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The role of spatial organization of Ca^{2+} release sites in the generation of arrhythmogenic diastolic Ca^{2+} release in myocytes from failing hearts

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Abstract

In heart failure (HF), dysregulated cardiac ryanodine receptors (RyR2) contribute to the generation of diastolic Ca^{2+} waves (DCWs), thereby predisposing adrenergically stressed failing hearts to life-threatening arrhythmias. However, the specific cellular, subcellular, and molecular defects that account for cardiac arrhythmia in HF remain to be elucidated. Patch-clamp techniques and confocal Ca^{2+} imaging were applied to study spatially defined Ca^{2+} handling in ventricular myocytes isolated from normal (control) and failing canine hearts. Based on their activation time upon electrical stimulation, Ca^{2+} release sites were categorized as coupled, located in close proximity to the sarcolemmal Ca^{2+} channels, and uncoupled, the Ca^{2+} channel-free non-junctional Ca^{2+} release units. In control myocytes, stimulation of β -adrenergic receptors with isoproterenol (Iso) resulted in a preferential increase in Ca^{2+} spark rate at uncoupled sites. This site-specific effect of Iso was eliminated by the phosphatase inhibitor okadaic acid, which caused similar facilitation of Ca^{2+} sparks at coupled and uncoupled sites. Iso-challenged HF myocytes exhibited increased predisposition to DCWs compared to control myocytes. In addition, the overall frequency of Ca^{2+} sparks was increased in HF cells due to preferential stimulation of coupled sites. Furthermore, coupled sites exhibited accelerated recovery from functional refractoriness in

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal procedures were approved and conducted in accordance with the guidelines issued by Institutional Animal Care and Use Committee of the Ohio State University, in compliance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The manuscript does not contain human data.

HF myocytes compared to control myocytes. Spatially resolved subcellular Ca^{2+} mapping revealed that DCWs predominantly originated from coupled sites. Inhibition of $\text{CaMK}\Pi$ suppressed DCWs and prevented preferential stimulation of coupled sites in Iso-challenged HF myocytes. These results suggest that $\text{CaMK}\Pi$ -(and phosphatase)-dependent dysregulation of junctional Ca^{2+} release sites contributes to Ca^{2+} -dependent arrhythmogenesis in HF.

Keywords

Ryanodine receptor; Ca^{2+} waves; Arrhythmia; Microdomains; Refractoriness; Ventricular myocyte

Introduction

In ventricular myocytes, most of the ryanodine receptors (RyR2s), sarcoplasmic reticulum (SR) Ca^{2+} release channels, are organized in clusters located in immediate proximity to sarcolemmal Ca^{2+} channels. These junctional RyR2 clusters comprise coupled Ca^{2+} release sites that initiate Ca^{2+} -induced Ca^{2+} release during the systolic action potential (AP) [21, 40, 43]. Structural and functional data indicate that there is a fraction of extra-junctional RyR2s that do not co-localize (couple) with the sarcolemmal Ca^{2+} channels [4, 14, 19, 40, 44, 46]. The non-junctional RyR2 clusters form (uncoupled/non-coupled) Ca^{2+} release sites and appear to play a secondary role by amplifying primary Ca^{2+} release. These populations of distinct Ca^{2+} release sites are exposed to different local ionic and signaling environments, including the $\text{Na}^+/\text{Ca}^{2+}$ “fuzzy space”, domain-specific $\text{CaMK}\Pi$ and reactive oxygen species signaling, and potential gradients in $[\text{Ca}^{2+}]_{\text{SR}}$ [1, 13, 18, 23, 47, 50]. As a consequence, the intrinsic properties and physiological modulation of coupled and uncoupled sites might be different. Indeed, recent studies have suggested that coupled sites are selectively influenced by $\text{CaMK}\Pi$ localized in the dyadic cleft [19, 20, 33].

Heart failure (HF) is a leading cause of death that occurs either as a result of pump failure or malignant arrhythmia [29, 49]. Although various mechanisms can underlie disrupted Ca^{2+} homeostasis in HF [39, 51, 52], dysfunction of RyR2s has consistently been reported in both animal models of HF and in human HF [8, 52]. It was shown that in HF, RyR2s become abnormally active, or ‘leaky’, owing to an accumulation of posttranslational modifications due to phosphorylation and oxidation [8, 10, 11, 45, 48, 52]. This dysregulated RyR2 activity has the potential to decrease systolic contraction while giving rise to diastolic Ca^{2+} waves (DCWs) and delayed after-depolarizations, precursors of triggered arrhythmias. Mechanistically, DCWs in HF myocytes were linked to accelerated recovery from a state of functional quiescence, i.e., refractoriness, that RyR2s normally enter following each systolic Ca^{2+} release [10, 17, 35]. Furthermore, shortened refractoriness in HF was attributed to abnormal $\text{CaMK}\Pi$ phosphorylation and oxidation of RyR2 [8, 10]. However, presently, very little is known about the contribution of anatomically and functionally distinct release sites in myocyte Ca^{2+} handling and the role of subdomain-specific Ca^{2+} signaling in arrhythmogenesis.

