Calcium-Dependent Arrhythmogenic Foci Created by Weakly Coupled Myocytes in the Failing Heart

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ABSTRACT

**Rationale:** Intercellular uncoupling and Ca mishandling can initiate triggered ventricular arrhythmias. Spontaneous Ca release activates inward current which depolarizes membrane potential ($V_m$) and can trigger action potentials in isolated myocytes. However, cell-cell coupling in intact hearts limits local depolarization and may protect hearts from this arrhythmogenic mechanism. Traditional optical mapping lacks the spatial resolution to assess coupling of individual myocytes.

**Objective:** We investigate local intercellular coupling in Ca-induced depolarization in intact hearts, using confocal microscopy to measure local $V_m$ and intracellular [Ca$^{2+}$] ([Ca$]$) simultaneously.

**Methods and Results:** We used isolated Langendorff-perfused hearts from control (CTL) and HF mice (HF induced by trans-aortic constriction). In CTL hearts, 1.4 % of myocytes were poorly synchronized with neighboring cells and exhibited asynchronous Ca$^{2+}$ transients (AS). These AS myocytes were much more frequent in HF (10.8% of myocytes, $p<0.05$ vs. CTL). Local Ca waves depolarized $V_m$ in HF but not CTL hearts, suggesting weaker gap junction coupling in HF-AS vs. CTL-AS myocytes. Cell-cell coupling was assessed by calcein fluorescence recovery after photobleach (FRAP) during [Ca$]$ recording. All regions in CTL hearts exhibited faster calcein diffusion than in HF, with HF-AS myocyte being slowest. In HF-AS, enhancing gap junction conductance (with rotigaptide) increased coupling and suppressed $V_m$ depolarization during Ca waves. Conversely, in CTL hearts, gap junction inhibition (carbenoxolone) decreased coupling and allowed Ca-wave-induced depolarizations. Synchronization of Ca wave initiation and triggered action potentials were observed in HF hearts and computational models.

**Conclusions:** Well-coupled CTL myocytes are effectively voltage-clamped during Ca waves, protecting the heart from triggered arrhythmias. Spontaneous Ca waves are much more common in HF myocytes and these AS myocytes are also poorly coupled, enabling local Ca-induced inward current of sufficient source strength to overcome a weakened current sink to depolarize $V_m$ and trigger action potentials.

**Keywords:** Heart failure, triggered activity, synchronization, Ca handling, gap junction, fluorescent recovery after photo-bleaching, extrasystole, premature ventricular contraction, premature ventricular beats, arrhythmia

Nonstandard Abbreviations and Acronyms:

- HF: heart failure
- CTL: control
- AP: action potential
- CaT: calcium transient
- CBX: carbenoxolone
- PVC: premature ventricular contraction
- $V_m$: membrane potential
- TAC: transverse aortic constriction
- NCX: Na$^+$/Ca$^{2+}$ exchanger
- FRAP: fluorescence recovery after photo-bleaching

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INTRODUCTION

In the heart, cardiomyocytes are mechanically and electrically coupled to produce coordinated contractions. However, in pathological conditions, ill-timed propagation of ectopic ventricular beats can contribute to fatal arrhythmias.\(^1\) Non-electrically driven Ca release can be a critical factor in the development of ventricular focal excitations, especially in heart failure (HF),\(^4,5\) where Ca handling dysfunction is common.\(^6\) Indeed, in HF there is increased diastolic sarcoplasmic reticulum (SR) Ca leak and Ca waves that are proarrhythmic.\(^5,8,12\) Diastolic SR Ca leak via ryanodine receptors (RyRs) can lead to propagating Ca waves in myocytes.\(^12,14\) These Ca waves drive extrusion of Ca from the myocyte via Na\(^+\)/Ca\(^2+\) exchange (NCX) current (INCX) that causes membrane potential (V\(_m\)) depolarization and delayed afterdepolarizations (DADs). If of sufficient magnitude, these DADs may trigger an action potential (AP) in the myocyte and potentially triggered activity or premature ventricular contraction (PVC) in the whole heart.\(^9,14\) Therefore, myocytes with spontaneous Ca release will present unsynchronized Ca dynamics to neighboring myocytes, but also may generate electrical asynchrony.

Detailed analysis of DADs and Ca-triggered APs has been largely conducted in isolated myocytes and has been mechanistically informative.\(^10,11,15-17\) However, in the intact heart, myocytes are electrically coupled to many neighbors, and that can greatly influence the effect that the Ca-dependent INCX has on local V\(_m\). Indeed, a single myocyte undergoing a Ca wave may produce a local inward INCX that would tend to depolarize itself and its neighbors, as a current source. But the large number of myocytes to which that cell is attached and electrically coupled, creates a significant current sink that may distribute that current source so broadly in space that the local depolarization, even in the initiating myocyte, is minimal.

A quantitative theoretical consideration of this issue by Xie et al.\(^1\) estimated the number of local myocyte DADs that would need to be synchronized to induce a PVC. The myocyte number in the normal heart model was very large, but the conditions associated with HF (e.g., increased NCX, reduced I\(_{K1}\) and gap junction uncoupling)\(^11,18-23\) decreased the number of myocytes required to overcome the normal source-sink mismatch that prevents a single myocyte Ca wave from triggering a PVC. Parallel whole heart optical mapping experiments with micro-injection of norepinephrine to induce locally synchronized SR Ca release under different HF-related conditions were in qualitative agreement with these modeling predictions.\(^24,25\) However, the spatial resolution of whole heart optical mapping, as is typically used to study these arrhythmogenic events,\(^24,28\) does not allow observation of local [Ca] or V\(_m\) at the level of single myocytes.

Several groups have used confocal [Ca] imaging of small groups of myocytes in the intact heart, and this allows the spatial resolution to measure individual myocyte behavior within a syncytium.\(^29-33\) We developed a confocal imaging platform capable of simultaneous [Ca] and V\(_m\) measurements in individual myocytes within intact Langendorff-perfused mouse hearts. This set-up also allows simultaneous measurement of [Ca], and direct measurements of local cell-cell-coupling via calcein fluorescence recovery after photobleach (FRAP).

We observed occasional individual myocytes in the heart that did not synchronize with all other visible myocytes during pacing-induced activation, and these asynchronous or rogue myocytes often exhibited independent spontaneous Ca transients (as waves). These Ca asynchronous myocytes were much more common in hearts in which HF had been induced by trans-aortic constriction. We hypothesized that these Ca asynchronous myocytes contribute to electrical asynchrony, especially in conditions such as HF. In control (CTL) hearts, myocytes exhibiting asynchronous Ca waves were well coupled to their neighbors, such that no depolarization was detectable during Ca waves. In contrast, asynchronous Ca waves in HF myocytes caused substantial depolarization during Ca waves (including in nearby cells), but these cells were only poorly coupled with neighboring myocytes. We conclude that regions with poorly coupled HF myocytes may allow local Ca-induced inward currents to overcome normal source-sink mismatching to propagate depolarization and triggered action potentials.
METHODS

Animals.
All animal procedures were approved by the Animal Care and Use Committee of the University of California, Davis and adhered to the NIH Guide for the Care and Use of Laboratory Animals. Experiments were conducted on normal adult male C57BL/6 mice (CTL: n=15) and mice in which HF had been induced by 8 weeks of transverse aortic constriction (TAC) by previously established techniques (HF: n=14). HF progression was verified by echocardiographic analysis and heart weight to body weight ratios upon cardiac excision. The HF mice used exhibited ~50% reduction in ejection fraction and fractional shortening, and wall thickening (Online Figure I).

