Optical Mapping of Sarcoplasmic Reticulum Ca$^{2+}$ in the Intact Heart:  
Ryanodine Receptor Refractoriness During Alternans and Fibrillation

Lianguo Wang1, Rachel C. Myles2, Nicole M. De Jesus1, Alex K.P. Ohlendorf1, Donald M. Bers1, and Crystal M. Ripplinger1

1Department of Pharmacology, School of Medicine, University of California, Davis, and; 2Institute of Cardiovascular & Medical Sciences, University of Glasgow, Glasgow, UK

Running title: Sarcoplasmic Reticulum Ca$^{2+}$ and Cardiac Alternans

Subject codes:
[132] Arrhythmias-basic studies

Address correspondence to:
Dr. Crystal M. Ripplinger
Department of Pharmacology
UC Davis School of Medicine
2219A Tupper Hall
One Shields Ave
Davis, CA 95616
Tel: 530-752-1569
Fax: 530-752-7710
crippinger@ucdavis.edu

In January 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.35 days.
ABSTRACT

Rationale: Sarcoplasmic reticulum (SR) Ca\(^{2+}\) cycling is key to normal excitation-contraction coupling but may also contribute to pathological cardiac alternans and arrhythmia.

Objective: To measure intra-SR free [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{\text{SR}}\)) changes in intact hearts during alternans and ventricular fibrillation (VF).

Methods and Results: Simultaneous optical mapping of V\(_{m}\) (with RH237) and [Ca\(^{2+}\)]\(_{\text{SR}}\) (with Fluo-5N AM) was performed in Langendorff-perfused rabbit hearts. Alternans and VF were induced by rapid pacing. SR Ca\(^{2+}\) and action potential duration (APD) alternans occurred in-phase, but SR Ca\(^{2+}\) alternans emerged first as cycle length was progressively reduced (217±10ms vs. 190±13ms, p<0.05). Ryanodine receptor (RyR) refractoriness played a key role in the onset of SR Ca\(^{2+}\) alternans, with SR Ca\(^{2+}\) release alternans occurring without changes in diastolic [Ca\(^{2+}\)]\(_{\text{SR}}\). Sensitizing RyR with caffeine (200μM) significantly reduced the pacing threshold for both SR Ca\(^{2+}\) and APD alternans (188±15ms and 173±12ms, p<0.05 vs. baseline). Caffeine also reduced the magnitude of spatially discordant SR Ca\(^{2+}\) alternans, but not APD alternans, the pacing threshold for discordance, or threshold for VF. During VF, [Ca\(^{2+}\)]\(_{\text{SR}}\) was high, but RyR remained nearly continuously refractory, resulting in minimal SR Ca\(^{2+}\) release throughout VF.

Conclusions: In intact hearts RyR refractoriness initiates SR Ca\(^{2+}\) release alternans, that can be amplified by diastolic [Ca\(^{2+}\)]\(_{\text{SR}}\) alternans and lead to APD alternans. Sensitizing RyR suppresses spatially concordant, but not discordant SR Ca\(^{2+}\) and APD alternans. Despite increased [Ca\(^{2+}\)]\(_{\text{SR}}\) during VF, SR Ca\(^{2+}\) release was nearly continuously refractory. This novel method provides insight into SR Ca\(^{2+}\) handling during cardiac alternans and arrhythmia.

Keywords: Fluo-5N, SR Ca\(^{2+}\), optical mapping, alternans, arrhythmia, ventricular fibrillation, sarcoplasmic reticulum, Nonstandard Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APD</td>
<td>action potential duration</td>
</tr>
<tr>
<td>APD(_{90})</td>
<td>action potential duration at 90% repolarization</td>
</tr>
<tr>
<td>[Ca(^{2+})](_{\text{I}})</td>
<td>intracellular Ca(^{2+})</td>
</tr>
<tr>
<td>[Ca(^{2+})](_{\text{SR}})</td>
<td>intra-sarcoplasmic reticulum free Ca(^{2+})</td>
</tr>
<tr>
<td>CDI</td>
<td>Ca(^{2+})-dependent inactivation</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca(^{2+})-induced Ca(^{2+}) release</td>
</tr>
<tr>
<td>CV</td>
<td>conduction velocity</td>
</tr>
<tr>
<td>DAD</td>
<td>delayed afterdepolarization</td>
</tr>
<tr>
<td>EAD</td>
<td>early afterdepolarization</td>
</tr>
<tr>
<td>ECC</td>
<td>excitation-contraction coupling</td>
</tr>
<tr>
<td>ECG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>I(_{\text{Ca}})</td>
<td>L-type Ca(^{2+}) current</td>
</tr>
<tr>
<td>ISO</td>
<td>isoproterenol</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>NCX</td>
<td>Na(^{+})-Ca(^{2+}) exchanger</td>
</tr>
<tr>
<td>PCL</td>
<td>pacing cycle length</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>S1</td>
<td>cycle length of pacing drive train</td>
</tr>
<tr>
<td>S2</td>
<td>cycle length of premature pacing stimulus</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>VF</td>
<td>ventricular fibrillation</td>
</tr>
<tr>
<td>V(_{m})</td>
<td>transmembrane potential</td>
</tr>
</tbody>
</table>
INTRODUCTION

