
The authors note that the following statement should be added to the Acknowledgments: “The authors acknowledge funding from Medical Research Council UK Program Grant MR/N002903/1.”

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www.pnas.org/cgi/doi/10.1073/pnas.2013263117
Arrhythmogenic late Ca\textsuperscript{2+} sparks in failing heart cells and their control by action potential configuration

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Edited by Mark T. Nelson, University of Vermont, Burlington, VT, and approved December 24, 2019 (received for review October 24, 2019)

Sudden death in heart failure patients is a major clinical problem worldwide, but it is unclear how arrhythmogenic early afterdepolarizations (EADs) are triggered in failing heart cells. To examine EAD initiation, high-sensitivity intracellular Ca\textsuperscript{2+} measurements were combined with action potential voltage clamp techniques in a physiologically relevant heart failure model. In failing cells, the loss of Ca\textsuperscript{2+} release synchrony at the start of the action potential leads to an increase in number of microscopic intracellular Ca\textsuperscript{2+} release events ("late" Ca\textsuperscript{2+} sparks) during phase 2–3 of the action potential. These late Ca\textsuperscript{2+} sparks prolong the Ca\textsuperscript{2+} transient that activates contraction and can trigger propagating microscopic Ca\textsuperscript{2+} ripples, larger macroscopic Ca\textsuperscript{2+} waves, and EADs. Modification of the action potential to include steps to different potentials revealed the amount of current generated by these late Ca\textsuperscript{2+} sparks and their subsequent spatiotemporal summation into Ca\textsuperscript{2+} ripples/waves. Comparison of this current to the net current that causes action potential repolarization shows that late Ca\textsuperscript{2+} sparks provide a mechanism for EAD initiation. Computer simulations confirmed that this forms the basis of a strong oscillatory positive feedback system that can act in parallel with other purely voltage-dependent ionic mechanisms for EAD initiation. In failing heart cells, restoration of the action potential to a nonfailing configuration improved the synchrony of excitation–contraction coupling, increased Ca\textsuperscript{2+} transient amplitude, and suppressed late Ca\textsuperscript{2+} sparks. Therapeutic control of late Ca\textsuperscript{2+} spark activity may provide an additional approach for treating heart failure and reduce the risk for sudden cardiac death.

Significance

Sudden cardiac death in heart failure is a major unsolved clinical problem that is linked to the development of a spontaneous arrhythmia. Early afterdepolarizations (EADs) are an arrhythmogenic mechanism, but the cellular trigger for EADs in heart failure is unclear. We show that the reduction in synchronous Ca\textsuperscript{2+} release early in the action potential (AP) of failing cardiac myocytes promotes the appearance of late Ca\textsuperscript{2+} sparks which can propagate, forming Ca\textsuperscript{2+} ripples and waves. These, in turn, produce an inward sodium–calcium exchange current which opposes AP repolarization. Restoration of AP phase 1 repolarization improved Ca\textsuperscript{2+} release synchrony and reduced late Ca\textsuperscript{2+} spark rate, suggesting a different approach to reducing the risk of sudden death in heart failure.

Author contributions: J.C.H. and M.B.C. designed research; E.D.F., N.W., M.H., and G.C. performed research; E.D.F., N.W., and M.B.C. analyzed data; E.D.F., J.C.H., and M.B.C. wrote the paper; and J.C.H. and M.B.C. assisted with funding acquisition.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1918649117/-/DCSupplemental.

were not triggered earlier during the AP (15, 22). The increased less uniform increase in Ca\textsuperscript{2+} are shown (CON 10/3 HF Representative currents at the indicated test potentials (mV) symbols. (Scale bars: 5 pA/pF, 10 ms.) (Fig. 1)

The increase in synchronous Ca\textsuperscript{2+} in HF. (CON, N = 16/6 CON; 14/5 HF. **

In this exemplar, I\textsubscript{net} was only ~0.18 pA/pF during the late AP re-polarization phase, and the average I\textsubscript{net} during AP repolarization was 0.26 ± 0.08 pA/pF (mean ± SD n/N = 15/4), showing that a small change in membrane currents will affect repolarization trajectory. It follows that any additional inward current of similar amplitude to I\textsubscript{net} could stop AP repolarization (SI Appendix, Fig. S5) and thereby initiate an EAD.