The objective of the present study was to define the subcellular determinants of arrhythmogenic DCWs by quantifying functional differences in Ca^{2+} signaling from

anatomically distinct sites (coupled vs. non-coupled) and their relative roles in the genesis of DCWs. Using a canine model of tachypacing-induced chronic HF, we show that excessive activation of coupled SR Ca^{2+} release sites underlies increased susceptibility of failing myocytes to arrhythmogenic DCWs.

Materials and methods

An expanded Methods section can be found in the Electronic Supplementary Material.

Canine model of heart failure

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were approved by Institutional Animal Care and Use Committee of the Ohio State University. Ventricular dysfunction was induced by right ventricular tachypacing, as described previously [10, 30]. Briefly, adult hound dogs (17–31 kg) of either sex were chronically instrumented with modified pacemakers (St. Jude Medical, MN) with the pacing lead (St. Jude Medical, MN) placed in the right ventricle apex. Tachypacing was performed at: 180 bpm for 2 weeks, 200 bpm for 6 weeks, and 180 bpm thereafter. This tachypacing protocol invariably induced HF evidenced by elevated concentration of brain natriuretic peptide, LV dysfunction, and functional impairment [10, 30] (electronic supplementary material, Table 1). Cellular studies were performed following 16 weeks of tachypacing unless otherwise stated.

Ventricular myocyte isolation

The dogs were anesthetized with pentobarbital sodium (50 mg/kg intravenously; Nembutal, Abbott Laboratories, IL), and the heart was rapidly removed and perfused with ice-cold cardioplegic solution containing the following (mM): NaCl 110, CaCl_2 1.2, KCl 16, MgCl_2 16, and NaHCO_3 10. Left circumflex artery was cannulated and used to perfuse both the left atria and left ventricle. Right atria and right ventricle were removed from the preparation. The heart was perfused for 10 min with a perfusion buffer containing (mM) NaCl 130, KCl 5.4, MgCl_2 3.5, NaH_2PO_4 0.5, Glucose 10, HEPES 5, and taurine 20 supplemented with 0.1 mM EGTA; this was followed by heart perfusion with the perfusion buffer containing 0.3 mM Ca^{2+} , 0.12 mg/ml of soybean trypsin inhibitor (Thermo Fisher Scientific, MA), and 1.33 mg/ml of type I collagenase (lot number 44C14804B, activity 265 U/mg, Worthington Biochemical Corp, NJ) for 30 min. Following enzymatic digestion, mid-myocardial section of the left ventricle was dissected from the heart and placed in shaking water bath at 37° C for additional 10 min. Single myocytes were obtained by filtering through nylon mesh followed 2–3 cycles of gravity sedimentation ($1\times g$ for 10 min). Cells were resuspended in the low Ca^{2+} (0.4 mM CaCl_2) perfusion buffer supplemented with 1% bovine serum albumin and plated on 12 mm diameter glass coverslips (CS-12R, Warner Instruments, CT) coated with mouse laminin. Myocytes were stored either at room temperature or at +4° C.

Ca^{2+} imaging

Cellular experiments were performed using an RC-25 open bath imaging chamber (Warner Instruments, CT) mounted on PM-6 magnetic platform (Warner Instruments, CT). The

chamber was continuously perfused with an external solution containing (in mM): 140 NaCl, 5.4 KCl, 2.0 CaCl₂, 0.5 MgCl₂, 5.6 glucose, and 10 HEPES (pH 7.4). In intact myocytes, Ca²⁺ transients were elicited by field stimulation through the pair of platinum electrodes using 4 ms square voltage pulses generated by SD9 stimulator (Grass Technologies/Astro-Med Inc, RI). Ca²⁺ dyes were loaded by incubating myocytes in a low Ca²⁺ (0.4 mM CaCl₂) external solution containing either 9 μM Fluo-3 AM (Molecular Probes/Thermo Fisher Scientific, MA) or 8 μM Rhod-4 AM (AAT Bioquest, CA) at room temperature for 20–30 min; following dye washout, 20–40 min was allowed for de-esterification of AM esters. In patch-clamped myocytes, Ca²⁺ transients were induced by a series (10–25) of voltage stimuli shaped as a typical control AP. Whole-cell patch-clamp configuration was established using an Axopatch 200B amplifier coupled to Digi-data1322A data acquisition system (Axon Instruments Inc./Molecular Devices, CA). Patch pipettes were filled with the following solution (mM): 90 K-aspartate, 50 KCl, 5 MgATP, 5 NaCl, 1 MgCl₂, 0.1 Tris GTP, 10 HEPES, and 0.1 Rhod-2 tri-potassium salt (Molecular Probes/Thermo Fisher Scientific, MA); pH 7.2. To inhibit CaMKII activity, myocytes were treated with the low Ca²⁺ external solution containing 1 μM KN-93 at room temperature for 30–50 min. Such treatment did not significantly affected Ca²⁺ current density recorded in control myocytes (Figure S5, electronic supplementary material). Intracellular Ca²⁺ imaging was performed using Olympus FluoView FV 1000 (Olympus America Inc., PA) and Nikon AIR (Nikon Instruments Inc., NY) confocal microscope systems, each equipped with 60x oil-immersion objective lens (NA 1.4). Rhod dyes were excited with 561 nm laser and fluorescence was collected at 570–620 nm wavelengths. Fluo-3 was excited with 488 nm line of argon laser and signal was collected at 500–600 nm wavelengths. In the line-scanning mode, images were acquired along the central axis of the myocytes at a speed of ~ 2 ms per line with pixel size ranging from 0.08 to 0.41 μm. In resonant scanning mode, images were recorded at the myocyte midsection at ~110 frames per second with pixel size of 0.27–0.41 μm. Fluorescence signals were normalized to the baseline cellular fluorescence (F₀).