In cardiac muscle, Ca\(^{2+}\) release from and reuptake into the sarcoplasmic reticulum (SR) plays a central role in contraction, and tight regulation of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) is required for proper excitation-contraction coupling (ECC).\(^1\) Experimental studies and computational modeling have revealed that SR Ca\(^{2+}\) handling can also contribute to arrhythmogenic behavior. In particular, abnormal intracellular Ca\(^{2+}\) handling has been demonstrated to underlie the development of cardiac alternans,\(^2\,8\) which is not only associated with lethal ventricular arrhythmia in patients,\(^9\,10\) but has also been shown to be mechanistically important in the development of re-entrant arrhythmias through the development of spatially discordant alternans.\(^11\,12\)

At the cellular level, beat-to-beat alternation in the amplitude of the intracellular Ca\(^{2+}\) transient has been shown to underlie repolarization alternans,\(^8\) which is in turn expressed as clinically observed T-wave alternans.\(^11\) Various subcellular mechanisms governing the development of intracellular Ca\(^{2+}\) alternans have been proposed, including alternating L-type Ca\(^{2+}\) current (I\(_{Ca}\)),\(^13\) alternating diastolic SR Ca\(^{2+}\) load,\(^3\,14\) and alternating refractoriness of the SR release channel (ryanodine receptor [RyR]).\(^4\,15\) Many of these detailed mechanistic investigations have been carried out in isolated cardiac myocytes, where total SR Ca\(^{2+}\) content can be measured with caffeine pulses\(^3\) or free intra-SR [Ca\(^{2+}\)] (\([\text{Ca}^{2+}]_{\text{SR}}\)) optically monitored with a low affinity Ca\(^{2+}\) indicator.\(^4\,15\) However, these single cell studies provide little insight into the spatially heterogeneous nature of SR Ca\(^{2+}\) cycling and how this impacts the emergence, severity and concordance of cardiac alternans in myocardial tissue. Thus, it is difficult to directly extrapolate findings in isolated cells to the intact heart. Furthermore, arrhythmogenic behavior such as spatially discordant alternans, and consequent ventricular fibrillation (VF), are inherently tissue-level phenomena and thus can only be studied in the intact heart.

Experimental investigations into the mechanisms of Ca\(^{2+}\) alternans in the intact heart and the role of Ca\(^{2+}\) in arrhythmogenesis have predominantly utilized wide-field optical mapping, which can record signals over multiple sites, but, until now, have been limited to dual mapping of transmembrane potential (V\(_{m}\)) and intracellular Ca\(^{2+}\), and so have been unable to directly examine SR Ca\(^{2+}\) kinetics. In this study, we report, for the first time simultaneous mapping of V\(_{m}\) and \([\text{Ca}^{2+}]_{\text{SR}}\) across the surface of the intact heart and use this novel approach to investigate the role of SR Ca\(^{2+}\) in cardiac alternans and VF. We further investigated the role of RyR refractoriness by sensitizing the RyR with low-concentration (200 μM) caffeine, and the combined effects of sensitized RyR and increased SR Ca\(^{2+}\)-ATPase (SERCA) activity through β-adrenergic receptor (β-AR) stimulation with isoproterenol (ISO, 100 nM).

METHODS

An expended Methods section is provided in the online-only Data Supplement.

All procedures involving animals were approved by the Animal Care and Use Committee of the University of California, Davis and adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Male New Zealand White rabbits (n=27) were anesthetized with an intravenous injection of pentobarbital sodium (50 mg/kg). Hearts were rapidly removed and Langendorff-perfused as described previously.\(^16\) An electrocardiogram (ECG) was recorded continuously, and pacing was from the base of the left ventricular (LV) epicardium.

To optically monitor intra-SR free \([\text{Ca}^{2+}]\), hearts were loaded with the low-affinity Ca\(^{2+}\) indicator fluo-5N AM (Invitrogen, Carlsbad, CA) for 60 min at room temperature.\(^17\) Hearts were subsequently stained with the voltage-sensitive dye RH237 (Invitrogen, Carlsbad, CA). All experiments were performed at 37°C. In a separate set of hearts (n=3), dual optical mapping of V\(_{m}\) with RH237 and intracellular Ca\(^{2+}\) with Rhod-2

DOI: 10.1161/CIRCRESAHA.114.302505  3
AM (Invitrogen, Carlsbad, CA) was performed as previously described.\textsuperscript{16} To induce alternans and ventricular arrhythmia, the pacing cycle length (PCL) was decremented in 10 ms steps until loss of 1:1 capture or induction of VF (Online Figure III). In a subset of animals, either low-concentration caffeine (200 μM, n=6) to sensitize RyR\textsubscript{18} or ISO (100 nM, n=3) to stimulate β-AR, was added to the perfusate.