An increase in intracellular Ca\textsuperscript{2+} moves the electrochemical gradient for NCX Ca\textsuperscript{2+} transport outward, causing an inward shift of I\textsubscript{NCX} (25) which can be up to ~1 pA/pF in magnitude (26). To examine whether the observed LCS activity could produce sufficient I\textsubscript{NCX} to oppose I\textsubscript{net}, we voltage-clamped cells with a failing AP which included steps to different holding potentials around the times (and V\textsubscript{m}) where EADs occur (Fig. 2C). During these V\textsubscript{m} steps, many LCS occurred which triggered Ca\textsuperscript{2+} ripples and Ca\textsuperscript{2+} waves. The average Ca\textsuperscript{2+} (blue trace) and evoked inward current (black trace) are shown in Fig. 2D. Lower. This inward current had a linear relationship to mean Ca\textsuperscript{2+} with a slope of ~1.5 pA/pF/μmol/L (SI Appendix, Fig. S6), compatible with the expected Ca\textsuperscript{2+} dependence of I\textsubscript{NCX} (26). The critical inward current (from Fig. 2B) that will balance I\textsubscript{NCX} and stop repolarization is indicated by the dotted line which, after projection onto the Ca\textsuperscript{2+} records, reveals the Ca\textsuperscript{2+} changes associated with this critical current density. It is notable that this current density is developed just before the onset of Ca\textsuperscript{2+} waves which appear as chevron patterns in line scans (5, 7), showing that LCS and/or Ca\textsuperscript{2+} ripples can be the initiating events for EADs.

Fig. 2E shows that multiple Ca\textsuperscript{2+} ripples, rather than Ca\textsuperscript{2+} waves, can also give rise to EADs in current-clamped cells. In this exemplar, the line scan Ca\textsuperscript{2+} image (upper panel) is rather chaotic with no obvious Ca\textsuperscript{2+} waves being present. However, after image processing, numerous LCS events are seen, some of which may provoke EADs (24). With this protocol, LCS activity increased, and EADs appeared (Fig. 2A). The high rate of LCS production (and appearance of Ca\textsuperscript{2+} ripples; see SI Appendix, Fig. S4) in these conditions clearly opposed the normal decline of Ca\textsuperscript{2+} during the Ca\textsuperscript{2+} transient.

In order to generate an EAD, the net cellular repolarizing current (I\textsubscript{net}) and/or repolarization reserve (11, 12) must be decreased and/or counteracted by depolarizing membrane current. While I\textsubscript{Ca} is the sum of all inward and outward currents, the rate of change of membrane potential (dV/dt) is directly proportional to I\textsubscript{net} because I\textsubscript{net} = -C\textsubscript{m}dV/dt (where C\textsubscript{m} is membrane capacitance). Applying this equation allows us to determine how much current is responsible for the repolarization phase of the AP, and Fig. 2B illustrates the magnitude and time course of I\textsubscript{net}.

The reduction in synchronous Ca\textsuperscript{2+} release in HF, seen as a less uniform increase in Ca\textsuperscript{2+} after the AP upstroke (Fig. 1E, arrow), was associated with an increase in the number of LCS (Fig. 1 G and H). This ~3-fold increase in LCS frequency can be explained by an increase in the number of Ca\textsuperscript{2+} release sites that were not triggered earlier during the AP (15, 22). The increased availability of Ca\textsuperscript{2+} release sites also promotes sequential activation of LCS, resulting in Ca\textsuperscript{2+} ripples (15), an example of which can be seen within the yellow dashed box in Fig. 1G.

**Linkage between LCS and Arrhythmogenesis**. LCS activity depends on SR Ca\textsuperscript{2+} content (15) which generally increases with AP frequency (10, 23). A pacing-pause protocol increases SR Ca\textsuperscript{2+} and may provoke EADs (24). With this protocol, LCS activity increased, and EADs appeared (Fig. 2A). The high rate of LCS production (and appearance of Ca\textsuperscript{2+} ripples; see SI Appendix, Fig. S4) in these conditions clearly opposed the normal decline of Ca\textsuperscript{2+} during the Ca\textsuperscript{2+} transient.

