought to arise when [Ca\textsuperscript{2+}]_{SR} exceeds a critical threshold level thereby directly activating RyR2s; however, direct experimental confirmation of this proposed mechanism is lacking.

**What New Information Does This Article Contribute?**

- Intra-SR [Ca\textsuperscript{2+}] during arrhythmogenic Ca\textsuperscript{2+} waves are recorded in cardiac myocytes isolated from post–myocardial infarction (MI) canine hearts prone to ventricular fibrillation (VF).
- Ca\textsuperscript{2+} waves do not arise immediately on [Ca\textsuperscript{2+}]_{i,ion} reaching its final Ca\textsuperscript{2+} level, but rather occur with a distinct time delay that is markedly shorter in myocytes from post-MI hearts.
- Increased predisposition toward Ca\textsuperscript{2+} waves in myocytes from post-MI hearts is due to diminished/shortened refractoriness of RyR2, caused by reduced ability of RyR2s to become deactivated by a decline in luminal Ca\textsuperscript{2+} after systolic SR Ca\textsuperscript{2+} release.
- Impaired refractory behavior of RyR2s is attributable to posttranslational modification of the RyR2 protein by both Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMKII)-dependent phosphorylation as well as oxidation.

Intracellular Ca\textsuperscript{2+} waves are known to play a key role in the pathophysiology of triggered ventricular arrhythmias. Although the SR Ca\textsuperscript{2+} load plays an important role in the genesis of Ca\textsuperscript{2+} waves, the specific mechanisms accounting for increased propensity toward Ca\textsuperscript{2+} waves in cardiac disease remain to be fully elucidated. In the present study, we monitored intra-SR Ca\textsuperscript{2+} levels during proarrhythmic Ca\textsuperscript{2+} waves in myocytes isolated from canine hearts with healed myocardial infarction prone to malignant arrhythmias. Our study demonstrated that the susceptibility for Ca\textsuperscript{2+} waves is determined by impairment of store-dependent deactivation of RyR2s, the mechanism that normally prevents SR Ca\textsuperscript{2+} release from occurring during the diastolic period. The shortened RyR2 refractoriness in post-MI myocytes is attributable to modifications of the channel protein by both CaMKII-dependent phosphorylation and thiol oxidation. Our findings provide a unifying conceptual framework for understanding Ca\textsuperscript{2+} handling in normal and diseased heart settings. Specifically, in normal myocytes, store-mediated deactivation of RyR2 stabilizes Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release that is intrinsically prone to self-regeneration. Impairment of this stabilizing mechanism in myocytes from diseased hearts results in decreased Ca\textsuperscript{2+} signaling refractoriness and increased propensity of myocytes for arrhythmogenic diastolic SR Ca\textsuperscript{2+} release.
Shortened Ca\textsuperscript{2+} Signaling Refractoriness Underlies Cellular Arrhythmogenesis in a Postinfarction Model of Sudden Cardiac Death

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Supplemental Material

Detailed Methods

Model Description

Prior to the exercise plus ischemia test, left ventricular (LV) contractile function was evaluated using echocardiography. In agreement with previous studies, myocardial infarction (MI) did not significantly alter LV contractile function, and no animal developed heart failure. No significant differences in LV ejection fraction (MI vs. no MI), LV diastolic internal diameter (MI vs. no MI) and LV systolic internal diameter (MI vs. no MI) were noted between the dogs that were susceptible to ventricular fibrillation (VF) following infarction compared to sham control (i.e., no infarction) dogs.

Sarcoplasmic reticulum (SR) Ca$^{2+}$ imaging.