Data analysis was performed using two commercially available analysis programs (BV\textsuperscript{Analyze}, Brainvision, Tokyo, Japan; and Optiq, Cairn, UK). \( V_m \) and \([\text{Ca}^{2+}]_{\text{SR}}\) datasets were spatially aligned and processed with a Gaussian spatial filter (radius 3 pixels). The spectral method, which has been used clinically for detecting micro-volt T-wave alternans,\textsuperscript{10} was used to detect the presence of significant APD and SR \( \text{Ca}^{2+} \) alternans as previously described.\textsuperscript{19} The magnitude of SR \( \text{Ca}^{2+} \) release alternans was calculated as 1 minus the ratio of the average small beat (S) release amplitude to the average large beat (L) release amplitude (1-S/L). Diastolic SR \( \text{Ca}^{2+} \) load alternans was calculated as the average difference between diastolic levels (D) of S and L beats divided by the average L amplitude (D/L) during a 1-2 sec recording (Figure 3B). Data are expressed as mean±standard deviation (SD) and were compared using Student's t-tests, paired where appropriate. \( P<0.05 \) was considered statistically significant.

**RESULTS**

*Simultaneous optical mapping of \( V_m \) and \([\text{Ca}^{2+}]_{\text{SR}}\).*

To simultaneously map \( V_m \) and \([\text{Ca}^{2+}]_{\text{SR}}\) signals at high spatial and temporal resolution, hearts were loaded with RH237 and Fluo-5N AM. Online Figure IA shows a schematic diagram of the optical setup for dual optical mapping of \( V_m \) and \([\text{Ca}^{2+}]_{\text{SR}}\). When hearts were loaded with either RH237 or Fluo-5N AM alone, no significant cross-talk or bleed-through of signal was observed in the other channel (Online Figure IB), confirming complete spectral separation between the \( V_m \) and \([\text{Ca}^{2+}]_{\text{SR}}\) signals. To validate \([\text{Ca}^{2+}]_{\text{SR}}\) signal kinetics and the dynamic spatial and temporal relationship between \( V_m \) and \([\text{Ca}^{2+}]_{\text{SR}}\), hearts were paced at a PCL of 300 ms. SR \( \text{Ca}^{2+} \) transients had the expected morphology of a rapid downstroke following the AP upstroke and a monotonic increase back to baseline levels as \( \text{Ca}^{2+} \) is pumped back into the SR (Figure 1A, i and iii). As expected from \([\text{Ca}^{2+}]_i\) measurements,\textsuperscript{16} the \( V_m \) upstroke preceded the \([\text{Ca}^{2+}]_{\text{SR}}\) downstroke by an average of 9.2±1.6 ms (n=7).

*Frequency-dependent \([\text{Ca}^{2+}]_{\text{SR}}\) changes.*

To test the response of \([\text{Ca}^{2+}]_{\text{SR}}\) to changes in heart rate, optical recordings were made during progressive increases in pacing rate (Figure 1B). Diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) increased rapidly (within 1-2 beats) to a new steady state as the PCL decreased, with alternans occurring at shorter PCLs (Figure 1B, iii). Once pacing was stopped, diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) quickly returned to the baseline level as a result of a larger release during the first sinus beat, which occurs after a pause (Figure 1B, iv). These traces were normalized as indicated in Figure 1B and normalized mean diastolic and systolic \([\text{Ca}^{2+}]_{\text{SR}}\) with changes in PCL are shown in Figure 1C (n=3).

*Pacing-induced APD and SR \( \text{Ca}^{2+} \) alternans.*

Alternans was induced by decrementing the PCL in 10 ms steps. An example of increasing alternans magnitude with decreasing PCL is shown in Figure 2. In this example, significant SR \( \text{Ca}^{2+} \) alternans was induced at PCL=220 ms, while significant APD alternans did not occur until 190 ms. The PCL threshold for SR \( \text{Ca}^{2+} \) alternans was significantly longer than for \( V_m \) alternans (217±10 vs. 190±13 ms, \( P<0.05 \)). APD and SR \( \text{Ca}^{2+} \) alternans normally occurred in-phase (large SR \( \text{Ca}^{2+} \) release corresponding...
to long APD and vice versa). Both APD and SR Ca\(^{2+}\) alternans progressively increased with decreasing PCL, as shown in the maps of spectral magnitude (Figure 2A, 2B) and in example traces (Figure 2C, 2D).