Ca-Vm coupling study in intact heart.
A custom-made Langendorff system was positioned on an Olympus FluoView™ 1000 confocal microscope (Olympus, Center Valley, PA, USA). Hearts from CTL (n=9) and HF (n=8) mice were superfused and retrogradely perfused via the aorta with oxygenated normal Tyrode’s solution (NT; in mmol/L: 128.2 NaCl, 4.7 KCl, 1.19 NaH2PO4, 1.05 MgCl2, 1.3 CaCl2, 20.0 NaHCO3, and 11.1 glucose, and gassed with 95% O2-5% CO2; pH=7.35±0.05). In most experiments the sino-atrial node and atrio-ventricular node was intentionally crushed to slow the intrinsic heart rate.

Hearts were simultaneously stained with the Vm indicator RH237 (Thermo Fisher Scientific, Waltham, MA; 10 μmol/L) and the Ca indicator Fluo-8 AM (Teflab, Austin, TX; 10 μmol/L) to investigate the Ca-Vm coupling at room temperature. Blebbistatin (Abcam Biochemicals, Cambridge, MA; 10-20 μmol/L) was used to eliminate motion artifacts during imaging. After 10–20 min stabilization of the heart, 4 field-of-view images under 40×/1.3 N.A. oil-immersion objective, pin-hole of 100nm, were acquired randomly from different areas on the ventricular free wall epicardium to evaluate the occurrence cardiomyocytes with asynchronous Ca waves. For such cells, line-scan mode imaging was then used under 60×/1.3 N.A. oil-immersion objective (0.414 μm/pixel) with scanning speed 2 μs/pixel for both Vm (excited at 559nm; emission at >700nm) and Ca (excited at 488nm; emission at 525±20 nm) channels. As the fluorescent intensity for RH237 decreases upon depolarization, these signals were displayed inverted (except as indicated). A typical simultaneous recording is presented in Figure 1A where action potentials (APs: red) and Ca transients (CaTs: gray) were well-coupled as indicated by time-aligned signals.

Validation of the recording methods is shown in Online Figure II-III. Recording of Vm and [Ca]i was sequential (9 ms delay). During separate excitation, there was negligible interference between Fluo-8 AM and RH237 signals (Online Figure II) indicating lack of crosstalk between these signals during experiments. Typical recordings of CaT under different pacing frequency and premature ventricular contractions detected in the individual myocytes from the intact heart are presented in Online Figure III.

Fluorescence recovery after photo-bleaching (FRAP) in intact hearts to functionally assess gap junction coupling.
Similar to the Ca-Vm study, isolated CTL (n=6) and HF (n=6) mouse hearts were also superfused and retrogradely perfused with oxygenated NT and stabilized for 10–20 min, but in this case hearts were stained with Rhod-2 AM (Thermo Fisher Scientific, Waltham, MA; 10 μmol/L; excited at 559nm; emission at 595±15nm) to assess [Ca] dynamics and Calcein AM (TEFLabs, Austin, TX; 10 μmol/L; excited at 488nm; emission at 525±20nm) to assess function of gap junctions (indicated by the recovery of calcine fluorescence in bleached myocytes). A representative control recording (Figure 1B) shows no change in the calcein channel (green) during beat-to-beat CaTs imaged with Rhod-2 AM channel (gray). FRAP experiments were conducted similar to those by Abbaci et, al. Briefly, 4 time sequence 2D-images (40×/1.3 N.A. oil-immersion objective: 512×512 pixels at resolution of 0.621μm/pixel) were acquired to measure baseline fluorescence (interval of 15s). Calcine photo-bleaching was conducted on a target myocyte via argon ion laser (488 nm) at 100% power for 100s. Imaging demonstrated that bleach was well-
confined to that myocyte in the x-y plane. Despite restricted confocal illumination, slight bleach in one or two adjacent myocytes beneath the target cell (in the z-direction) cannot be ruled out. While this may limit estimates of diffusion coefficients, the effects are similar across all FRAP experiments, and should not influence relative FRAP. A time lapse series was then acquired to record calcein FRAP in the target myocyte using 1% power at indicated time intervals for CTL and HF hearts (200-300 cycles). Detailed analyses are described in supplemental materials.

**ECG recordings.**
ECG was recorded by a pair of 4mm Ag/AgCl pads during the stabilization time before loading the dyes in this study.

**Experimental protocol.**
In both the Ca-V_m coupling study and gap junction FRAP experiments, the gap junction uncoupler carbenoxolone (CBX; Sigma-Aldrich, St. Louis, MO; 25μmol/L) or I_{k1} blocker BaCl_2 (Sigma-Aldrich, St. Louis, MO; 5 μmol/L) were delivered by aortic perfusion into hearts. The gap junction modifier rosiglitazone (ZP123; 50nmol/L), which is reported to increase gap junctional conductance, was perfused into HF hearts. Ca-V_m coupling experiments were also conducted on the isolated myocytes.

**Cardiomyocyte isolation, staining and confocal imaging.**
Cardiac ventricular myocytes were isolated from adult male C57bL/6 mice (CTL, n=2) as previously described. Briefly, hearts were removed and retrogradely Langendorff perfused with type 2 collagenase (Worthington Biochemical Corporation, Lakewood, NJ) to isolate cardiac ventricular myocytes, with gradual [Ca]o increase to 1 mmol/L at room temperature. Freshly isolated myocytes were plated on laminin-coated glass cover slip for 15 min before dye loading (with 10 μmol/L Fluo-8 AM for 15 min followed by 20 min wash out in Tyrode’s). Cells were then stained with 10 μmol/L RH237 for 5 min and washout. Imaging was then applied under 60×/1.42 N.A. oil-immersion objective using Olympus FluoView™ 1000 confocal microscopy system. All settings are as described in Ca-V_m intact heart study.

**Computational modeling.**
We use our previous detailed stochastic AP and Ca handling myocyte model, with ~20,000 individually stochastic SR junctional SR Ca release units per myocyte, and 10,000 myocytes in a 2-dimensional array (1.5 x 1.5 cm) coupled via physiological gap junctional conductance. Some of the rabbit model parameters were adjusted to reflect HF phenotype (e.g. reduced levels of some K+ currents, increased NCX and increased RyR sensitivity) and increased SERCA2 activity to mimic mouse vs. rabbit differences and spontaneous Ca-transients in some cells. Further model details are presented in the Online Supplement.

**Statistical analysis.**
Quantitative data are shown as mean±SEM. Fisher’s exact test and one-way ANOVA with Bonferroni post-hoc tests were applied where appropriate. A value of p<0.05 was considered statistically significant.