Fluo-5N fluorescence was converted to [Ca$^{2+}$] according to the following equation: 

$$[\text{Ca}^{2+}] = K_d \times \frac{F - F_{\text{MIN}}}{F_{\text{MAX}} - F},$$

where $K_d$ was 400 $\mu$mol/L. $F_{\text{MIN}}$ was determined by application of 10 mmol/L caffeine. $F_{\text{MAX}}$ was determined by application of 10 mmol/L of [Ca$^{2+}$] and 0.1% saponin in the presence of 20 mmol/L of BDM. Cytochalasin D (15-40 $\mu$mol/L) was used in experiments presented in Figure 2 and 3 (main manuscript) to minimize movement artifacts. It should be noted that since the SR-entrapped Fluo-5N tracks free [Ca$^{2+}$]$_{\text{SR}}$, the Flu-5 fluorescence signal is not expected to exactly mirror the simultaneously measured fluorescent signal from the cytosolic Ca$^{2+}$ indicator, Rhod-2, which is proportional to the sum of the total Ca$^{2+}$ released from the SR and Ca$^{2+}$ influx through the Ca$^{2+}$ channels. Thus the differences in the restitution of the luminal Fluo-5N and cytosolic Rhod-2 signals (Figure 3A, main manuscript) can be ascribed to: a) effective buffering of free SR Ca$^{2+}$ by calsequestrin; and b) contribution of the L-type Ca$^{2+}$ current to the cytosolic signal, since initial recovery of the Ca$^{2+}$ current from inactivation occurs faster than restitution of Ca$^{2+}$ release (Online Figure IV).

Myocyte treatment with antioxidant mercaptopropionylglycine

To assess the role of redox modifications in mediating VF-associated alterations in Ca$^{2+}$ signaling myocytes were incubated for 30 min in external solution containing 0.75 mmol/L 2-mercaptopropionylglycine (MPG), a cell-permeable reactive-oxygen species (ROS) scavenger. Our choice to use MPG as a reducing agent in the present study was based on the following reasons: 1) we previously showed that MPG was effective in alleviating ROS-mediated changes in SR Ca$^{2+}$ leak in VF and heart failure myocytes; 4,5 2) MPG did not have apparent non-specific effects on intracellular Ca$^{2+}$ handling. 4 In the present study we confirmed the apparent lack of non-specific effects of MPG on Ca$^{2+}$ cycling: MPG did not affect amplitude of Ca$^{2+}$ transients recorded in control myocytes in the presence of isoproterenol (Iso), a $\beta$-adrenergic agonist (Online Figure VI). MPG treatment also did not affect the frequency of diastolic Ca$^{2+}$ waves (DCWs) recorded in control myocytes field stimulated at 0.3 Hz in a presence of 100 nmol/L Iso [0.12±0.03 waves per cycle in the absence (n=29) vs. 0.12±0.02 waves per cycle in the presence of MPG (n=37)]. Consistent with the lack of antioxidant effect on frequency of DCWs in control, we did not observe alterations in the rate of ROS production in control myocytes in the presence of 100 nmol/L Iso (Online Figure VII).

Using caffeine as a sensitizer of ryanodine receptors (RyR2s) to cytosolic Ca$^{2+}$

Caffeine is considered to be a classical RyR2 agonist that acts by increasing the sensitivity of RyR2 to cytosolic Ca$^{2+}$. Recently Kong et al., 7 reported that the effects of high concentrations of caffeine are mediated by changing the sensitivity of recombinant RyR2 to luminal Ca$^{2+}$. However a subsequent study of the effects of caffeine on rabbit cardiac RyR2 indicated that the effects of caffeine at <2.5 mmol/L are mediated by sensitization of the RyR2 to cytosolic Ca$^{2+}$ 8. Therefore we used concentrations of caffeine below 1 mmol/L (0.4-1 mmol/L) to study the effects of sensitizing RyR2s to cytosolic Ca$^{2+}$ on arrhythmogenesis.
Sulfhydryl labeling assay
The content of free thiols in RyR2s was determined using the monobromobimane (mBB) fluorescence method. Briefly, LV tissue samples were obtained from VF hearts perfused for 30 min with control buffer or with buffer containing 0.75 mmol/L MPG. Control perfusion buffer contained (mmol/L): 130 NaCl, 5.4 KCl, 3.6 MgCl₂, 0.05 CaCl₂, 0.5 Na₂HPO₄, 10 Glucose, 5 HEPES, 20 Taurine (pH 7.4). Samples were incubated with 20 mmol/L mBB for 1 hour in the dark at room temperature. To determine the maximal range of mBB signal some samples were treated for 30 min with either 10 mmol/L dithiothreitol, a reducing agent, or 0.2 mmol/L 2,2'-dithiodipyridine, an oxidizing agent. Subsequently proteins were subjected to SDS-PAGE. mBB fluorescence was normalized to the RyR2 levels quantified using Coomassie Blue staining of gels run in parallel. mBB fluorescence was acquired using Epichemi II Darkroom system (UVP Bioimaging Sys., CA). Images were analyzed using ImageJ software (U.S. National Institutes of Health).

