Figures and figure supplements

Ryanodine receptor dispersion disrupts Ca^{2+} release in failing cardiac myocytes

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Figure 1. dSTORM imaging enables quantification of RyR localization within Ca\(^{2+}\) release units (CRUs). RyR imaging was performed with antibody labelling of isolated and fixed rat ventricular cardiomyocytes. (A). Imaging of RyRs with confocal microscopy (left panel) or Structured Illumination Microscopy (SIM, centre panel) revealed a predominantly striated pattern of RyR localization across cells, but individual CRUs could not be discerned (magnified regions in lower panels). dSTORM imaging provided markedly improved spatial resolution enabling identification of RyR clusters (scale bars = 5 \(\mu\)m). (B). Quantification of RyR localization was performed by fitting raw images to a 30 \(\times\) 30 nm grid (Baddeley et al., 2009), and performing thresholding to create binary images; an RyR was counted as present if > half the area of a 30 nm square was suprathreshold. CRUs were defined as collections of RyR clusters with an edge-to-edge distance < 150 nm (Macquaide et al., 2015) (red boundaries) or < 100 nm (Baddeley et al., 2009; Hou et al., 2015). (Scale bar = 2 \(\mu\)m). DOI: https://doi.org/10.7554/eLife.39427.003
Figure 2. RyRs are dispersed in failing cardiomyocytes. Alterations in nanoscale RyR organization were examined in cardiomyocytes from rats with post-infarction heart failure (HF). Representative images show that macroscale

Figure 2 continued on next page
**Figure 2 continued**

Organization of RyRs was similar in HF and Sham-operated controls (A, upper panels). However, nanoscale examination revealed that RyR clusters were broken apart in HF. For the magnified regions in (A), conversion from raw dSTORM to binary images is shown in the middle and lower panels (saturation levels indicated by high-low look-up table). Mean measurements showed fewer RyRs per cluster in failing cells, with an increased fraction of small clusters (B). Dispersion of RyR clusters into smaller fragments resulted in an increased overall number of clusters (D), reduced inter-cluster distances (E) and inclusion of more clusters in each CRU (F). Overall CRU composition became less solid in failing cells (G), assessed by convex-hull analysis, as the average CRU contained fewer RyRs (C). See Figure 2—source data 1 for analysis of 100 nm vs 150 nm CRU inclusion criterion (n_cells = 46, 50 in Sham, HF; *P < 0.05 vs Sham).

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Figure 2—figure supplement 1. HF in post-infarction rats is not associated with altered expression of RyR, BIN1, or Junctophilin-2. Western blotting was performed on homogenates of left ventricles from post-MI HF rats and Sham-operated controls. Representative immunoblots are shown at left, with mean values at right, normalized to Sham. (JPH2 = junctophilin 2; nhearts = 5, 5 in Sham, HF; P = NS).
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Figure 3. Failing cardiomyocytes exhibit increased ‘silent’ RyR leak. Total RyR-mediated Ca\(^{2+}\) leak was assessed in SR microsomes using fura-2 fluorescence (A). Vesicular Ca\(^{2+}\) uptake was initiated by addition of ATP, and halted by addition of thapsigargin. SR Ca\(^{2+}\) leak was estimated as the thapsigargin-induced rate of rise of [Ca\(^{2+}\)], normalized to releasable SR content (rise in [Ca\(^{2+}\)] induced by the RyR opener 4-chloro-m-cresol, CMC). While the rate of SR Ca\(^{2+}\) uptake was reduced in HF relative to Sham (B), total RyR leak was increased (C) even with a slight reduction in the releasable Ca\(^{2+}\) store (D) (n = 8 from 3 Sham hearts, 7 from 3 HF hearts; *=P < 0.05 vs Sham). To assess whether elevated SR leak in failing cells could be attributed to Ca\(^{2+}\) sparks, line-scan confocal imaging of resting cardiomyocytes was employed (E). Ca\(^{2+}\) spark mass was increased in HF relative to Sham (F), due to augmented spark geometry (Figure 5B). However, since spark frequency tended to be reduced (G), overall Ca\(^{2+}\) spark-mediated leak was similar in Sham and HF (H). These results are consistent with increased ‘silent’ non-spark-mediated SR leak in HF cardiomyocytes. (FWHM = full width at half maximum, FDHM = full duration at half maximum; n\(_{\text{cells}}\) = 43 in Sham, 50 in HF; *=P < 0.05 vs Sham).