**Technical limitations.**
We did not perform photo-bleaching of myocytes adjacent to asynchronous myocytes because subsequent bleaching of a neighboring cell would complicate results and interpretation. Thus, FRAP measurements in both asynchronous and adjacent cells were not performed. As with most Ca/V_m imaging in intact hearts, quantitative calibration of V_m and [Ca], was not possible, therefore we cannot compare absolute baseline diastolic V_m or [Ca], levels of AS with SY cells.
RESULTS

Asynchronous Ca release in failing ventricular myocardium.

In the intact heart, healthy ventricular myocytes are normally well coupled and exhibit well-synchronized local electrical activity (AP) and Ca transients (CaTs; Figure 1A). However, a small number of myocytes (even in CTL hearts) exhibited asynchronous CaTs which appeared not to affect neighboring myocytes. Such rogue cells could be single asynchronous myocytes, coupled asynchronous myocytes, or whole areas of asynchronous myocytes. Figure 1C shows consecutive 2D confocal images of CaTs recorded from the intact ventricle, with a Ca asynchronous myocyte (cell3) highlighted by a yellow box. Figure 1D is a line scan image along the green dotted line through five neighboring cells in Figure 1C. Individual myocyte spatially integrated CaTs are shown for the asynchronous myocyte (cell3) and the adjacent myocyte that is synchronized with the other neighboring myocytes (cell4). Cell 3 presented spontaneous Ca waves (arrows in Figure 1C and 1D) while the neighboring cells (both in the longitudinal and transverse directions) were at the overall rhythm. Such Ca asynchronous (AS) ventricular myocytes were observed in both CTL and HF mouse hearts but with dramatically different incidence. Only 1.4% of myocytes in fields studied were Ca asynchronous in CTL hearts while 10.8% of cells were Ca asynchronous in HF hearts (Figure 1E).

Ca waves cause depolarization in asynchronous myocytes only in HF hearts.

Importantly, in CTL hearts, spontaneous Ca waves in asynchronous myocytes did not result in detectable local V_m depolarizations (presumably because V_m in those myocytes was effectively clamped by gap junctional coupling to the neighboring myocytes). However, in HF, we observed two distinct types of Ca waves; one occurred without V_m changes (like CTL) and the other associated with detectable V_m depolarizations. Figure 2A is a series of consecutive 2D images where the decrease of RH237 fluorescence (depolarization) was detected in the same cells simultaneous with increased [Ca^{2+}]_i (measured with Fluo-8) during spontaneous and synchronized Ca waves. The signal from a selected cluster of myocytes (yellow box) in Figure 2A, was integrated, normalized and plotted in Figure 2B, for both [Ca^{2+}]_i and V_m. Even at the low scan rate attainable in 2D, the changes coincide in time. A full recording is in the Online Video.

To increase temporal resolution for such Ca waves and V_m depolarizations, we used line scanning (Figure 2C-D). The CTL myocyte in Figure 2C (left) exhibits a clear Ca wave, but no detectable depolarization, while the HF myocyte (right) exhibits a Ca wave that induced a clear depolarization which peaks with some delay. This suggests that V_m depolarization was induced by the Ca wave. Such Ca wave-induced V_m depolarization was only observed in Ca AS myocytes in HF hearts (not in CTL hearts). These Ca-induced depolarizations were seen in the majority of HF-AS myocytes (87.6% in HF-AS cells vs. 0% in CTL-AS, p<0.01, Figure 2E). It should be emphasized that in contrast to the intact heart, in isolated myocytes, Ca waves always induced V_m depolarization, in CTL and HF groups (Online Figure IV). This agrees with previous isolated myocyte studies, where SR Ca release drives an inward I_{NCX} to initiate delayed afterdepolarizations (DADs). This inward I_{NCX} surely occurs in both cases in Figure 2C, but in CTL the inward current is well-absorbed by the cells to which it is coupled via gap junctions. Conversely, the HF myocytes may have weaker gap junctional coupling, allowing local depolarization.

In another HF heart (Figure 2D) asynchronous Cell2 exhibits a Ca wave that does not propagate at all to Cell1. However, the consequent depolarization in Cell2 does propagate to Cell1. Moreover, this depolarization is sufficient to trigger an AP in both myocytes near the end of this trace (manifest also on the Ca trace as a spatially synchronous Ca transient). Note that the depolarization level in Cell1 (prior to the AP) is only around half of that seen in Cell2, when normalized to AP peak vs. diastole (i.e. 78.5% of AP level in the AS Cell2 vs. 37.6% in neighboring Cell1). We conclude that these AS HF myocytes have significant, yet reduced coupling to neighboring cells.
Ca asynchronous myocytes in HF hearts are poorly, but reversibly coupled via gap junctions.

To directly measure gap junction coupling in identifiable asynchronous HF myocytes we measured [Ca], and FRAP of calcine simultaneously. Figure 3A shows photobleach of a synchronous myocyte in a CTL heart (indicated by arrow in Bleach panel). In CTL hearts, calcine FRAP in synchronous myocytes recovered rapidly (halftime t½ ~3-4 min) and was complete by 30 min (CTL-SY; Figure 3A-C). This was also true for asynchronous myocytes in CTL hearts (CTL-AS). In contrast, in HF hearts, even synchronous myocytes took >45 min (HF-SY), and asynchronous HF myocytes (HF-AS) had severely limited overall FRAP (Figure 3C). The mean extent of recovery from the groups are compared in Figure 3C & 4C. Despite slower rate, HF-SY cells eventually recovered comparably to CTL hearts (CTL-SY; ns); whereas the extent of FRAP in HF-AS myocyte was much lower. This suggests that there may be gradations in coupling among myocytes in a region, with HF-AS myocytes being the most isolated.

To test whether this variable coupling can be modulated acutely by reagents known to influence gap junction coupling, we also measured calcine FRAP in CTL-SY myocytes treated with the gap junction blocker CBX (25 µM). Figure 4A-B shows that CBX reduced FRAP dramatically in CTL-SY myocytes, reaching only the steady state level seen in HF-AS myocytes (Figure 4B-C). We also tried to promote coupling in HF hearts using rotigaptide (ZP123, 50nM), which has been reported to enhance gap junctional conductance. Figure 4A-B shows that ZP123 restored FRAP kinetics in both HF-SY and even HF-AS myocytes back to a level comparable to CTL-SY myocytes (Figure 4B-C). This suggests that the poor coupling in HF myocytes could be acutely restored, even in HF-AS myocytes.

In addition to calcine FRAP extent, we analyzed FRAP kinetics by fitting either a single or bi-exponential curve to each record (selected by unbiased goodness of fit). Online Figure VA shows the FRAP data normalized by final recovery amplitude. Most CTL heart myocytes (11 of 14) were best fit with a single exponential, but some required a second slow component (Online Figure VB). In contrast, in the 3 groups of HF hearts shown, most required a bi-exponential fit. For all bi-exponential fits (CTL and HF) the slower component τ (4-30 min) was dominant, and the fast τ (1-2 min) was a minor fraction and similar among groups; Online Figure VC-D). The dominant slow time constant was much faster for CTL myocytes (5 min) than any of the HF groups (23-29 min; Figure 4D).

Ca-dependent depolarization is modulated by gap junction coupling and I_{k1}.