Data analysis

To classify image pixels into coupled/non-coupled Ca²⁺ release groups, median time to 30% of amplitude of spatially averaged Ca²⁺ transients (T₃₀) was determined and pixels with the response time less than T₃₀ were assigned into early response (coupled) group (e.g. Figure 1, blue rectangles); image regions with response time longer than T₃₀ were categorized into the delayed-response (uncoupled) group (Fig. 1, red rectangles). If line-scan image region categorized into coupled or uncoupled group had widths of less than half of the sarcomere length (<0.8 μm), it was removed from the corresponding group and marked with the gray rectangles, as illustrated in Fig. 1. Image pixels with a response time equal to T₃₀ were considered as “intermediate” and also marked with the gray rectangles (Fig. 1).

Diastolic Ca²⁺ sparks were detected using the variance stabilization and local baseline subtraction approach described by Bankhead et al. [6]. Briefly, the square root transform was applied to the images for variance stabilization followed by a sequence of image smoothing performed using cubic B-spline filter with 2⁽ⁿ⁻¹⁾—1 zeros inserted between filter coefficients at each smoothing iteration (*n* is a number of smoothing iterations). Baseline (Ca²⁺ transient) signal was approximated using five smoothing iterations; two smoothing

iterations were used for the Ca^{2+} spark images. Following baseline signal subtraction, the Ca^{2+} spark images were thresholded using threshold of five standard deviations. End-diastolic Ca^{2+} spark properties were analyzed using Ca^{2+} sparks detected during last 600 ms of diastolic interval (cycle length 2000 ms).

A similar approach was used to detect Ca^{2+} waves: six and two smoothing iterations were used for baseline and Ca^{2+} wave images, respectively. The Ca^{2+} wave images were thresholded using a 3.8 standard deviation threshold and reconstructed to include image pixels with signal >mean plus two standard deviations. *K*-mean clustering of the areas of detected objects was used to separate the Ca^{2+} waves from the Ca^{2+} sparks.

Images were analyzed using MATLAB (2014b, The MathWorks, Inc., MA). An original code for Ca^{2+} sparks detection was obtained from Bankhead et al. [6]. Aggregated data were analyzed using R software environment (R Foundation for Statistical Computing, <http://www.R-project.org>). Results are expressed as the mean $\pm$ SEM. Statistical significance between two groups was defined by Student's *t* test *p* values of <0.05.

All materials were obtained from Sigma-Aldrich (St. Lois, MI, USA) unless specified otherwise.

Results

Differential regulation of coupled and uncoupled Ca^{2+} release sites by β -adrenergic receptor stimulation in control myocytes

Based on activation time following electrical stimulation, Ca^{2+} release sites in cardiac myocytes can be categorized as early and delayed, corresponding to L-type Ca^{2+} channel-coupled and uncoupled sites, respectively [19, 20, 41] (see Materials and Methods, and Figure S1, electronic supplementary material). We examined the properties of diastolic Ca^{2+} sparks originated at coupled and uncoupled sites in AP-stimulated cardiac myocytes (at 0.5 Hz) under baseline conditions and in the presence of the β -adrenergic receptor (β -AR) agonist, isoproterenol (Iso). As shown in Fig. 1a, the frequency of Ca^{2+} sparks originating from coupled sites was not different from the frequency of sparks originated from uncoupled sites in the absence of Iso. Treatment of myocytes with Iso (100 nM) resulted in a disproportional increase in the frequency of uncoupled site compared to coupled sites (Fig. 1b). Of note, uncoupled site spark frequency also had a markedly broadened Iso dose-dependency compared to that of coupled sites (Fig. 2). For example, Iso at 10 nM produced no further stimulation of coupled site sparks but increased uncoupled site frequency by approximately threefold (Fig. 2b). At the same time, coupled and uncoupled site sparks exhibited similar amplitudes and spatio-temporal properties (Table 1).

SR Ca^{2+} content was measured in AP-stimulated myocytes at different Iso concentrations by rapid application of 15 mM caffeine (Fig. 2c). Notably, the SR Ca^{2+} content reached maximum at 3 nM Iso and did not further increase at higher Iso concentrations. Taken together, these data suggest that the preferential stimulation of uncoupled sites by Iso is attributable to site-specific modulation of RyR2s, independent of SR Ca^{2+} content.

To assess the role of protein phosphatases in site-specific regulation of Ca^{2+} release by β -AR stimulation, we examined the effect of Iso in the presence of the protein phosphatase inhibitor okadaic acid (OA, 1.2 μM). OA increased the frequency of sparks compared to the effects of Iso alone (Fig. 3). Interestingly, OA exerted more pronounced effects on coupled Ca^{2+} release sites (108 vs. 52% increase in the activity uncoupled sites), indicating that the activity of coupled Ca^{2+} release sites is tightly regulated by protein phosphatases in control myocytes.