Model Analysis of the Role of I\textsubscript{Ca} and I\textsubscript{NCX} in EADs. The importance of I\textsubscript{NCX} for EAD generation has also been demonstrated in murine cells by heterozygous NCX knockdown and in failing rabbit ventricle by acute NCX blockade (27, 28). To further test the idea that sufficient I\textsubscript{NCX} can be generated by LCS to trigger an
During repolarization, an initial $V_{m}$ inflection at $\sim -20$ mV generates EADs (Top), and numerous LCS create oscillatory Ca$^{2+}$ release (Bottom). (B) Calculation of $I_{net}$ for a typical HF AP. The filled region indicates where the greatest number of LCS occur (A and Fig. 4E) and corresponds to an $I_{net}$ of $\sim 0.18$ pA/pF. Any balancing current that counteracts $I_{net}$ (i.e., $-I_{net}$, Bottom) will stop repolarization (SI Appendix, Fig. S5). (C) Failing AP with voltage-clamp steps used to probe Ca$^{2+}$ and currents underlying EADs. (D) During the AP steps, LCS-triggered Ca$^{2+}$ waves (Top) and inward currents (black trace) appear. Dashed line shows the inward current that balances $I_{net}$ in B and could therefore stop repolarization. This current occurs when LCS are about to initiate Ca$^{2+}$ waves. (E) Example of apparently chaotic Ca$^{2+}$ during EADs in an HF cell (Top). Image processing reveals many LCS and Ca$^{2+}$ ripples during $V_{m}$ oscillations (red trace). The filled regions show where the depolarizing current ($I_{net}$) is increasing, and this corresponds to increasing Ca$^{2+}$ (blue) and LCS activity (orange bars).

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Discussion

Reactivation of ICa and INa to overcome the repolarization reserve and initiate EADs has been widely considered to be the primary mechanism for EAD generation (illustrated by the inner feedback loop shown in Fig. 5), e.g., refs. 11, 31, and 32. Indeed, when SR Ca\textsuperscript{2+} release was inhibited by blocking LTCCs, it was still possible to evoke EADs by a simulated ICa (33). Here we show that LCS-activated inward NCX current can initiate EADs and, we suggest, act as a coupled oscillator to further increase the risk for development of multiple EADs (outer loop in Fig. 5). This Ca\textsuperscript{2+} oscillator arises from two connected mechanisms: (1) Diffusion of Ca\textsuperscript{2+} from an initiating LCS may trigger additional LCS due to the gain inherent in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR). This SR load-dependent process manifests as propagating Ca\textsuperscript{2+} ripples and, if enough LCS sites are available, more synchronous and larger Ca\textsuperscript{2+} waves. (2) LCS will increase inward NCX current to depolarize Vm (34) while Vm couples back onto LCS activity via the Vm dependence of LTCC gating (which may explain the Vm dependence of LCS frequency shown in Fig. 4E). The stochastic nature of LCS provides an explanation for the sudden appearance of arrhythmogenic EADs, and, once started, an EAD will promote additional LCS and EADs due to the increased SR Ca\textsuperscript{2+} load arising from ICa reactivation.

The computer model clearly showed that both the electrical (Vm) and Ca\textsuperscript{2+} oscillators can couple and synergize (Fig. 4A). While the phase relationship between LCS-dependent NCX currents and dVm/dt directly supports the idea that INCX can initiate Ca\textsuperscript{2+} dominant oscillations, ICa reactivation is also important (35) because late LTCC openings are a potent trigger for LCS (15). However, when Ca\textsuperscript{2+} levels are lower, and Ca\textsuperscript{2+} ripples and waves cannot form, EADs can still arise from instability in the repolarization process due to recruitment of noninactivated inward currents in the presence of insufficient repolarization reserve (11). In the latter case, smaller numbers of LCS may still play a lesser role in EAD initiation, and this is reminiscent of the role played by diastolic Ca\textsuperscript{2+} sparks in cardiac pacemaking by sino-atrial node cells (36).