We further tested whether the Ca wave-induced depolarizations in HF-AS myocytes (Figure 2) are sensitive to gap junction modifiers, analogous to calcine diffusion (Figure 3-4). Figure 5A shows a HF-AS myocyte that exhibited repetitive Ca waves and depolarizations, independent of surrounding myocytes. ZP123 (50 nM) did not suppress the Ca waves, but nearly abolished the associated depolarizations. Thus, restoring cell-cell coupling through gap junctions attenuated the ability of Ca waves to induce depolarization. Conversely, in a CTL myocyte exhibiting Ca waves that do not produce detectable depolarization (Figure 5B, left), partial gap junction block with 25 µM CBX allowed the Ca waves to trigger local depolarization (right). This, together with the results above indicate that reduced cell-cell coupling is a prerequisite for local Ca waves to induce significant depolarization, by limiting the current sink ordinarily created by well-coupled myocardium.

The inward rectifier potassium channel that carries I_{k1} is critical in stabilizing the resting membrane potential, and is likely a major contributor to the current sink effect of myocytes around a cell that exhibits a Ca wave and inward I_{NCX}. If that is the case, inhibiting I_{k1} should also enhance depolarizations in myocytes exhibiting Ca waves. Figure 5C shows that partial block of I_{k1} with 5 µM BaCl_{2} promoted the ability of Ca waves in CTL hearts to produce depolarization, consistent with this hypothesis.
Ca wave-induced depolarization influences adjacent asynchronous myocytes.

In a few cases, we observed adjacent HF-AS myocytes in a single image. Ca waves in the two myocytes in Figure 6 seemed independent (left), but they still may interact, like in Figure 2D. We speculate that the two closely coupled beats in Cell1 may have depolarized Cell2 (which can enhance Ca loading) and facilitate the second Ca wave in Cell2. When this area was paced at 1 Hz (right panel), Cell1 followed the pacing, but the Ca transients were not spatially synchronized as in other myocytes, but rather propagated as Ca waves. This suggests that Cell 1 did not have normal AP-induced synchronized Ca transients (but \( V_m \) was not measured in this heart). Cell2 was similar, except that the slow [Ca] decline at the first beat may have been responsible for the very limited Ca transients at the second beat. So these myocytes seem only weakly coupled to their surrounding myocardium.

Premature ventricular contraction (PVC) initiated from Ca waves in HF hearts.

A pair of adjacent HF-AS myocytes in Figure 7A showed Ca waves, where the second wave (in lower cell) triggers an AP that causes synchronous SR Ca release throughout both in that myocyte and the adjacent myocyte. This is more like what happens with robust Ca release in isolated myocytes, where the current sink that is present in the intact heart is not present (Online Figure IV right panel). The \( V_m \) trace (Figure 7B bottom part) has a motion artifact, right after the AP peak, at the Ca transient peak (likely due to insufficiently blocked contraction). \( V_m \) traces are more prone to these artifacts vs. [Ca\(^{2+}\)], because RH237 is restricted to the membranes. \( V_m \) depolarization can still be appreciated from the top part of cell which was less affected by contraction.

If these events at the local level of 10-20 myocytes can impact overall cardiac rhythm, we might expect more arrhythmic events in HF hearts. Electrical noise prevented simultaneous ECGs during dual confocal imaging, so we could not directly test whether events we happen to capture in small fields of ~20 myocyte (Figure 7A-B) directly caused PVCs. However, we did see increased incidence of PVCs in HF vs. CTL hearts in ECG recordings before switching on confocal recording (12/14 HF hearts vs. 1/15 CTL hearts; \( p<0.01; \) Figure 7C and D). Runs of ventricular tachycardia (VT) were also detected in HF hearts, but not in the CTL hearts (Figure 7E and F). Thus, these HF hearts have greater vulnerability to PVCs and VT.

Computational modeling.

A working mechanistic hypothesis that emerges from these novel results is depicted in Figure 7G. In HF, there is a relatively high proportion of AS or rogue myocytes (10.8%) that can produce a local SR Ca release-dependent depolarization that can spread locally via reduced (but viable) cell-cell coupling. Furthermore, these local regions (pink) may be poorly coupled to the whole heart, and that could allow these foci to generate sufficient magnitude of current source to overcome the myocardial current sink that would prevent this type of triggered activity in well-coupled normal hearts. To test whether a mechanism like this is plausible, we used computational simulations that are founded in detailed cellular and tissue properties.\(^{41-43}\)

Figure 8A shows 2-dimensional [Ca]-\( V_m \) simulations of 10,000 physiologically coupled myocytes, where 78 myocytes at the center are Ca-loaded, with intra-SR [Ca] sufficiently high to drive spontaneous Ca waves (due to the stochastic properties of 20,000 independent release channel clusters; white circle in [Ca], images). At 127 ms overlapping Ca waves cause a small local depolarization from ~80 to ~71 mV, but this transient depolarization does not propagate. When we now reduce gap junctional coupling in this small group of myocytes by 8-fold (based on the ~6-fold reduction in FRAP kinetics in HF-SY, and much more dramatic FRAP reduction in HF-AS [Figure 3-4]) a full-blown propagating AP occurs (Figure 8B).
The local depolarization is larger and occurs earlier (see 96 ms) and by 105 ms a propagating AP ($V_m=+27$ mV) is induced. If we broaden the region of reduced conductance (white circle in $V_m$ images in Figure 8C), while keeping the same 78 Ca-loaded myocytes as above, the AP starts sooner (see 105 ms). Notably, the number of myocytes needed to initiate a tissue PVC is smallest (21) for panel C (Figure 8E), and only half as many as in panel B. While additional in-depth simulations could further clarify conditions most conducive, these demonstrate the plausibility of this local triggering mechanism.

DISCUSSION

Extrasystoles, DADs, triggered activity, and PVCs are important causes of fatal ventricular arrhythmias, including in HF patients.3,44-46 However, many aspects of the underlying mechanisms are not well understood in quantitative mechanistic detail.

Poorly coupled and asynchronous myocytes in CTL and HF hearts.

We show here that in normal hearts a tiny fraction of ventricular myocytes (1.4%) are Ca-asynchronous (AS), but that this is much higher in HF (to 10.8%). Since our confocal view includes 10-20 myocytes, many CTL fields showed no AS myocytes, but HF fields typically showed one or more AS myocytes. Confocal microscopy allowed detection at this spatial scale, which is not possible with the usual whole-heart optical mapping approach.

This novel observation has several functional consequences. First, it disturbs local propagation pathways (both intracellular and extracellular) for the normally propagating AP depolarization and repolarization wavefronts. Second, the higher density of such myocytes in HF increases the probability of functional islands involving numerous cells (and consequent tortuosity) that could further perturb conduction in HF, independent of gap junction coupling or interstitial fibrosis in synchronous regions. Third, the incomplete nature of the uncoupling (as shown here) means that the poorly coupled AS myocytes can (and do) interact with the well-coupled tissue in their region. As current sinks, these regions could further slow conduction around them. Conversely, these islands could provide sufficient current source strength to cause PVCs, in a manner analogous to the normal pacemaker situation in the sino-atrial node (Figures 7G and 8). That is, a group of partially uncoupled asynchronous myocytes could be sufficiently isolated to create sufficient source current (as a triggering island) to overcome the source-sink mismatch that normally prevents single myocytes from triggering a propagating depolarization.1 Thus, the observed Ca waves in these AS regions may have a small but finite potential to drive PVCs, especially in HF where more myocytes have the gross phenotype and may also be surrounded by relatively weak coupling. Fourth, the penetrance of local Ca wave-induced depolarizations to initiate PVCs would be aided by relative synchronization among these partially isolated islands, for which we find evidence (Figures 2D and 6).