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Figure 4. CRU dispersion provides the structural basis for silent RyR leak in HF. A mathematical model of the dyad was employed to examine the effects of CRU dispersion on Ca\textsuperscript{2+} sparks and non-spark mediated RyR leak. (A). As dSTORM imaging indicated an increased fraction of small CRUs in HF (Figure 2C), small idealized CRUs were initially modelled with as few as 4 RyRs. Simulated Ca\textsuperscript{2+} sparks (300 consecutive simulations) were never detected for the smallest CRUs, based on an experimentally determined spark detection threshold of ΔF/F\textsubscript{0} = 0.4. Higher probability of visible spark generation (fidelity) was observed for larger CRUs. (B). Real CRU geometries obtained by dSTORM imaging were employed to simulate sparks from larger dyads. Four configurations were modelled with varying numbers of constituent RyR clusters, but similar total RyR number (~55). While the single-cluster CRU exhibited high Ca\textsuperscript{2+} spark fidelity, lower probability of spark generation was observed in dispersed, multi-cluster CRUs (fidelity indicated by colour scale). These data support that CRU rearrangement during HF promotes silent RyR leak, due to an increased fraction of both small CRUs as well as larger CRUs with dispersed, irregular configurations.

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Figure 4—figure supplement 1. Development and characterization of the mathematical dyadic model. (A) Graphical representation of the computational domain of the model. The junctional SR (jSR) was shaped to encompass idealized RyR geometries or dSTORM-based CRU contours. (B) To understand the role of the jSR geometry in determining the maximum inter-cluster distance permissive of functional coupling, and thus CRU definition, we spread two square 16-RyR clusters at variable distances up to 144 nm edge-to-edge (i.e. 4 RyR lengths). Coupling could occur at distances up to ~150 nm but only when the jSR was contiguous between clusters. (C) Similar effects could be observed in the realistic 7-cluster geometry, where a fully contiguous jSR was sufficient to functionally couple clusters at distances between 100 and 150 nm.

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Figure 5. Ca\textsuperscript{2+} spark kinetics are slowed in HF. (A) Representative line-scan images of Ca\textsuperscript{2+} sparks in Sham and HF, selected from the cell-wide scans presented in Figure 3E. Temporal profiles (right panels) show that spark kinetics were generally tightly constrained in Sham, with low values for both time to peak (TTP) and duration (full duration at half maximum, FDHM). Although many sparks were also brief in HF cells, a subset of sparks exhibited slowed kinetics. (B) Distributions of measurements for TTP and FDHM were right-shifted in HF, and mean values were significantly increased. Spark magnitudes tended to be larger in HF than Sham. (\(n_{\text{sparks}} = 130, 100\) from 75, 72 cells in Sham, HF; *\(P < 0.05\) vs Sham).

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Figure 6. RyR dispersion during HF results in slowing of Ca\(^{2+}\) sparks. To examine whether altered CRU morphology could slow Ca\(^{2+}\) spark kinetics in HF, spark profiles were simulated for a variety of dSTORM-derived RyR configurations. (A) Sparks were triggered by opening a single RyR (circled) which was randomly placed in consecutive simulations (example RyR opening trajectories are shown in the upper panels, with a family of spark time-courses illustrated below). Time to opening was registered for each RyR in the CRU, and the resultant time course of the Ca\(^{2+}\) spark was plotted until the final RyR closure, at which point the simulation was stopped for computational efficiency. Opening times were similar for individual RyRs within a solid, single cluster CRU, and the overall temporal profile of elicited sparks showed rapid kinetics which were rather consistent between consecutive simulations. By contrast, delayed and variable opening times were observed for individual RyRs in multi-cluster CRUs. This resulted in variable and slowed Ca\(^{2+}\) spark kinetics with these CRU configurations, as indicated by temporal spark profiles (A), a right-shifted distribution of time-to-peak measurements (B) and mean data (C). (*=P < 0.05 vs single-cluster CRU).