Increased Ca^{2+} spark activity at coupled Ca^{2+} release sites in HF myocytes

HF is associated with increased frequency of Ca^{2+} sparks and Ca^{2+} waves that become particularly pronounced and promote arrhythmogenesis during β -AR stimulation of failing myocytes [8, 32]. Consistent with the previous reports [7, 10, 45], Ca^{2+} spark frequency was significantly increased in HF myocytes compared to control at baseline (i.e., the absence of Iso; Figure S4 electronic supplementary material). To probe the subcellular mechanism of arrhythmogenesis in adrenergically stressed failing hearts, we examined the contributions of coupled (early) vs. uncoupled (delayed) sites to HF-dependent alterations in Ca^{2+} handling in Iso-challenged HF myocytes with the same protocol that we used for control myocytes above. In agreement with the previous reports [10, 26], the propensity for Ca^{2+} waves and the overall Ca^{2+} spark frequency were significantly higher in HF myocytes than in control cells (Fig. 4a). This increase in overall Ca^{2+} spark frequency in HF myocytes was attributable to an increased frequency of coupled sites (Fig. 4b). Whereas the frequency of uncoupled sites was not different between HF and control myocytes, coupled site spark frequency increased ~ twofold to a level similar to that for uncoupled sites in both HF and control cells. These differences in frequency were associated with no significant differences in most of the spatio-temporal properties of Ca^{2+} sparks (Table 1). Of note, in the absence of Iso end-diastolic Ca^{2+} spark frequencies under AP-clamp pacing were not different between control and HF myocytes (data not shown).

Accelerated recovery from refractoriness of Ca^{2+} release in HF myocytes

We previously demonstrated that shortened RyR2 refractoriness facilitates synchronization of aberrant diastolic Ca^{2+} release and promotes arrhythmogenesis in failing cardiomyocytes [8, 10, 15]. To evaluate the contributions of coupled and uncoupled sites to this aberration, we measured site-specific restitution of SR Ca^{2+} release using a standard two-pulse protocol. Consistent with our previous findings [8], restitution of globally measured SR Ca^{2+} release ($dF/dt \text{ max}$) occurred significantly faster in HF myocytes than in control cells (Fig. 5). Functional recovery of coupled sites occurred manifestly faster in HF myocytes than in control cells (Fig. 5b). At uncoupled sites, Ca^{2+} release showed no signs of refractoriness even at the shortest time intervals examined in HF or control cells and no significant differences in the functional recovery were revealed at these sites between the groups. Thus, accelerated recovery of global SR Ca^{2+} release in HF myocytes could be attributed predominantly to shortened refractoriness of coupled Ca^{2+} release sites. Considered with the results on site-specific alterations in Ca^{2+} spark frequency (Fig. 5), these findings suggest that HF is associated with preferential facilitation of coupled sites as compared to uncoupled sites.

Preferential regulation of coupled Ca^{2+} release sites by CaMKII

Enhanced functional activity of Ca^{2+} release in HF settings, including this model of canine HF, has previously been attributed to excessive phosphorylation of RyR2 by CaMKII [2, 10, 31, 38]. To assess the role of CaMKII in site-specific regulation of Ca^{2+} activity, we utilized the CaMKII inhibitor KN-93 (1 μM). Consistent with our previous results [10], this treatment significantly reduced the propensity to DCWs in field-stimulated HF myocytes in the presence of Iso (Fig. 6a, b). Moreover, inhibition of CaMKII also resulted, on average, in a twofold decrease in the frequency of Ca^{2+} spark recorded in HF myocytes (Fig. 6c). As illustrated in Fig. 6d, inhibition of CaMKII in HF myocytes preferentially suppressed Ca^{2+} sparks at the coupled release sites. In agreement with the results of field stimulation experiments described above, KN-93 also preferentially suppressed the activity of coupled Ca^{2+} release sites in HF myocytes stimulated by a standard AP waveform under AP-clamp (Figure S6 electronic supplementary material). Further support for the critical role of CaMKII in site-specific regulation of Ca^{2+} release was obtained in experiments utilizing the selective auto-camtide-3 derived inhibitory peptide (AC3) [3]. In these experiments, the addition of AC3 to the pipette solution significantly suppressed the frequency of DCWs and preferentially inhibited the activity of coupled Ca^{2+} release sites in AP-clamped HF myocytes (Figure S7 electronic supplementary material).

If increased coupled site activity in HF cells is, indeed, due to preferential modulation by CaMKII, then it could be possible to mimic the HF phenotype by local stimulation of CaMKII at coupled sites in control cells. Therefore, we used the L-type Ca channel agonist Bay K8644 to stimulate CaMKII at coupled sites [22]. As demonstrated in Figure S8 (electronic supplementary material), BayK8644 applied in the presence of Iso caused a significant increase in Ca^{2+} influx associated with a noticeable reduction in Ca^{2+} current inactivation in AP-clamped control myocytes. At the same time, BayK8644 significantly increased the frequency of DCWs in paced control myocytes (Figure S9 A–C electronic supplementary material). Analysis of diastolic Ca^{2+} sparks showed that Bay K8644 predominantly increased the activity of coupled Ca^{2+} release sites (Figure S9 D electronic supplementary material). Taken together, these data support the idea that CaMKII mediates its pro-arrhythmic effects in HF myocytes by selectively affecting RyR2 properties at the early Ca^{2+} release sites.