Isolated myocytes vs. whole heart.

In isolated myocytes, Ca-waves and more controlled SR Ca release events produce a robust inward current that is carried almost exclusively by $I_{NCX}$, and which can bring $V_m$ to threshold to trigger a full-blown AP.11,12,15,47 In HF, Na+/Ca$^{2+}$ exchange is increased and $I_{K1}$ is reduced, such that a smaller Ca release is sufficient to trigger an AP (more inward current and less stabilizing outward $I_{K1}$).11,18,23 Indeed, the quantitative connection between myocyte SR Ca release, DADs and triggered APs is well understood. However, SR Ca release in a single well-coupled myocyte in the heart is not expected to cause appreciable depolarization because of the huge current sink provided by all of the myocytes to which it is electrically well-coupled via gap junctions. Theoretical calculations support this notion and allowed inference of the
number of local myocytes that would be required to have synchronous SR Ca release events to cause a PVC.\(^1\)

In support of this idea, in CTL-AS myocytes we did not detect depolarization during the Ca wave, which agrees with prior confocal whole heart V_m imaging.\(^{30}\) So in normal hearts, coupling is sufficiently robust to essentially voltage-clamp a single cell exhibiting a Ca wave, thus protecting the heart from this PVC mechanism. However, a key and remarkable finding here was that most HF-AS Ca waves caused measurable depolarization in the actual myocyte exhibiting the Ca wave, and further, that this local depolarization could even depolarize nearby neighboring myocytes appreciably (e.g. Figure 2). The higher \(I_{NCX}\) and lower \(I_{K1}\) in HF would enhance the intrinsic depolarizing strength of a given Ca wave.\(^{11}\) In addition, our calcein FRAP studies also show that cell-cell coupling is reduced in HF hearts (even HF-SY), consistent with the reduced Cx43 expression, lateralization and altered phosphorylation reported in HF.\(^{19-22,48}\) The reduced coupling is especially profound where HF-AS sites are observed (Figure 3-4). But importantly, these HF-AS myocytes are not totally uncoupled (such that they would do no active harm). In fact, ZP123 can acutely restore coupling in those regions (Figure 4) and suppress local depolarization (Figure 5A), suggesting that function (vs. expression) of gap junctions may be critical. This scenario, where combined increase of \(I_{NCX}\), reduction of \(I_{K1}\) and regional reduction of gap junction conductance, promote Ca-dependent triggered activity in HF hearts agrees with both theoretical predictions\(^{7}\) (and Figure 8) and experimental observations.\(^{24,25}\) Moreover, the \(I_{NCX}\) increase would increase the current source, while reduced \(I_{K1}\) and gap junction conductance would limit the current sink to promote arrhythmic activity. The extent and location of reduced coupling might also enhance the source strength by functionally enlarging the volume of cells that contribute to the source. Our data here may help to further constrain theoretical models to strengthen the depth of our understanding of these events.

Synchronization of spontaneous Ca waves among myocytes also helps to achieve sufficient source current in vulnerable islands. There are likely two intrinsic factors that promote such local synchrony. First, synchrony is aided by electrotonic coupling, where the electrical coupling seen in Figure 2D can also enhance synchrony of Ca waves.\(^{49,50}\) Second, there is a characteristic RyR restitution time in myocytes and hearts that synchronizes Ca wave timing after a prior beat.\(^{33,51,52}\) The increased RyR sensitivity and leakiness in HF\(^8\) may also increase the likelihood and amplitude of the Ca waves in HF-AS myocytes. These factors enhance the arrhythmogenic strength of areas around HF-AS myocytes.

Consequences and range of varied cell-cell coupling.

The FRAP time constants among synchronous regions were 7.5 times longer in HF-SY vs. CTL-SY (Figure 4D). This suggests a large reduction in gap junction conductance with maintained local synchronization, and is consistent with a large gap junction safety margin for normal propagation. Even asynchronous myocytes in CTL hearts (CTL-AS) which were rare, were well-coupled, such that no depolarization was apparent in those cells. So, Ca waves in control hearts are unlikely to be consequential. In contrast, most HF-AS myocytes were very poorly coupled, even compared to HF-SY (Figure 3C) such that an even slower time constant for more complete FRAP was unresolved. However, these HF-AS cells are electrically coupled locally (Figure 2 and 6) and the regional severity of uncoupling may be the basis of the arrhythmogenic vulnerable islands discussed above and exemplified in Figure 7G and 8). Thus, the extent of local uncoupling required to create these dangerous loci may be extreme vs. that in CTL hearts or even in most of the HF heart. And many of these potential foci may occur around the heart, based on how common these are in random regions of 15-20 epicardial myocytes sampled by our method.

The severity of uncoupling in HF-AS myocytes might eventually transition into complete uncoupling, and lose influence on neighboring cells. But as we showed, ZP123 can improve the coupling of the HF-AS cells (Figure 4) and allow them to be once again voltage-clamped by neighboring cells (Figure 5A). This also suggests that the gap junctions are recoverable, and that the cells are still physically
able and have enough Cx43 available to couple better. We cannot determine from these experiments whether this involves altered post-translational modifications or subcellular localization of Cx43.

Conceivably, eventual shut down of these low-conductance junctions involves the steep $V_m$-dependence of gap junctional conductance (reduced at high $\Delta V_m$ between cells),$^{53}$ activation of ATP-sensitive K$^+$ current ($I_{\text{KATP}}$) and elevated $[\text{Ca}^{2+}]_i$. That is, the higher resistance of the junction may favor a larger transcellular $\Delta V_m$ gradient, which would further reduce conductance. And if the $[\text{ATP}]/[\text{ADP}]$ ratio falls sufficiently to open $I_{\text{KATP}}$ channels these HF-AS cells could be clamped near $E_K$, thereby further increasing transcellular $\Delta V_m$ gradient and junctional resistance. Elevated $[\text{Ca}^{2+}]_i$ can decrease the gap junctional conductance in ventricular myocytes.$^{55}$ This regulation takes time, so Ca waves per se might not cause this, but Ca loading (in the sick cell context) could hasten electrical isolation as well.

In conclusion, in HF we find a much higher density of Ca asynchronous myocytes that are poorly coupled electrically to the surrounding myocardium. These HF-AS myocytes may contribute to small islands that could initiate triggered beats, overcoming the normal source-sink current mismatch that provides a safety margin in normal hearts.

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DISCLOSURES
None.