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Figure 6—figure supplement 1. Effect of focal plane on Ca\textsuperscript{2+} spark detection and kinetics. To examine whether out-of-focus Ca\textsuperscript{2+} release events could artefactually alter assessment of Ca\textsuperscript{2+} spark parameters, simulated Ca\textsuperscript{2+} release profiles were compared in ‘linescans’ collected at various depths of the 2 × 2 \( \mu \text{m} \) computational domain (54 RyR single cluster model). Vertical, stepwise displacement from the central plane resulted in a rapid decrease in measured spark amplitude (A). Indeed, events > 0.5 \( \mu \text{m} \) displaced from the focal plane were undetectable. While displacement of the focal plane also slowed apparent Ca\textsuperscript{2+} spark kinetics, such effects were negligible within the range where sparks were detectable (B).

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Figure 7. Slow Ca\textsuperscript{2+} sparks promote slow, desynchronized Ca\textsuperscript{2+} transients in HF. (A) Representative confocal linescan images of Ca\textsuperscript{2+} transients in field-stimulated cells (stimulus illustrated as a horizontal line). The overall Ca\textsuperscript{2+} transient was slowed in HF compared to Sham, as indicated by plots of spatially-averaged Ca\textsuperscript{2+} transients (IA, right panel), and measurements of half rise time (TTF\textsubscript{50}, (B)) and time to peak (C). Slowed Ca\textsuperscript{2+} transient kinetics included de-synchronization of Ca\textsuperscript{2+} release across HF cells, as indicated by profiles of local TTF\textsubscript{50} (lower panels in A). The standard deviation of these values, defined as the dyssynchrony index (Louch et al., 2006), showed a right-shifted distribution in HF compared to Sham (D). To examine the relationship between slowed Ca\textsuperscript{2+} spark kinetics and de-synchronized Ca\textsuperscript{2+} transients in HF, local Ca\textsuperscript{2+} transients were examined within 2 \textmu m regions of the linescan centered at the location of recorded sparks. Paired representative recordings of sparks and local Ca\textsuperscript{2+} transients are shown in (E and F), respectively, corresponding to indicated positions in A (vertical arrows). Local Ca\textsuperscript{2+} release at 'slow' spark locations (rise time > 13 ms) was protracted during the action potential, in comparison with local transients with 'fast' sparks in both HF and Sham (F). This association is demonstrated by clustering of locations with slow Ca\textsuperscript{2+} spark and local transient kinetics in 'heat maps' (G), and links slowing of Ca\textsuperscript{2+} transient kinetics to de-synchronization in HF.
Figure 7 continued

Ca\textsuperscript{2+} release kinetics at the level of the single CRU and whole cell. (Ca\textsuperscript{2+} transients: n\textsubscript{cells} = 43 in Sham, 57 in HF; n\textsubscript{fast sparks} = 18 in Sham, 19 in HF; n\textsubscript{slow sparks} = 18 in HF, *=P < 0.05 vs Sham).

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Figure 7—figure supplement 1. T-tubule structure is disrupted during HF. Isolated cardiomyocytes were stained with di-8-ANEPPS and examined by confocal microscopy (A). Zoomed regions enlarged in (B). In agreement with previous work employing the same post-infarction rat model (Frisk et al., 2016), we observed marked remodeling of t-tubular structure in HF. Skeletonized images (C, D) indicate that this reorganization included a loss of transverse t-tubule elements (colored white) and the appearance of additional longitudinal tubules running in the axial, perpendicular orientation (colored red). Scale bar = 5 μm.

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