L-type Ca^{2+} channels as potential targets for CaMKII

L-type Ca^{2+} channels are known as potential targets for CaMKII phosphorylation which, in general, has been shown to increase channel activity [12]. Increased Ca^{2+} entry into junctional domains could stimulate coupled Ca^{2+} release sites, thus contributing to the observed CaMKII-dependent alterations in Ca^{2+} release in HF myocytes. To assess whether this potential mechanism contributed to the remodeling of intracellular Ca^{2+} signaling in HF, we examined the functional characteristics and phosphorylation status of the L-type Ca^{2+} channels in HF vs. control myocytes. As summarized in Table S2 (electronic supplementary material), peak Ca^{2+} current in HF myocyte was either decreased (at the baseline) or unchanged (in the presence of 100 nM Iso) when compared to the currents in control myocytes. In addition, inactivation of the Ca^{2+} currents was not significantly different between control and HF myocytes. These functional effects are not consistent with increased

CaMK Π -dependent phosphorylation of the L-type Ca $^{2+}$ channels in HF myocytes. We also performed Western blot analysis of phosphorylation statuses of Cav1.2 α 1C and β 2 subunits extracted from the membrane fractions of control and HF samples. Phosphorylation levels of these subunits of L-type Ca $^{2+}$ channels were not significantly different in control and HF (Figure S10, electronic supplementary material). Thus, our data do not support a critical role for CaMK Π -dependent phosphorylation of the L-type Ca $^{2+}$ channels in the generation of arrhythmogenic Ca $^{2+}$ waves in HF myocytes.

The role of coupled sites in initiation of DCWs in HF myocytes

To directly examine the role of coupled and uncoupled sites in the generation of arrhythmogenic Ca $^{2+}$ waves in HF myocytes, we mapped Ca $^{2+}$ wave initiation using fast 2D confocal Ca $^{2+}$ imaging. First, we categorized Ca $^{2+}$ release sites as coupled and uncoupled according to their activation time on AP stimulation (Fig. 7a). Then, we visualized initiation and spread of DCWs. Having assigned recorded Ca $^{2+}$ wave initiation sites into 'in focus' and 'out-of-focus' groups (see electronic supplementary materials, Methods and Fig. 2), we overlaid 'in focus' DCW initiation sites with the 'onset map' (Fig. 7b, f). As illustrated in Fig. 7c DCW initiation sites for the Ca $^{2+}$ wave shown in panel 7B coincided with the early Ca $^{2+}$ release sites (onset time of 20–40 ms). On average, 45% (10 out of 22) of recorded initiation sites of Ca $^{2+}$ waves were assigned to out-of focus group. Analysis of the 'in focus' initiation sites showed that the majority of pixels comprising the initial area Ca $^{2+}$ wave corresponded to coupled Ca $^{2+}$ release sites (Fig. 7d). These data suggest that coupled Ca $^{2+}$ release sites underlie the initiation of DCWs in HF myocytes.

Discussion

In the present study, we investigated the role of subcellular compartmentalization of Ca $^{2+}$ signaling in catecholamine-dependent arrhythmogenesis in failing canine cardiac myocytes. In particular, we used a well-characterized canine model of tachypacing-induced HF to determine how chronic HF affects diastolic activities of sarcolemmal Ca $^{2+}$ channel-coupled and -uncoupled Ca $^{2+}$ release sites in adrenergically challenged cardiomyocytes prone to arrhythmogenic DCWs. Our main findings are as follows: (1) upon β -AR stimulation of control cells with Iso uncoupled sites exhibited higher diastolic activity (measured as frequency of topographically mapped Ca $^{2+}$ sparks) than coupled sites, and this site-specific modulation was lost in HF cells that presented indiscriminate facilitation of both coupled and uncoupled sites. (2) The shortened overall Ca $^{2+}$ release refractoriness underlying the increased arrhythmogenic potential of HF cells was specifically attributable to accelerated functional restitution of coupled sites; (3) initiation of DCWs in HF cells was mapped preferentially to coupled sites; and (4) the site-specific effects of Iso in control and HF myocytes depended on protein phosphatase and CaMK Π activities. These findings provide new insights into subcellular and molecular mechanisms of cardiac arrhythmias in the failing heart.

Coupled and uncoupled RyR2 clusters

In cardiac myocytes, Ca $^{2+}$ handling shows substantial spatial heterogeneity [37,42]. RyR2s are typically grouped in release units composed on average of 14–63 channels [5, 25, 40].

While most of SR Ca^{2+} release units face sarcolemmal Ca^{2+} channels in the juxtaposed t-tubule membrane, thus forming couplons [21, 40, 43], a certain fraction of release units is not associated with the Ca^{2+} channels [4, 14, 24, 27, 28, 44, 46]. Coupled and uncoupled RyR2s can functionally be distinguished by different response times (i.e., fast and delayed, respectively) during AP activation [19, 20, 24, 27, 28]. They can be further quantified by correlating Ca^{2+} release rise times with distances to the nearest t-tubule membrane stained with a membrane dye [19, 24]. Consistent with previous reports [16, 19, 24], we found a significant fraction of uncoupled sites in canine myocytes examined in this study, thus revealing a substantial contribution of non-junctional sites to the generation of the Ca^{2+} transient. Notably, in HF myocytes, this fraction was not significantly altered (Figure S2, electronic supplementary material).