REFERENCES

1. Xie Y, Sato D, Garfinkel A, Qu Z, Weiss JN. So little source, so much sink: Requirements for afterdepolarizations to propagate in tissue. Biophys J. 2010;99:1408-1415
2. Gilmour RF, Jr., Moise NS. Triggered activity as a mechanism for inherited ventricular arrhythmias in german shepherd dogs. J Am Coll Cardiol. 1996;27:1526-1533

DOI: 10.1161/CIRCRESAHA.117.312050


38. Xing D, Kjolbye AL, Nielsen MS, Petersen JS, Harlow KW, Holstein-Rathlou NH, Martins JB. Zp123 increases gap junctional conductance and prevents reentrant ventricular tachycardia during myocardial ischemia in open chest dogs. *J Cardiovasc Electrophysiol.* 2003;14:510-520


43. Sato D, Bers DM. How does stochastic ryanodine receptor-mediated Ca leak fail to initiate a Ca spark? *Biophys J.* 2011;101:2370-2379


48. Ai X, Zhao W, Pogwizd SM. Connexin43 knockdown or overexpression modulates cell coupling in control and failing rabbit left ventricular myocytes. *Card iovasc Res*. 2010;85:751-762
52. Satoh H, Blatter LA, Bers DM. Effects of [Ca2+], SR Ca2+ load, and rest on Ca2+ spark frequency in ventricular myocytes. *Am J Physiol*. 1997;272:H657-668
FIGURE LEGENDS

Figure 1. Ca asynchronous myocytes in the intact hearts. A). Confocal 2D and line scan images of Langendorff perfused intact mouse heart simultaneously stained with Fluo-8 AM (gray) and RH237 (red). Integrated signals from line scan images show precise time alignment of beat-to-beat AP and Ca transients. B). Images of intact hearts simultaneously stained with Calcein AM (green) and Rhod-2 AM (gray). Line scan imaging showed no effects of Ca transients on calcein fluorescence. C & D). An asynchronous myocyte (cell 3,yellow arrow) showed spontaneous Ca waves, while adjacent myocytes (cells 1, 2, 4 & 5) synchronously follow the heart rhythm. E). Asynchronous myocytes were detected under baseline condition in both groups of hearts. The percentage of AS cells was calculated as number of AS cells normalized to total cells in the field of view. In HF hearts 10.8% of the 1,204 cells monitored were asynchronous (130 HF-AS). In CTL hearts only 1.4% of the 1,143 myocytes observed were asynchronous (16 CTL-AS myocytes); p<0.05 by Fisher’s exact test.

Figure 2. Ca waves induce depolarization of membrane potential in HF hearts. A). 2D confocal image captured Ca wave-induced Vm depolarization in the intact hearts. At time point 1668 ms, spontaneous Ca release is indicated by increased Fluo-8 fluorescent intensity (white) simultaneously with Vm depolarization (decrease of RH237 fluorescence, red). B). Fluorescent intensity of each signal in A) was integrated and plotted vs. time. C). Line scan of asynchronous (AS) myocytes exhibiting Ca waves in CTL and HF hearts. Depolarization was only observed in HF (n=23) but not WT hearts. D). HF-AS cells not only depolarize themselves, but also neighboring cells. Such depolarization was greater in the AS cell (cell2: 78.5% of its AP amplitude) vs. neighbor cell1 (37.6% of its AP amplitude). E). None of the observed CTL-AS cells exhibited depolarization during Ca waves, whereas 87.5% of the HF-AS cells observed exhibited detectable depolarization as Ca waves occurred.

Figure 3. Gap junction FRAP experiments in the WT and HF intact hearts. A). Representative images of WT normal myocytes (CTL-SY) and HF normal (HF-SY) and asynchronous myocytes (HF-AS) at baseline condition, right after photo-bleaching and 33 and 50 min later. Full time course of Calcein FRAP for CTL hearts is 33 min (200 images at 10 s steps) and for HF hearts is 50 min (300 recordings at 10s steps). B). Calcein FRAP protocol indicates fluorescence prior to bleach (Fb) at end of bleach (Fo; bleach extent was 50-60% of Fb for each case) and during recovery (Ft). C) Recovery time course R, was calculated as (Ft–Fo)/(Fb–Fo) for CTL-SY (n=14, black), CTL-AS (n=10, blue), HF-SY (n=8, green) and HF-AS (n=10, red) myocytes. Total recovery was only significantly different for HF-AS myocytes among the groups measured here. Overall recoveries for CTL-SY, CTL-AS and HF-SY were to 88%, 84% and 85% of Fb, respectively. The incomplete recovery is likely due in part to bleach of calcein trapped in organelles (e.g. mitochondria), which would not recover during FRAP.

Figure 4. CBX reduces cell-cell coupling in CTL hearts and ZP123 rescued weakened coupling in HF hearts. A). Representative images of a CTL-SY myocyte, treated with CBX (CTL-SY+CBX) and a HF-AS myocyte treated with ZP123 (HF-AS+ZP123), at times indicated. B). CBX dramatically attenuated calcein FRAP recovery in CTL-SY myocytes (CTL-SY+CBX, n=8). FRAP time course for CTL-SY from Figure 3B is incuded for comparison. At right, ZP-123 enhanced calcein FRAP in both HF-SY (HF-SY+ZP123, n=7, green) and HF-AS cells (HF-AS+ZP123, n=9, red). HF-SY and HF-AS curves from Figure 3B are included for comparison. C). Total calcein FRAP recovery at steady state are shown for the groups indicated were compared (as described in text). D) Predominant time constant (τ) of FRAP for the groups shown (further analysis is in Online Figure V).

Figure 5. CBX, ZP123, and BaCl2 modulated the incidence of Ca wave induced depolarizations. A). Representative HF-AS myocyte images showing that 50 nM ZP123 (right panel, 7/7 cells) severely diminished Ca wave-induced Vm depolarization in its absence (left panel). B). In CTL hearts, 25 µM CBX...
(right, 12/17 cells) induced Ca wave dependent V_m depolarization normally not seen (left). C). BaCl_2 (5 µM) also promoted Ca wave dependent V_m depolarization in CTL-SY myocytes (10/12 cells) (not seen at baseline, left).

**Figure 6. Synchronized initiation of Ca waves under pacing in HF-AS myocytes.**
Line scan images and integrated signals show Ca wave initiation in two adjacent asynchronous myocytes in an intact HF heart. At baseline, random Ca waves were observed from both myocytes, while under pacing, the Ca waves initiated synchronously from the two adjacent myocytes, as indicated by the dashed lines.

**Figure 7. Ca wave-dependent extrasystoles, PVCs and ventricular tachycardia in HF hearts.**
A). Line scan of a Ca wave induced extrasystole in two adjacent HF-AS myocytes in an intact HF heart ([Ca]_i top, V_m bottom). Spontaneous Ca wave in top myocyte induced depolarization which was not sufficient to reach AP threshold, whereas the Ca wave in the bottom myocyte depolarized V_m to AP threshold, resulting in an extrasystole. B). Integrated signals from Ca and V_m channels in A). First green arrow indicates Ca wave and its subsequent depolarization. Second green arrow indicates an extrasystole with full AP induced after the second depolarizing Ca wave. At the time of the AP, a artifactual increase of the RH237 fluorescence (downward V_m deflection) due to contraction. C). Representative PVC detected in the ECG from a HF heart. D). PVC , with incidence in CTL (rare) and HF hearts (most hearts). E). Representative example of a run of VT detected in the ECG from a HF heart. F). VT was never seen in CTL hearts but evident in several HF hearts (HF). G). Mechanism schematic for ectopic foci vulnerable island resulted from HF-AS myocytes in HF hearts. A few AS cells with simultaneous Ca waves provide a current source sufficient to depolarize surrounding cells, which are also susceptible to asynchronous Ca activities. These surrounding cells would be somewhat depolarized and also more excitable, because lower gap junctional conductance, creating a larger arrhythmogenic current source. Reduced conductance also lowers the effective current sink that protects the normal heart from such focal arrhythmias.