Single RyR2 Channel Measurements
Single RyR2s were reconstituted by fusing canine LV heavy SR microsomes into planar lipid bilayers and single channel currents were recorded with an Axopatch 200A (Molecular Devices, CA) patch clamp amplifier currents at room temperature (21–23°C), as described previously. Data were digitized at 5–10 kHz and filtered at 2 kHz. Acquisition and analysis of data were performed by using PCLAMP 10 software (Molecular Devices). Experimental solutions contained (in mmol/L): 350 CsCH₃SO₃, 0.02 CaCl₂, 3 MgATP, and 20 HEPES (pH 7.4) on the cytosolic (cis) side of the bilayer, and 20 CsCH₃SO₃, 0.02 CaCl₂, and 20 HEPES (pH 7.4) on the luminal (trans) side of the bilayer.

Western Blotting
For Western blot analyses, myocytes were field stimulated at 0.5 Hz for 1 min, then RIPA buffer supplemented with phosphatase, protease and calpain inhibitors was added directly to the cells and then, the samples were instantly frozen with liquid nitrogen. Cell lysate proteins (40 μg) were subjected to 4 to 20% gradient SDS-PAGE and blotted onto nitrocellulose membrane. Phosphorylation of RyR2s was assessed using standard procedures. Anti-phospho-RyR2-Ser-2808 and Ser-2814 antibodies were kindly provided by Dr. X. Wehrens (Baylor College of Medicine, TX). Anti-phospho-RyR2-S2030 antibody was raised against (CG) TIRGRLLS(PO4)LVEKVTYLKKCONH₂ (Phosphosolutions, CO). Total RyR2 protein content was assessed in the same samples with anti-RyR2 antibodies (ThermoFisherScientific, MA) on a different blot and used as a control for loading. Protein bands were visualized using the Super Signal Pico Kit. Scans were quantified with Image J software (NIH).
Online Figure I. Recovery of SR-entrapped fluo-5N fluorescence from photo-bleaching is not different between control and VF myocytes.

Control and VF myocytes were loaded with fluo-5N. After permeabilization with saponine, 30 µmol/L ruthenium red was added to block ryanodine receptors activity. About 35% of myocyte area was bleached using 86% of laser power for 3.8 s. Fluo-5N fluorescence was acquired using laser power at 0.5%. Kinetics of fluo-5N fluorescence recovery after photo-bleaching (FRAP) were analyzed and compared in control and VF myocytes. A, Representative images of control and VF myocytes are shown before bleaching (a), just after bleaching (b), and at time point when recovery reached steady-state level (c). Bleaching areas are shown in red. B, Average time-course of fluo-5N fluorescence recorded during FRAP experiments in nine control and eleven VF myocytes. Arrows indicate beginning of bleaching period. FRAP data were fitted by monoexponential functions (shown in red).
Online Figure II. Protein level of calsequestrin (CSQ2) is not altered in VF. A, Representative Western blots showing levels of CSQ2 and GAPDH in control and in VF hearts. CSQ2 antibody was from ABR Affinity BioReagents, CO and GAPDH antibody was from Abcam, MA. B, Graph demonstrates that average levels of CSQ2 normalized to GAPDH recorded in four control and four VF samples are not significantly different (P>0.3).
Online Figure III. Reduced [Ca2+]SR is associated with increase in time delay between SR Ca2+ depletion and SCW initiation in control cells.