**Role of diastolic [Ca\(^{2+}\)]\(_{SR}\) during alternans.**

Diastolic [Ca\(^{2+}\)]\(_{SR}\) has the ability to change on a beat-to-beat basis in response to increasing heart rate, as shown in Figure 1B. Changes in diastolic [Ca\(^{2+}\)]\(_{SR}\) can also contribute to Ca\(^{2+}\) alternans; with a larger diastolic SR Ca\(^{2+}\) load facilitating a larger SR Ca\(^{2+}\) release on the subsequent beat and a lower load leading to a smaller release.\(^3\) To investigate the role of diastolic [Ca\(^{2+}\)]\(_{SR}\) in contributing to alternans in the intact heart, the emergence of alternation in diastolic [Ca\(^{2+}\)]\(_{SR}\) was compared to alternation of SR Ca\(^{2+}\) release. SR Ca\(^{2+}\) release alternans occurring without changes in diastolic [Ca\(^{2+}\)]\(_{SR}\) were routinely observed (Figure 3B, i). SR Ca\(^{2+}\) release alternans typically occurred before diastolic [Ca\(^{2+}\)]\(_{SR}\) alternans (i.e., at longer PCLs), with significant SR Ca\(^{2+}\) load alternans only occurring at shorter PCLs (Figure 3D, 3E). Due to heterogeneity of SR Ca\(^{2+}\) handling throughout the heart, SR Ca\(^{2+}\) release alternans both with and without diastolic SR Ca\(^{2+}\) load alternans were routinely observed occurring simultaneously in the same heart at the same PCL (200 ms in the example of Figure 3A, 3B, 3D). Thus in the intact heart, diastolic [Ca\(^{2+}\)]\(_{SR}\) alternans are not required for SR Ca\(^{2+}\) release alternans to occur. We suggest that RyR refractoriness may initiate SR Ca\(^{2+}\) release alternans at longer PCLs, and might cause alternans of both diastolic [Ca\(^{2+}\)]\(_{SR}\) and APD as PCL shortens further.

**Role of RyR refractoriness on SR Ca\(^{2+}\) and APD alternans.**

RyR release kinetics play a key role in ECC and under normal conditions, the number of RyRs opening during CICR varies little from beat-to-beat, resulting in consistent SR Ca\(^{2+}\) release.\(^1\) However, when a shorter diastolic interval occurs, such as during a premature stimulus, a smaller SR Ca\(^{2+}\) release is observed due to incomplete recovery of RyRs from refractoriness. Caffeine sensitizes RyRs to Ca\(^{2+}\), and at high concentration, it can cause SR Ca\(^{2+}\) release even when the SR is refractory to activation by \(I_{Ca}\).\(^20\) Therefore, we tested whether low-concentration caffeine (200 μM) could modify RyR refractoriness and shift the alternans threshold to a shorter PCL. As expected, caffeine significantly reduced diastolic [Ca\(^{2+}\)]\(_{SR}\) after 20 min of treatment (Figure 4C, p<0.05). To quantify RyR recovery from refractoriness, we evaluated the ratio of the S2-induced SR Ca\(^{2+}\) release amplitude to the S1 release amplitude (S2/S1 ratio) at S2 coupling intervals ranging from 500-180 ms before and after sensitization of RyR with caffeine. Caffeine resulted in a significant increase in the S2/S1 ratio at S2 coupling intervals of 200 and 180 ms (p<0.05, Figure 4A, 4B), suggesting a decrease in RyR refractoriness with caffeine treatment. As expected, caffeine did not alter the time course of SR Ca\(^{2+}\) recovery (τ, Figure 4D).

In line with these data, RyR refractoriness was found to play a key role in the onset of APD and SR Ca\(^{2+}\) alternans. Figure 5 demonstrates the effect of caffeine (200 μM) on the emergence and severity of APD and SR Ca\(^{2+}\) alternans. The spectral magnitude of both APD and SR Ca\(^{2+}\) alternans at a PCL of 180 ms was reduced following caffeine treatment (Figure 5A, 5B vs. 5C, 5D). Figures 5E-5G show that caffeine significantly reduced the magnitude of SR Ca\(^{2+}\) release alternans at PCLs of 200-160ms (Figure 5E), the magnitude of diastolic SR Ca\(^{2+}\) load alternans at PCLs of 180-160ms (Figure 5F), and the pacing threshold for induction of both SR Ca\(^{2+}\) and APD alternans (baseline: 217±10 ms and 190±13 ms vs. caffeine: 188±15 ms and 173±12 ms, p<0.05 for SR Ca\(^{2+}\) and APD, respectively [Figure 5G]).

To further assess the role of RyR refractoriness on alternans and the interplay between RyR refractoriness and SERCA function, β-AR stimulation was performed with ISO (100 nM). Like caffeine, β-AR stimulation sensitizes RyR, but unlike caffeine, it also significantly increases SERCA function. As expected, ISO reduced SR Ca\(^{2+}\) release time (time to nadir) from 56.3±3.5 to 42.2±1.8 ms, and SR Ca\(^{2+}\) recovery (τ) decreased from 72.8±6.3 to 58.6±4.8 ms (p<0.05 for both, Online Figure VIB and C). ISO decreased the magnitude and pacing thresholds of SR Ca\(^{2+}\) and APD alternans from 247±6 to 173±12 ms (p<0.05) and 200±10 to 153±12 ms (p=0.06), respectively (Online Figures VII and VIII). Importantly,
increased SERCA function with ISO prevented diastolic SR Ca\(^{2+}\) load alternans even at very rapid pacing rates, yet alternans of SR Ca\(^{2+}\) release still occurred due to encroachment on RyR refractoriness (Online Figure VIII B-D).