**Figure 8. Mathematical modeling of PVCs due to regionally low GJ conductance.** A). Homogeneously normal GJ conductance (top V_m, bottom [Ca]_i). The 78 myocytes within the circle in [Ca]_i image (1.5 mm diameter) are Ca-loaded (and therefore spontaneously active). B). GJ conductance within this white circle is 8-fold lower than normal. C). Area of low GJ conductance is extended beyond the 78 rogue myocytes to twice that area. D). Zoomed region from pane B, showing oscillatory Ca waves. E). The minimum number of myocyte required to initiate a PVC under each condition. Number of spontaneous myocytes used in A-C (78) is indicated by dashed line.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Triggered arrhythmias in disease are associated with intercellular uncoupling and myocyte sarcoplasmic reticulum (SR) Ca\(^{2+}\) mishandling.

- Spontaneous SR Ca\(^{2+}\) release can trigger depolarization and action potentials in isolated myocytes, but strong electrical coupling limits this in intact hearts.

- How spontaneous SR Ca\(^{2+}\) release facilitates triggered activity in diseased intact hearts is not well understood.

What New Information Does this Article Contribute?

- Using confocal imaging of subcellular [Ca\(^{2+}\)]\(_i\) and voltage, we observed \(\sim 1\%\) of myocytes in intact heart exhibit spontaneous SR Ca release and fail to synchronize with the bulk ventricle.

- In heart failure (HF), these asynchronous rogue myocytes are \(\sim 10\)-fold more prevalent and Ca\(^{2+}\) waves cause local depolarizations in intact hearts, implying poor electrical coupling.

- Direct measurements of gap junction coupling by fluorescence recovery after photobleach (FRAP of calcein) demonstrated very poor coupling of these rogue HF myocytes (vs. those in normal hearts).

- In HF these poorly coupled myocytes allow local Ca\(^{2+}\) release-induced depolarization to depolarize neighboring myocytes.

- The combination of spontaneous Ca\(^{2+}\) releases and reduced local coupling may form local island of cells (a current source) which can propagate to produce focal activity (driving the sink) to promote arrhythmias.

- A novel mechanism is introduced (validated by computational modeling), by which a modest number of rogue myocytes in local poorly-coupled ventricular regions may overcome the traditional source-sink mismatch that protects healthy hearts.

In HF, triggered ventricular arrhythmias are often associated with Ca\(^{2+}\) mishandling and gap junction uncoupling. In normal hearts, individual myocytes are extremely well-coupled to their neighbors. Thus, a spontaneous diastolic SR Ca\(^{2+}\) release that can depolarize an isolated single myocyte (even causing an action potential) is prevented via effective voltage clamp by the rest of the heart. This coupling protects the normal heart from this delayed afterdepolarization triggered arrhythmia mechanism. In contrast, in HF we show that spontaneous SR Ca\(^{2+}\) release in single rogue myocytes can depolarize themselves (indicating that they are poorly coupled to their neighbors). But they also depolarize their neighbors, despite that weak coupling. The latter implies that the neighbors are also poorly coupled to the rest of the heart, creating a potential perfect storm for triggered arrhythmias. That is, a small region can generate sufficient source current (not dissipated because of relative isolation from the whole heart) that it can drive a triggered event that can escape and propagate to the whole heart (as a premature ventricular contraction). This pathological situation is analogous to how spontaneous pacemaking cells in the sinoatrial node (a small, poorly coupled region) can activate the entire heart during the normal heartbeat.
FIGURE 3

A

Baseline  Bleach  33 min  50 min

CTL-SY

n~=20 cells /FOV

100μm

HF-SY

HF-AS

B

C

Calcein FRAP

$R_t = \frac{(F_t - F_0)}{(F_B - F_0)} \times 100\%$

time

F_B

F_t

F_0

Bleach

ctl-SY

ctl-AS

HF-SY

HF-AS

Time (min)
FIGURE 5

A
HF-AS Baseline Condition

126 μm

HF-AS+50nM ZP123

143 μm

Ca

100 ms

Vm

2

1

1.28

1.15

B
CTL-SY Baseline Condition

150 μm

CTL-SY+25μM CBX

103 μm

Ca

100 ms

Vm

1.8

1.15

C
CTL-SY Baseline Condition

113 μm

CTL-SY+5μM BaCl2

97 μm

Ca

100 ms

Vm

1.6

1.5

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FIGURE 8

A. Homogeneous, physiological GJ conductance

B. Regionally (small area) reduced GJ conductance

C. Regionally (large area) reduced GJ conductance

D. Diagram showing minimum number of cells to initiate a PVC

E. Bar graph showing the minimum number of cells to initiate a PVC:

- Normal GJ conductance (Homogeneous): 158 cells
- Reduced GJ Conductance (Small area): 40 cells
- Reduced GJ Conductance (Large area): 21 cells

(78 cells as in A-D)
Calcium-Dependent Arrhythmogenic Foci Created by Weakly Coupled Myocytes in the Failing Heart
Di Lang, Daisuke Sato, Yan Jiang, Kenneth S Ginsburg, Crystal M Ripplinger and Donald M Bers

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Supplementary Methods

FRAP data analysis:
Raw Calcein fluorescence intensity $F(t)$ was averaged over the whole target myocyte at each time point $t$ during recovery. It was necessary to account for photodegradation and/or loss of dye by leakage due to the relatively long time required for recovery. Therefore, a pre-correction procedure was applied to the raw signals.

a). Pre-correction:
For this purpose a cell near the edge of the field of view was chosen as a reference. The reference cell intensity $F_{\text{ref}}(t)$ at any time $t > 0$ was a fraction of the reference cell intensity at the beginning of recovery $F_{\text{ref}}(0)$. The difference between $F_{\text{ref}}(0)$ and $F_{\text{ref}}(t)$ was due to the photodegradation and/or loss of dye by leakage during recording. Therefore, an photodegradation rate could be calculated as in equation 1:

$$\text{PhotoDegradationRate}(t) = \frac{F_{\text{ref}}(0) - F_{\text{ref}}(t)}{F_{\text{ref}}(t)};$$  \hspace{1cm} (1)