Control myocytes were loaded with fluo-5N. Different levels of SR Ca2+ loading were achieved by stimulating cells at 1 and 0.2 Hz. A, Stimulation protocol and corresponding time-courses of fluo-5N fluorescence. Average end-diastolic levels of fluo-5N fluorescence (B), average time to SCW (C), and SCW incidence during 20s time period after the last stimulus (D) were obtained for control myocytes (n=11) field-stimulated at 1 and 0.2 Hz.
Online Figure IV. L-type Ca current (I_{Ca}) recovery from inactivation is not different in control and VF myocytes.

A, Schematic voltage protocol and corresponding traces of I_{Ca} in control and VF myocytes. B, Average data showing kinetics of recovery of the amplitude of I_{Ca} were obtained from eight control and seven VF myocytes. Data were fit to two exponentials with constants of 85±37 and 576±44 ms in control, 98±21 and 674±34 ms in VF. C and D, Kinetics of recovery from inactivation of the peak I_{Ca} and amplitude of Ca transients (CaT) are presented for control and VF myocytes.
Online Figure V. MPG, a reducing agent, normalizes RyR2 activity and increases free thiol content of RyR2s in VF hearts. 
Representative recordings (A) and summary data (B) of single RyR2 activity obtained from control (n=8), VF (n=7), and VF plus MPG (n=8) samples. Representative Coomassie blue-stained gels (upper panels) and corresponding monobromobimane (mBB) fluorescence intensity (lower panels) of RyRs from VF hearts perfused for 30 min with solution contained no MPG (reference) or 0.75 mmol/L MPG. Samples were treated with 0.2 mmol/L 2,2'-dithiodipyridine (DTDP) or 5 mmol/L dithiothreitol (DTT) to determine maximal changes produced by oxidation. D, Relative free thiol content of RyRs from reference vs. MPG samples obtained by normalizing mBB fluorescence to RyR amount determined using Coomassie Blue staining of the gels run in parallel. *P<0.05. Data were obtained from 3 samples for each condition.. *P<0.05 vs control, † P<0.05 vs VF.
Online Figure VI. Mercapto-propionyl-glycine (MPG) does not affect intracellular Ca\textsuperscript{2+} transient in control myocytes.

Control myocytes were loaded with Rhod-2 Ca\textsuperscript{2+}-sensitive dye.

A. Time-course of Rhod-2 fluorescence recorded during 0.3 hz field stimulation in the presence 100 nmol/L isoproterenol (Iso) in untreated and MPG-treated (0.75 mmol/L, 30 min) control myocytes. B. Average data obtained for the amplitude of Ca transients from twenty eight untreated and twenty four MPG-treated control myocytes.
Online Figure VII. Reactive-oxygen species (ROS) production rate is not affected by beta-adrenergic stimulation in control myocytes.

Control myocytes were loaded with CFDA, a ROS-sensitive dye. A, Time-course of CFDA fluorescence and CFDA fluorescence rate recorded in control myocyte in the absence and in the presence of 100 nmol/L isoproterenol (Iso). Cell was electrically stimulated at 0.3 Hz. Data were acquired every 16 s. At the end of experiment 10 mmol/L H$_2$O$_2$ was applied. CFDA fluorescence was normalized to the maximal value recorded in the presence of H$_2$O$_2$. B, Average data obtained from five control myocytes. CFDA fluorescence recorded just before and at 10th min of Iso application was used in analyses.
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