Spatial heterogeneity of alternans and spatial discordance.

A key advantage of mapping-based techniques is the ability to record spatially heterogeneous phenomena, which may play an important role in the development of arrhythmia. Indeed, at the whole heart level, \(V_m\) alternans may become spatially discordant, where one area of tissue exhibits a long-short APD sequence and another area has the opposite, short-long sequence. Discordant alternans is associated with large spatial gradients of repolarization and can initiate re-entrant arrhythmias\(^{11, 21}\). In this study, spatial heterogeneity in the magnitude of alternans (of both APD and SR Ca\(^{2+}\)) was routinely observed with some areas seemingly more prone to the development of alternans than others (alternans emerge in these areas at longer PCLs and have a larger magnitude as the PCL is decreased). As demonstrated in Figures 2A and 2B, local onset of SR Ca\(^{2+}\) alternans was first observed in the central region of the mapping field of view at PCL=220 ms. APD alternans followed at a PCL of 190 ms and emerged at the same location. Interestingly, when spatially discordant alternans developed at PCL=150 ms, the area of largest alternans magnitude remained similar to the original onset location. Spatial heterogeneity of alternans magnitude was also clearly observed in Figure 5A, and remained even after caffeine treatment (Figure 5C). Additional examples of spatial heterogeneity of APD and SR Ca\(^{2+}\) alternans magnitude are shown in Online Figure V. Spatially discordant alternans were observed in 12 of 14 hearts with discordance emerging at the same PCL (140±12 ms). Not surprisingly, VF could only be induced in those hearts that first exhibited spatial discordance, supporting the role of discordant alternans in the initiation of VF.

Spatially discordant alternans and the role of RyR refractoriness.

To investigate the role of RyR refractoriness in contributing to spatially discordant alternans, the threshold and magnitude of discordant alternans were compared before and after 200 \(\mu\)M caffeine. Figure 6 shows an example of spatially discordant alternans from a heart paced at 140 ms under control conditions (Figure 6A-6B) and after caffeine (Figure 6C-6D). Spatially discordant APD and SR Ca\(^{2+}\) alternans were induced with distinct phases separated by black nodal lines (Figure 6A, 6C) both before and after caffeine. Example traces of APD and SR Ca\(^{2+}\) from two locations show opposite phases on either side of the nodal line (Figure 6B, 6D, black vs. white box), although APD and SR Ca\(^{2+}\) alternans remained in-phase with one another (i.e., large SR Ca\(^{2+}\) transient corresponds to long APD and vice versa). While 200 \(\mu\)M caffeine reduced the spectral magnitude of SR Ca\(^{2+}\) alternans at PCL=140ms (69±41 vs. 37±32 A.U., p<0.05), it did not reduce the spectral magnitude of APD alternans (20±21 vs. 19±12 A.U., p=0.9, Figure 6E), nor did it reduce the pacing threshold for induction of either spatially discordant APD or SR Ca\(^{2+}\) alternans (Figure 6F). Caffeine also did not affect the pacing threshold for induction of VF (118±8 vs. 113±6 ms, Figure 6G). These data suggest that mechanisms other than RyR kinetics, such as restitution of APD and/or conduction velocity (CV), may become more critical for the transition from spatially concordant to spatially discordant alternans and the transition of alternans to VF. Indeed, an analysis of CV restitution kinetics during alternans indicates that the transition from spatial concordance to spatial discordance occurs only after CV restitution is evoked (Online Figure IV).

Comparison of [Ca\(^{2+}\)]\(_{SR}\) and [Ca\(^{2+}\)]\(_{i}\) during VF.

To further investigate the role of [Ca\(^{2+}\)]\(_{SR}\) and [Ca\(^{2+}\)]\(_{i}\) during VF, hearts were loaded with either RH237 and Fluo-5N AM for dual imaging of \(V_m\) and [Ca\(^{2+}\)]\(_{SR}\), or RH237 and Rhod-2 AM for \(V_m\) and [Ca\(^{2+}\)]\(_{i}\). In 8 of 14 hearts, rapid pacing induced non-sustained VF. Figure 7A shows an example of a heart with dual imaging of \(V_m\) and [Ca\(^{2+}\)]\(_{SR}\) during ventricular pacing and VF. When normalized to the amplitude of the SR Ca\(^{2+}\) transient during ventricular pacing, very minimal SR Ca\(^{2+}\) release occurred during VF (<10%
of normal SR Ca\(^{2+}\) release amplitude, Figure 7A, iii), suggesting near complete RyR refractoriness during VF. Additional signal locations during this same VF episode are shown in Online Figure IX. On the other hand, dual imaging of \(V_m\) and [Ca\(^{2+}\)], (Figure 7B) shows that during VF, [Ca\(^{2+}\)], oscillated with a peak-to-peak amplitude that is approximately 30% of the normal intracellular Ca\(^{2+}\) transient amplitude during pacing (Figure 7B, iii). Although the absolute [Ca\(^{2+}\)]\(_{SR}\) cannot be quantified with Fluo-5N in the intact heart, a comparison of the fluorescence during VF and following spontaneous cardioversion to sinus rhythm showed that, during VF, [Ca\(^{2+}\)]\(_{SR}\) is approximately 30% higher than normal diastolic levels (Figure 8, iii).