The role of release site heterogeneity in the generation of arrhythmogenic Ca^{2+} release in HF

HF is associated with increased diastolic activity of RyR2s (i.e., “leak”) secondary to their increased phosphorylation and oxidation [8, 11]. Using the same canine model of HF that was employed here, we previously demonstrated that changes in RyR2 function translate into impaired Ca^{2+} signaling refractoriness that accounts for increased predisposition to arrhythmogenic DCWs in paced HF myocytes [10]. Normally, refractoriness prevents CICR from spontaneous activation during the diastolic period. When refractoriness is impaired (shortened), Ca^{2+} sparks that arise synchronously throughout the cell upon reloading of the SR give rise to DCWs. Here, we specifically examined the role of coupled and uncoupled RyR2s in generation of DCWs. We found that aberrant Ca^{2+} release arises preferentially from fast, i.e., coupled, Ca^{2+} release sites. This conclusion is based on the following evidence: first, the increase in overall release site activity (sparks) in HF cells was entirely attributable to increased spark frequency at fast sites (Fig. 4); two-pulse restitution experiments demonstrated that the shortened overall release refractoriness associated with increased arrhythmogenic potential of HF cells can be ascribed to shortened refractoriness of coupled sites (Fig. 5); and finally, mapping of subcellular Ca^{2+} directly demonstrated the predominant role of fast sites in the genesis of DCWs (Fig. 7). Thus, these experiments show for the first time that arrhythmogenic diastolic Ca^{2+} release in failing myocytes arises from RyR2s localized in couplons adjacent to sarcolemmal Ca^{2+} channels. In line with these results, Dries et al. [20] recently showed that in normal pig myocytes challenged by Iso, Ca^{2+} waves tend to arise at coupled sites.

Given the similar functional activities of coupled and uncoupled sites in HF myocytes in this study (Fig. 4), it is not obvious why DCWs arise preferentially at coupled sites. The observed site specificity of Ca^{2+} wave generation could be ascribed to particulars of subcellular architecture as well as the distribution of Ca^{2+} buffers and Ca^{2+} transport systems including SERCA at the coupled vs. uncoupled sites. For example, the localization of coupled sites in close proximity to the T-tubule membrane, but farther away from sites of re-uptake, could facilitate lateral spread of Ca^{2+} and the cross activation of neighboring sites required for the transition of localized release to a propagating Ca^{2+} wave. Further studies including modeling with accurate representation of the spatial organization of Ca^{2+} release

are needed to delineate the factors accounting for the specific role of coupled site in DCW generation.

Mechanism of site-specific modulation of RyR2s in normal and HF myocytes

β -AR stimulation is a critical factor in the initiation of Ca^{2+} -dependent arrhythmias. In general, it is thought that β -AR stimulation causes arrhythmia by increasing the SR Ca^{2+} load (via PKA-dependent phosphorylation of phospholamban) and facilitating RyR2 phosphorylation by PKA and CaMK Π . However, the specific subcellular and molecular mechanisms remain to be clarified. A key finding of this study is that while in control myocytes, Iso resulted in preferential stimulation of uncoupled sites, in HF cells, Iso increased spark frequency at both coupled and uncoupled sites. Importantly, inhibiting protein phosphatase activity altered the preferential Iso-dependent stimulation of uncoupled sites in control myocytes leading to an indiscriminate facilitation of coupled and uncoupled sites (Fig. 3). Thus, the site-specific modulation of RyR2 observed in normal (but not HF) cells could be attributed to elevated protein phosphatase activity at junctional sites. This site-specific modulation is likely to involve direct effects on RyR2 through changing its phosphorylation status rather than indirect effects via phospholamban/SERCA-dependent changes in SR cisternae Ca^{2+} content (Fig. 2c). This control mechanism is evidently lost in HF myocytes that exhibit Iso-dependent facilitation of activities of both coupled and uncoupled sites. Consistent with this notion, binding of protein phosphatases (PP1 and PP2A) to RyR2 is reportedly decreased in failing hearts [2, 9, 36]. In further support of the role of altered compartmentalization of RyR2 phosphorylation mechanisms in HF, the β -AR-mediated increase in coupled site activity in HF myocytes was sensitive to inhibition of CaMK Π (Fig. 6). Previously, we and others showed that Ca^{2+} -dependent arrhythmogenesis was associated with increased CaMK Π phosphorylation of RyR2s [2, 10, 31, 38]. Our present results show for the first time that arrhythmogenesis in HF myocytes is specifically attributable to CaMK Π -dependent facilitation of coupled sites.

Besides phosphorylation of RyR2, the observed site-specific effects of CaMK Π on SR Ca^{2+} release in HF myocytes could involve phosphorylation of the L-type Ca^{2+} channels [12]. However, we found no functional or biochemical evidence for enhanced CaMK Π -dependent phosphorylation of Ca^{2+} channels in HF (Table S2 and Figure S10 electronic supplementary material). Therefore, this pathway is unlikely to play a significant role in mediating local pro-arrhythmic remodeling of Ca^{2+} signaling in the HF model employed in the current study.