Ca\textsuperscript{2+} Ripples, Ca\textsuperscript{2+} Waves, and Oscillator Coupling. Although Ca\textsuperscript{2+} waves (Fig. 2D) have been implicated in EAD genesis (37), it is notable that the fluctuations in Ca\textsuperscript{2+} shown in Fig. 2A and E are not typical Ca\textsuperscript{2+} waves (as seen in Fig. 2D) (6, 7); instead, the Ca\textsuperscript{2+} fluctuations came from low-amplitude Ca\textsuperscript{2+} ripples. In such cases, the local propagation of Ca\textsuperscript{2+} release (short-range Ca\textsuperscript{2+} ripples) may not be able to initiate cell-wide Ca\textsuperscript{2+} waves because the effective amplification of Ca\textsuperscript{2+} release by CICR is insufficient for full Ca\textsuperscript{2+} wave support. This lack of sufficient amplification
could be explained by the stochastic nature of LCS, coupled with local SR refractoriness (15, 38), decreasing the recruitment of adjacent LCS sites. Nevertheless, some synchronization in Ca\(^{2+}\) ripple initiation must occur for average Ca\(^{2+}\) to oscillate. The mathematical basis for the emergence of macroscopic Ca\(^{2+}\) oscillations from large numbers of independent LCS oscillators is beyond the scope of this study, but Kuramoto model analysis has shown that macroscopic oscillations can develop even when coupling across individual oscillators is weak (39). The relative importance of the Ca\(^{2+}\) and V\(_m\) oscillators in EAD generation will be variable because they depend on many factors such as the actual V\(_m\) trajectory, K\(^+\) current availability, the level of SR Ca\(^{2+}\) loading in the cell, and \(I_{Ca}\) availability, as well as RYR2 Ca\(^{2+}\) sensitivity, as illustrated in Fig. 5. In connection with this point, increased LTCC activity and SR load associated with \(\beta\)-adrenergic stimulation (10) would almost certainly increase the risk for LCS-stimulated EADs. It should be noted that the LCS activity seen in our confocal line scans reflects only a small fraction of the actual number of LCS occurring in the entire cell; the confocal line scan surveys ~2% of the cell volume, so ~1 LCS/ms (Fig. 2E) would correspond to ~50 LCS/ms cell-wide, and this much larger number underlies the measured NCX current that can initiate the EAD. This estimate is in reasonable agreement with the computer model used here which predicts that 107 LCS/ms may generate 0.26 pA/PF NCX current (29).

Since LTCC activity is common to both the V\(_m\) and Ca\(^{2+}\) oscillators, the idea that LTCC gating modification could be a therapeutic target for EAD prevention (33) becomes even more attractive. In addition, if modifying the late component of \(I_{Ca}\), is able to reduce net Ca\(^{2+}\) influx into the cell, SR Ca\(^{2+}\) content might be reduced (40), and this would also inhibit LCS activity (15). The improvement in Ca\(^{2+}\) signaling produced by applying a normal AP to HF myocytes is remarkable. This suggests that new therapies should be developed with the aim of improving early Ca\(^{2+}\) release by restoring phase I repolarization and/or restoring t-tubule regularity. This would reduce LCS frequency and thereby reduce the risk for potentially lethal LCS-triggered arrhythmias as well as mitigate the defective excitation–contraction coupling seen in HF (41).

**Materials and Methods**

More extensive details are available in SI Appendix. Briefly, all experiments were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and institutional approval by the University of Bristol ethics committee. We used an established coronary artery ligation model that leads to heart failure in adult New Zealand White rabbits (3–3.5 kg) which were daily monitored for health status. The target endpoint for HF was an ejection fraction of 40% (as measured by echocardiography SI Appendix, Table S1). This model, due to repolarizing current behavior, can be used to gain insight into repolarization reserve with human relevance (42). It was not possible to wait for arrhythmias to start in this model (as they would be fatal), but EADs can be provoked in vitro by suitable interventions. Rather than use pharmacological manipulation to provoke EADs, we chose a pacing-pause method to minimize other possible perturbations of cellular function.

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Fig. 5. Flow diagram for two interacting positive-feedback mechanisms that can drive \(V_{m}\) and Ca\(^{2+}\) oscillations during EADs. The red inner cycle represents the well-established V\(_m\) oscillator, wherein an increase in \(dV_{m}/dt\) (caused by a relative increase in depolarizing currents compared to repolarizing currents, e.g., text in orange box at Lower Right) leads to voltage-dependent reactivation of \(I_{Ca}\), which in turn causes further depolarization. The blue outer cycle represents the stochastic LCS-mediated Ca\(^{2+}\) oscillator mechanism for EAD initiation. Reduced early Ca\(^{2+}\) release and/or increased SR load increases LCS production, and the resulting increase in \(K^{+}\) influx tends to depolarize the membrane which then feeds onto \(I_{Ca}\) to trigger additional LCS. Green arrows indicate possible factors contributing to EAD initiation. \(\beta\)-AR, \(\beta\)-adrenoreceptor.