**DISCUSSION**

In this study, we developed the first methodology for simultaneous optical mapping of \(V_m\) and SR Ca\(^{2+}\) at high spatial-temporal resolution in the intact heart. The voltage-sensitive dye RH237 and low-affinity Ca\(^{2+}\) indicator Fluo-5N AM were used together to map \(V_m\) and [Ca\(^{2+}\)]\(_{SR}\), respectively with high fidelity and no fluorescent cross-talk. We used this novel approach to investigate the role of SR Ca\(^{2+}\) in cardiac alternans and VF. The results demonstrate that 1) APD and SR Ca\(^{2+}\) alternans predominantly occur in-phase with one another, but SR Ca\(^{2+}\) alternans emerge first, at longer PCLs. As expected, both APD and SR Ca\(^{2+}\) alternans worsen with decreasing PCL. 2) RyR refractoriness plays a key role in the onset of SR Ca\(^{2+}\) alternans. This was demonstrated by the observation that SR Ca\(^{2+}\) release alternans often occur in the absence of diastolic [Ca\(^{2+}\)]\(_{SR}\) alternans and that release alternans tend to emerge at longer PCLs, with diastolic [Ca\(^{2+}\)]\(_{SR}\) alternans occurring as PCL decreases. Accordingly, sensitizing RyR with low-concentration caffeine (200 \(\mu\)M) reduced the pacing threshold for onset as well as the magnitude of SR Ca\(^{2+}\) and APD alternans. Even when SERCA function is sufficiently increased (with 100 nM ISO) to prevent alternans of diastolic [Ca\(^{2+}\)]\(_{SR}\), release alternans were not prevented due to encroachment on RyR refractoriness. 3) Spatially discordant alternans of both APD and SR Ca\(^{2+}\) emerge at even shorter PCLs. Although caffeine reduces the magnitude of spatially discordant SR Ca\(^{2+}\) alternans, it does not prevent the onset of discordance, nor does it reduce the magnitude of spatially discordant APD alternans. 4) During VF, [Ca\(^{2+}\)]\(_{SR}\) is substantially higher than during normal rhythms, but the RyRs remain almost continuously refractory with minimal Ca\(^{2+}\) release from the SR. This key finding may have important clinical implications for the treatment of VF and for preventing spontaneous re-initiation of arrhythmic events.

**Dual optical mapping of \(V_m\) and [Ca\(^{2+}\)]\(_{SR}\).**

Over the last few decades, dual optical mapping of \(V_m\) and intracellular Ca\(^{2+}\) in intact hearts has provided a wealth of information toward a more complete understanding of normal cardiac ECC and the mechanisms of ventricular arrhythmias.\(^{16, 22, 23}\) In mammalian hearts, the majority of the intracellular Ca\(^{2+}\) transient is comprised of Ca\(^{2+}\) release from and reuptake into the SR (approximately 70% in the rabbit heart).\(^{24}\) Thus, the ability to precisely discern whether changes in intracellular Ca\(^{2+}\) are due to transmembrane Ca\(^{2+}\) flux or SR Ca\(^{2+}\) release/reuptake provides important insight into the mechanisms of ECC and Ca\(^{2+}\)-mediated arrhythmias. Low-affinity fluorescent Ca\(^{2+}\) indicators, such as Fluo-5N and Mag-Fluo4, can be used for this purpose. These indicators have dissociation constants (\(K_d\)) in the range of 10-400 \(\mu\)M, and exhibit minimal fluorescence in the cytosol compared to the SR lumen and the fluorescence decreases upon SR Ca\(^{2+}\) release.\(^{25}\) Several groups have recently reported using these low-affinity Ca\(^{2+}\) indicators to optically monitor [Ca\(^{2+}\)]\(_{SR}\) in isolated myocytes to investigate fractional SR Ca\(^{2+}\) release\(^{25}\) and the mechanisms of Ca\(^{2+}\) alternans.\(^{4, 15}\) Furthermore, Mag-Fluo4, combined with a pulsed local field fluorescent microscope, has been used to record [Ca\(^{2+}\)]\(_{SR}\) from a single location on the epicardial surface of the intact mouse heart.\(^{17, 26}\) Although these studies have provided new and important information on SR Ca\(^{2+}\) release and reuptake during normal and pathological circumstances, until now, methods to image SR Ca\(^{2+}\) activity across the entire surface of the intact heart had not been developed. This spatial information is vitally important for further understanding the heterogeneous nature of SR Ca\(^{2+}\) cycling in the heart and
the role of SR Ca\(^{2+}\) in spatially distinct arrhythmic phenomena such as focal arrhythmia sources, spatially discordant alternans, and VF.