As another interesting possibility, we recently showed that local $\text{Na}^+/\text{Ca}^{2+}$ signaling involving neuronal Na^+ channel plays an important role in the genesis of Ca^{2+} -dependent arrhythmia [34]. Stimulation of neuronal Na^+ channels by CaMK Π results in increased Na^+ influx and hence increased Ca^{2+} level in the junctional space. Thus, CaMK Π -dependent activation of neuronal Na^+ channel could account for increased arrhythmogenic potential of coupled sites in the settings of leaky RyR2s in HF. Further studies are required to define the specific molecular factors involved in differential regulation of coupled and uncoupled sites in normal and diseased hearts.

Comparison with previous results

Previously, Dries et al. [19] showed selective modulation (activation) of coupled sites in porcine myocytes, which was dependent on CaMKII and was lost following infarction. While being in apparent agreement about the role of coupled sites in arrhythmogenesis, our results and those of Dries et al. seem to have some important differences. In our study, β -AR stimulation selectively increased the activity of uncoupled as opposed to coupled sites in control myocytes; HF led to increased activity of coupled sites. Thus, it appears that in our study, the normal regulation of coupled sites is negative rather than positive (as in Dries et al.), such that loss of this inhibitory influence in HF results in increased activity of coupled sites and enhanced arrhythmogenesis. The specific reasons behind these divergent results are not clear. They could involve species differences as well as differences in experimental conditions, including differences in pacing rate. Consistent with the latter possibility, in porcine myocytes, spark rate at coupled vs. uncoupled sites was not different at slow pacing rates [19]. Further experiments are needed to explain these differences and define the specific mechanisms of compartmentalized regulation of RyR2s in normal and diseased hearts.

Supplementary Material

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Fig. 1. Site-specific regulation of Ca^{2+} release by β -adrenergic receptor (β -AR) stimulation. **a** and **b**: **a** Representative traces of 'typical' control action potentials (APs) used as a voltage command and line-scan images with corresponding profiles of Ca^{2+} transients recorded in control myocyte under baseline (**a**, $n = 14$) and in the presence of 100 nM isoproterenol (Iso, **b**, $n = 18$), a β -AR agonist. **b** Line-scan images corresponding to the part of the image marked with yellow box in panel a illustrate work-flow of data analysis. Time to 30% of the spatially averaged amplitude of Ca^{2+} transient was detected (white line) and pixels with the response time less than median time to 30% of the amplitude (T30, red dashed line) were assigned into early (coupled) group (blue rectangles). Image regions reaching 30% of the amplitude with the response time more than T30 were categorized into delayed (uncoupled) group (red rectangles). The remaining pixels marked with gray rectangles (see "Materials and methods" for detailed description). This pixel classification was used to group end-diastolic Ca^{2+} sparks, **c** Average frequency of Ca^{2+} sparks observed in coupled and uncoupled regions

Fig. 2.

Sensitivity of Ca^{2+} sparks and myocyte SR Ca^{2+} content to Iso. The dose-dependent effects of Iso on the frequency of Ca^{2+} sparks recorded throughout the myocyte (**a**, all sparks) and recorded at coupled and uncoupled Ca^{2+} release sites (**b**). **c**, Iso effects on the SR Ca^{2+} content. Each data point represents data collected from 4 to 18 control myocytes

Fig. 3.

Phosphatase inhibitor eliminates site-specific effects of Iso. **a:** *a* representative traces of ‘typical’ control APs used as a voltage command and line-scan images with corresponding profiles of Ca^{2+} transients recorded in control myocyte in the presence of 100 nM Iso plus 1.2 μ M okadaic acid (OA), a protein phosphatase inhibitor, **a:** *b* Line-scan images corresponding to the part marked with yellow box of the image shown in *panel a*; the images illustrate the onset of Ca^{2+} transient, classification of the line-scan image regions into early and delayed, and end-diastolic Ca^{2+} activity. **b** Average frequency of Ca^{2+} sparks recorded throughout the myocytes, at the coupled and uncoupled regions in control myocytes in the presence of 100 nM Iso alone (data presented in Figs. 1 and 2) and in the presence of Iso plus OA ($n = 11$)

Fig. 4.

HF preferentially increases activity of the coupled Ca^{2+} release sites. **a:** *a*, Representative traces of ‘typical’ control APs used as a voltage command and line-scan images with corresponding profiles of Ca^{2+} transients recorded in HF myocyte in the presence of 100 nM Iso. **a:** *b*, Line-scan images corresponding to the part, marked with *yellow box*, of the image shown in *panel a*; the images illustrate the onset of Ca^{2+} transient, classification of the line-scan image regions into early and delayed, and end-diastolic Ca^{2+} activity. **b** Average frequency of Ca^{2+} sparks recorded throughout the myocytes, and at the coupled and uncoupled regions in control (**c**, $n = 18$) and heart failure (HF, $n = 10$) myocytes

Fig. 5.