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**Fig. 4.** Applying a normal AP to HF cells improves excitation–contraction coupling and reduces LCS frequency. (A) Ca\(^{2+}\) transient upstroke velocity, amplitude (orange), and Ca\(^{2+}\) release synchrony all increase in an HF cell (Left) when voltage-clamped with a normal AP (Right). (Lower) Improved synchrony in Ca\(^{2+}\) release. (B) Mean latency for Ca\(^{2+}\) release and (C) Ca\(^{2+}\) transient duration were reduced in HF cells clamped with a normal AP. (D) Processed Ca\(^{2+}\) images show fewer LCS in HF after applying normal AP. (E) Number of LCS detected in HF with failing AP waveform (HF AP, 404 LCS) and with a normal AP (+Norm AP, 217 LCS) from 13 HF cells binned by \(V_{m}\) during the AP repolarization. **P < 0.01, paired t test \(n/N = 13/5\).
Cardiac Myocyte Isolation. Left ventricular epicardial myocytes were obtained from rabbit hearts after full anesthesia (50 mg/kg sodium pentobarbital i.v.) and subsequent diastolic dissociation was carried out using 1 mg/ml collagenase I ( Worthington), 0.05 mg/ml protease (type XIV Sigma), and 0.1 mmol/L CaCl₂, as described previously (22). Guinea pig, rat, mouse, and zebrafish myocytes were isolated using similar methods to those described previously (22, 43, 44), and methods for zebrafish myocyte isolation are given in SI Appendix.

Electrophysiology. Electrophysiology experiments were performed in a modified Tyrode solution (containing, in mmol/L: 133 NaCl, 5 KCl, 1 NaHPO₄, 10 (2-hydroxyethyl) 1-piperazineethanesulfonic acid (HEPES), 10 glucose, 1.8 CaCl₂ from rabbit hearts after full anesthesia (50 mg/kg sodium pentobarbital i.v.) and pH 7.4 with NaOH) at 36 ± 1 °C. Patch pipettes were pulled from borosilicate glass using a P80 micropipette puller (Sutter Instruments). Pipettes were filled with an intracellular solution containing, in mmol/L: 120 aspartic acid, 20 KCl, 10 HEPES, 10 NaCl, 5 glucose, 5 MgATP, 0.05 Fluo-4 pentapotassium salt, with KOH added to adjust to pH 7.2. Tip resistance was typically 1.6–2.0 MΩ when filled with this solution. Membrane potential and currents were recorded using an Axopatch 1D amplifier (Molecular Devices), Power1401 digitizer (Cambridge Electronic Design), and Signal data acquisition software (version 6.04, Cambridge Electronic Design). Cell membrane capacitance was measured by step depolarizations to −75 mV from a holding potential of −80 mV for 25 ms. Series resistance was compensated by −70%. Liquid junction potential (10 mV) was subtracted from recordings.

Confocal Imaging. Ca²⁺ sparks and transients were recorded in line scan mode (45) from the fluo-4 loaded cells using an inverted confocal microscope (LSM 880, Zeiss) with a 1.4 NA 63× oil immersion lens. Excitation light was provided by a 488-nm argon laser, and fluorescence emission was collected at 492–600 nm. Ca²⁺ line scans were recorded with the pinhole set to <2 Air units, at a pixel size of 0.1–0.2 μm/pixel, and with a scan speed of 1 ms per line. GaAsP photode- tectors were used to increase the sensitivity of Ca²⁺ spark detection. The t-tubule system was imaged by labeling the sarcolemma with di-B-ANEPS from a stock 1 mmol/L solution (in anhydrous dimethyl sulfoxide) added directly to the cell-recording chamber (final concentration 1 μmol/L for 2–3 min. Excitation was at 488 nm, and emission was collected at >600 nm.

Statistical Analysis. Statistical analyses were performed at the level of the cell, and statistics on replicates of n individual independent cell experiments from N animals are given in the text as n±x. Data were tested for normality using the Shapiro-Wilk test (Prism7, Graphpad); in any cases where data were skewed, the test was reapplied to log-transformed data. Paired or unpaired t-tests were performed on normally and log-normally distributed data. Results are presented as mean ± SEM. The limit of statistical confidence was P < 0.05.

Data Availability Statement. All data and computer codes are available upon request from the authors.

ACKNOWLEDGMENTS. We thank Drs. H. Cheng (BHF PG/15/55/22277 to J.C.H.), A. H. R. (BHF PG/15/55/22277 to J.C.H.), and R. J. Richardson for supplying some of the myocytes used for SI Appendix, Fig. 51 and Prof. G. L. Smith (University of Glasgow) for help and advice on HF model development.