This study utilized dual optical mapping techniques to monitor, for the first time, \(V_m\) and \([Ca^{2+}]_{SR}\) simultaneously at high spatial and temporal resolution. We developed an optical setup using the voltage-sensitive indicator RH237 and the low-affinity Ca\(^{2+}\) indicator Fluo-5N AM that results in minimal fluorescent cross-talk between \(V_m\) and \([Ca^{2+}]_{SR}\) signals (Online Figure IB). More importantly, detailed analysis of signal kinetics and the dynamic temporal relationship between \(V_m\) and \([Ca^{2+}]_{SR}\) shows that SR Ca\(^{2+}\) transients have the expected morphology of a rapid downstroke, indicating SR Ca\(^{2+}\) release following the AP upstroke and a monotonic increase back to baseline levels as Ca\(^{2+}\) is pumped back into the SR (Figure 1A, i and iii). As expected, diastolic \([Ca^{2+}]_{SR}\) rapidly increases or decreases in response to changes in heart rate (Figure 1B-1C), consistent with studies in isolated cardiac myocytes.\(^{25}\) The average \(V_m-[Ca^{2+}]_{SR}\) delay of 9.2±1.6 ms measured with this approach is comparable to \(V_m-[Ca^{2+}]\) delays reported in previous studies.\(^{16, 22}\) These data indicate that dual optical mapping of \(V_m\) and \([Ca^{2+}]_{SR}\) in the intact heart is indeed a reliable tool for study of ECC and the mechanisms of cardiac arrhythmias.

**Mechanisms of SR Ca\(^{2+}\) alternans.**

Cardiac alternans play an important role in ventricular arrhythmias and have been studied extensively both clinically and experimentally.\(^{11, 27-29}\) Clinically, pacing- or exercise-induced T-wave alternans have been reported as a highly sensitive marker for sudden cardiac death.\(^{11, 27}\) The search for the mechanisms underlying T-wave alternans has revealed that intracellular Ca\(^{2+}\) alternans is a key determinant.\(^{8, 11, 29}\) Consistent with previous studies,\(^{4, 15}\) our results show that SR Ca\(^{2+}\) transients alternate as heart rate increases (Figure 2). While APD alternans are typically in-phase with SR Ca\(^{2+}\) alternans, APD alternans emerge at significantly faster heart rates (Figure 2E, 5G). Importantly, APD alternans emerge from the same location as do the earliest and strongest SR Ca\(^{2+}\) alternans (Figure 2A, 2B), indicating that SR Ca\(^{2+}\) alternans likely contribute to the development of APD alternans.

Diastolic \([Ca^{2+}]_{SR}\) varies little from beat to beat under normal conditions, indicating a balance between SERCA uptake and RyR release during each ECC cycle. An imbalance between SERCA uptake and RyR release may result in beat-to-beat alternation of diastolic \([Ca^{2+}]_{SR}\) and/or SR Ca\(^{2+}\) release. Studies performed in normal isolated myocytes show that either alternating diastolic \([Ca^{2+}]_{SR}\)\(^{3, 14}\) or alternating refractoriness of RyR\(^{4, 15}\) may contribute to SR Ca\(^{2+}\) alternans. In the intact heart, however, our results indicate that SR Ca\(^{2+}\) release alternans routinely occur without changes in diastolic \([Ca^{2+}]_{SR}\) (Figure 3B, i). While diastolic SR Ca\(^{2+}\) load alternans can also occur, they tend to emerge at faster heart rates, after the onset of release alternans (Figure 3E). Indeed it is logical that SR Ca\(^{2+}\) release alternans (when large enough) would cause alternation of diastolic \([Ca^{2+}]_{SR}\).\(^{4}\) A larger SR Ca\(^{2+}\) release can reduce net Ca\(^{2+}\) entry through \(I_{Ca}\) due to Ca\(^{2+}\)-dependent inactivation (CDI), and increase Ca\(^{2+}\) efflux via NCX, thus causing net cellular Ca\(^{2+}\) loss and secondary diastolic \([Ca^{2+}]_{SR}\) alternans. In this way, diastolic \([Ca^{2+}]_{SR}\) alternans may amplify Ca\(^{2+}\) release and APD alternans. Furthermore, at fast heart rates, SERCA may no longer be able to fully sequester all the Ca\(^{2+}\) released before the onset of the next action potential, thus further contributing to alternans of diastolic \([Ca^{2+}]_{SR}\). However, our data demonstrate that in the intact heart, diastolic \([Ca^{2+}]_{SR}\) alternans is not a requirement for SR Ca\(^{2+}\) release alternans to occur, suggesting that other mechanisms, such as RyR refractoriness may contribute to the onset of SR Ca\(^{2+}\) alternans.