HF speeds up recovery of coupled Ca^{2+} release sites. *a* Restitution of Ca^{2+} transient was measured in control and HF myocytes using two-pulse protocol. *Upper panels* show line-scan images of Rhod-2 fluorescence during Ca^{2+} release activation in response to the first depolarizing pulse (control pulse) and in response to the second pulse that occurred with 0.5, 0.8, and 1.2 s delay, respectively. Ca^{2+} release sites were classified as early (*blue rectangles*) and delayed (*red rectangles*) as described in Fig. 1. *Black traces* show restitution of spatially averaged Ca^{2+} transients, while blue and red traces illustrate restitution of Ca^{2+} release at coupled and uncoupled sites. *b* Time-course of Ca^{2+} release recovery recorded in control and HF myocytes was analyzed for all, coupled and uncoupled Ca^{2+} release sites and expressed as time-dependent restitution of gain function. Gain function for all and coupled release sites was calculated as the ratio of maximum rate of changes of fluorescence (maximum Ca^{2+} release flux) to the peak density of Ca^{2+} current; the gain for the uncoupled sites was calculated as the ratio of maximum Ca^{2+} release fluxes recorded at uncoupled and coupled sites, respectively. Data were obtained from 4 control and 5 HF myocytes, respectively. * $p < 0.05$

Fig. 6. CaMKII inhibition preferentially suppresses activity of coupled Ca^{2+} release sites in HF myocytes. **a** Line-scan images of HF myocyte periodically stimulated at 0.5 Hz in the presence of 1 μM Iso alone, and in the presence of 1 μM Iso plus 1 μM KN-93, a CaMKII inhibitor. *Upper trace* illustrates timing of field stimulation. **b** Average data for the effect of KN-93 on frequency of diastolic Ca^{2+} waves per diastolic interval (DI), and on the fraction of cells with the Ca^{2+} waves. Data were obtained from 19 (Iso 1 μM alone) and 20 (Iso + KN93) HF myocytes. **c** Line-scan images of HF myocyte periodically stimulated at 0.5 Hz in the presence of 10 nM Iso alone, and in the presence of 10 nM Iso plus 1 μM KN-93. *Left panels* illustrate classification of Ca^{2+} release sites into early (coupled, *blue rectangles*) and delayed (uncoupled, *red rectangles*). **d** Summary of the effects of KN-93 on frequency of

Ca²⁺ spark recorded from all, coupled and uncoupled Ca release sites. Data were obtained from 20 (Iso 10 nM alone) and 17 (Iso + KN93) HF myocytes

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Fig. 7.

Ca^{2+} waves in HF myocytes originate preferentially from coupled Ca^{2+} release sites. **a:** *a-c*, XY images of HF myocyte during onset of Ca^{2+} transient in response to electrical stimulation in the presence of 100 nM Iso. **a:** *d* Time-course of changes in spatially averaged Rhod-4 fluorescence. **a:** *e* Activation map of systolic Ca^{2+} release illustrates time to 30% of the amplitude of spatially averaged Ca^{2+} transient expressed for each pixel. Nuclear area was excluded from the analysis. **b:** *a-c* XY images of HF myocyte exemplify time-dependent evolution of a diastolic Ca^{2+} wave. **b:** *d* Myocyte map shows initiation and

propagation of the Ca^{2+} wave. Asterisk indicates site of Ca^{2+} wave initiation. **b: e** Image illustrates initial stages of Ca^{2+} wave propagation. *White area* indicates initiation site of the Ca^{2+} wave. The image corresponds to the white square ($15 \times 15 \mu\text{m}$) shown in panel **b: d**. Scale bars in **a** and **b** correspond to $10 \mu\text{m}$. **b: f** Juxtaposition of the initial site of the Ca^{2+} wave with the Ca^{2+} release activation map (*white square* in panel **a: e**). **c** Distribution of cell image pixels during onset of Ca^{2+} transient (for the cell shown in **a** and **b**). **d** Summary data for localization of the initiation sites of Ca^{2+} waves calculated for 9 HF myocytes

Table 1

Properties of end-diastolic Ca^{2+} sparks recorded in control and HF myocytes in the presence of 100 nM Iso

Parameter	All sparks		Coupled		Non-coupled	
	Control	HF	Control	HF	Control	HF
Frequency ($100 \mu\text{m}^{-1} \text{s}^{-1}$)	7.7 ± 1.2	$11.9 \pm 1.6^*$	5.6 ± 1.1	$9.7 \pm 1.8^*$	$9.2 \pm 1.2^{\&}$	13.2 ± 1.7
Peak (F/F_0)	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	$1.4 \pm 0.1^{\&}$
FWHM (μm)	3.3 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.2 ± 0.2	3.2 ± 0.1	$3.8 \pm 0.2^{*\&}$
FDHM (ms)	23 ± 1	23 ± 1	24 ± 1	20 ± 1	23 ± 1	$25 \pm 1^{\&}$
TTP (ms)	20 ± 1	19 ± 1	20 ± 1	20 ± 3	22 ± 2	19 ± 1
Max Rate ($\text{F}/\text{F}_0/\text{ms}$)	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.09 ± 0.01	0.09 ± 0.01

Data presented as mean $\pm$ sem. Data were collected from 18 control and 10 HF myocytes, respectively

Peak Ca^{2+} spark amplitude, *FWHM* half-width of Ca^{2+} spark, *FDHM* duration of Ca^{2+} spark at $1/2$ of amplitude, *TTP* time to peak of Ca^{2+} spark

* $p < 0.05$ vs. control,

& $p < 0.05$ vs. coupled