The adjusted fluorescence intensity of the target cell at time $t$, $F_{\text{adj}}(t)$, was calculated as the raw intensity $F(t)$, scaled up by fraction $\text{PhotoDegradationRate}(t)$ as in equation 2:

$$F_{\text{adj}}(t) = F(t) \times \left(1 + \text{PhotoDegradationRate}(t)\right) = F(t) \times \frac{F_{\text{ref}}(0)}{F_{\text{ref}}(t)};$$  \hspace{1cm} (2)

b). Percentage recovery calculation:
The fluorescent intensity gradually recovered after photobleach procedure due to the Calcein diffusion from adjacent cells into the target cell, which is measured as the intensity difference between $F_{\text{adj}}(t)$ and $F_{\text{adj}}(0)$. The percent recovery $R(t)$ at time $t$ was then calculated as such recovery relative to the bleached fluorescent intensity indicated as $F_{\text{base}}$ - $F_{\text{adj}}(0)$.

$$R(t) = \frac{F_{\text{adj}}(t) - F_{\text{adj}}(0)}{F_{\text{base}} - F_{\text{adj}}(0)} \times 100;$$  \hspace{1cm} (3)

In the figures, since photodegradation correction is applied before further analysis, Equation 3 is simplified to $R_t = (F_t-F_0)/(F_b-F_0)$ and expressed as percent.

c). Recovery time constant measurement:
Recovery functions are plotted in Figures 3-4.
Recovery data were fit to exponential association models to establish time constants. A one-phase (equation 4) or two-phase model (equations 5 and 6) was chosen, with the two-phase model used if it was more robust according to an extra sum-of-squares $F$ test.

$$R(t) = R(\infty) \times \left(1 - e^{-\frac{t}{\tau}}\right);$$  \hspace{1cm} (4)

$$R(t) = R(\infty) \times w_{\text{fast}} \times \left(1 - e^{-\frac{t}{\tau_{\text{fast}}}}\right) + R(\infty) \times w_{\text{slow}} \times \left(1 - e^{-\frac{t}{\tau_{\text{slow}}}}\right);$$  \hspace{1cm} (5)

with weights $w_{\text{fast}} > 0$ and $w_{\text{slow}} > 0$ such that

$$w_{\text{fast}} + w_{\text{slow}} = 1.$$  \hspace{1cm} (6)
Mathematical modeling:
In order to simulate realistic Ca waves in cells and propagating APs in tissue, we use a physiologically detailed model of a rabbit ventricular myocyte used in our previous studies. Ionic currents and Ca transport from the Shannon-Bers model\(^1\) as tuned by Mahajan et al.\(^2,3\) was combined with subcellular Ca cycling model of Restrepo et al.\(^4\) that includes subcellular stochastic properties of 19,305 individual SR Ca release units (CRUs) per myocyte. In each CRU there are 5 Ca compartments; cytosolic, submembrane, cleft space, network SR, and junctional SR. CRUs are coupled by Ca diffusions in cytosol and the network SR. There are 19,305 CRUs (65×27×11) in each cell, each containing 100 RyRs (1,930,500 RyRs per cell). The RyR channel, which is sensitive to both cytosolic Ca ([Ca]\(_i\)) and SR Ca ([Ca]\(_{SR}\)), is described by a 4-state Markov model as refined in Sato et al.\(^5-8\) Ca wave are not observed with normal parameters. Therefore, we increased the SERCA pump strength 50% (to partly account for the several-fold higher SERCA activity in mouse vs. rabbit myocytes) and increased RyR sensitivity to Ca (as is known to occur in HF). To mimic SR Ca loading and promote Ca waves as observed HF-AS myocytes, the SERCA pump strength was further increased.

To model tissue we use cable equations and couple cells by a \(V_m\) diffusion coefficient (\(D_v\)) that functions as gap junction (GJ) conductance and mimics normal conduction velocity:

\[
\frac{\partial V}{\partial t} = -\frac{I_{ion}}{C_m} + D_v \left( \frac{\partial^2 V}{\partial x^2} + \frac{\partial^2 V}{\partial y^2} \right),
\]

where \(C_m=1\ \mu F/cm^2\) is membrane capacitance, \(D_v=4\times10^{-4}\ cm^2/ms\ (D_v=5\times10^{-5}\ cm^2/ms\ for\ reduced\ GJ\ conductance),\) and where \(I_{ion}\) is the total transmembrane current. The cable equation was integrated using an operator splitting approach, with space step \(\Delta x=0.015\ cm,\) and with a variable time step in the range \(dt=0.01~0.1\ ms.\) The program codes are written in C++ and simulated in a High-Performance Computing cluster (24 nodes, Intel Xeon E3-1270, 3.4GHz, 32GB RAM) and Amazon Web Services.

Modifications from original model\(^5\) were to update or make it more HF- or mouse-like:

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<td>NCX (V_{\text{max}}) (up in HF)</td>
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<tr>
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<td>Ca diffusion coefficients</td>
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<tr>
<td>0.5 gkr</td>
<td>(I_{Kr}) conductance (not in mouse)</td>
<td>1.5 (v_{\text{max}})</td>
<td>SERCA (V_{\text{max}}) (4x for HF-AS cells).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

More detailed descriptions of the model can be found in the following references.

References
5. Sato D, Bers DM. How does stochastic ryanodine receptor-mediated Ca leak fail to initiate a Ca spark? Biophys J. 2011;101:2370-2379
**Online Figure I.** Heart failure induced in mice by transverse aortic constriction (TAC). A) Operated (HF) hearts were significantly hypertrophied. B) M-mode echocardiographic images from CTL and HF mice, showing hypocontractility. Ejection fraction (C) and fractional shortening (D) were decreased in HF, while end-diastolic interventricular posterior wall (IVPWd; E) and interventricular septal thickness (VSD, F) were increased in HF vs CTL hearts (all p<0.05).

**Online Figure II.** Absence of crosstalk between Fluo-8AM and RH237. Sequential excitation was applied to a heart loaded with both Fluo-8 and Rh237 as in the experiments. For either channel (Ca or AP) excited alone, there was no signal detected from the other channel.
Online Figure III. Line scan images of representative Ca events in individual cells of intact hearts, with spatial integrals. A. CaTs during 1Hz, 10Hz and 5 Hz pacing. B. Ca alternans was observed at 2 Hz. C. An example premature ventricular contraction (PVC).
Online Figure IV. Spontaneous Ca activity induced $V_m$ depolarization in isolated myocytes from CTL mouse hearts, unlike $V_m$ in CTL whole heart. Left: Ca waves were accompanied by membrane potential depolarization, which was delayed, and whose level was positively correlated with the size of Ca wave. Right (a different myocyte): depolarization induced by a Ca wave reached the AP threshold, as evidenced by a Ca transient and contraction which were both fast rising and time-aligned throughout the myocyte.
Online Figure V. A). Calcein recovery curves of CTL-SY (black, n=14), HF-SY (green, n=8), HF-SY+ZP123 (dark green, n=7) and HF-AS+ZP123 (dark red, n=9) myocytes, normalized so that final FRAP is 1. B). Properties of recovery curve fits in CTL and HF groups. Most myocytes in CTL hearts were best fit with one exponential (11/14), whereas most cells in HF hearts, even those treated with ZP123, were better fit using two components. C). In all biexponential fits from CTL or HF experiments, the slower component dominated. D). The fast (non-dominant) time constant was similar among all CTL and HF experimental groups.