**Role of RyR refractoriness in SR Ca\(^{2+}\) alternans.**

An important property of RyRs is that they have a period of refractoriness following each opening. Under steady-state conditions, the number of individual RyRs opening during CICR varies little from beat-to-beat, resulting in consistent SR Ca\(^{2+}\) release (Figure 1). However, as heart rate increases and the diastolic interval between beats becomes shorter, the recovery of RyRs from refractoriness limits SR Ca\(^{2+}\) release. If only a subset of RyRs have fully recovered from refractoriness, less Ca\(^{2+}\) will be released. On the next
beat, the portion of RyRs that were not available for release on the previous beat have now fully recovered
from refractoriness, resulting in a larger SR Ca\(^{2+}\) release. This alternation of SR Ca\(^{2+}\) release can lead to
intracellular Ca\(^{2+}\) alternans.

In this study, RyR refractoriness was demonstrated and quantified using an S1-S2 pacing protocol, where a
premature stimulus (S2) induces a smaller SR Ca\(^{2+}\) release compared to the previous S1-induced release (Figure 4A). As expected, sensitization of RyR with low-concentration caffeine\(^{15, 30}\) results in a significant increase in the S2-induced SR Ca\(^{2+}\) release (Figure 4A-4B), indicating a decrease in RyR refractoriness with caffeine. Interestingly, sensitization of RyR decreases the induction threshold and magnitude of not only SR Ca\(^{2+}\) alternans, but also APD alternans (Figure 5), indicating that RyR refractoriness plays a key role in the onset of both SR Ca\(^{2+}\) and APD alternans. β-AR stimulation also sensitizes RyR, but unlike caffeine, β-AR stimulation significantly increases SERCA activity (Online Figure VIC) via phosphorylation of phospholamban. Accordingly, β-AR stimulation with ISO reduced the magnitude and induction threshold for both SR Ca\(^{2+}\) and APD alternans (Online Figures VII and VIII), likely via the combined effects of reduced RyR refractoriness and increased SERCA activity. This increase in SERCA activity prevented alternans of diastolic [Ca\(^{2+}\)]\(_{\text{SR}}\) even at the fastest pacing rates (Online Figure VIII B and D), yet SR Ca\(^{2+}\) release alternans still occurred due to encroachment on RyR refractoriness (Online Figure VIII B and C), but as with caffeine, release alternans occurred at a faster pacing rate compared to baseline due to sensitization of RyR.

Spatially discordant alternans.

Experimental studies have revealed complex spatio-temporal patterns of cardiac alternans, including spatially discordant alternans which are particularly arrhythmogenic due to the extreme gradients of repolarization generated.\(^{11}\) Several mechanisms contributing to spatially discordant alternans have been identified, including CV restitution, spatial heterogeneities of Ca\(^{2+}\) cycling, and intercellular uncoupling.\(^{31}\) Due to the significant role RyR refractoriness plays in contributing to the onset of SR Ca\(^{2+}\) and APD alternans, we sought to determine if RyR refractoriness also contributes to the emergence or severity of spatially discordant alternans.

Spatially discordant APD and SR Ca\(^{2+}\) alternans were routinely induced both before and after caffeine treatment (Figure 6A, 6C). While caffeine reduced the severity of discordant SR Ca\(^{2+}\) alternans (as measured by the average spectral magnitude at PCL=140 ms, Figure 6E), it did not reduce the severity of discordant APD alternans, the induction threshold for discordance (Figure 6F), or the threshold for induction of VF (Figure 6G). These data suggest that other dynamical mechanisms such as restitution of CV may govern the onset and severity of spatially discordant alternans rather than SR Ca\(^{2+}\) handling.\(^{12, 21, 32}\) Indeed, an analysis of CV restitution kinetics during alternans revealed that the transition to spatial discordance occurs only after CV restitution is evoked (Online Figure IV). Thus, although RyR refractoriness plays a key role in the onset of concordant alternans, the transition to and maintenance of discordant alternans may rely more heavily on dynamical instabilities.

Role of SR Ca\(^{2+}\) and RyR refractoriness during VF.

The role of intracellular Ca\(^{2+}\) during VF has been studied previously.\(^{33-35}\) In those studies, diastolic intracellular Ca\(^{2+}\) overload was routinely observed, which may set the stage for spontaneous SR Ca\(^{2+}\) release upon termination of VF. Indeed, previous reports on the mechanisms of post-shock arrhythmias following successful defibrillation of VF suggest that spontaneous SR Ca\(^{2+}\) release, leading to late phase 3 early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs), may be responsible for VF re-initiation.\(^{36, 37}\) Our results demonstrating near-complete RyR refractoriness and an increase in [Ca\(^{2+}\)]\(_{\text{SR}}\) during VF support these findings. Upon spontaneous termination of VF, [Ca\(^{2+}\)]\(_{\text{SR}}\) is approximately 30% higher than during normal sinus rhythm (Figure 8, iii). Because the likelihood of
spontaneous SR Ca\(^{2+}\) release increases with increasing [Ca\(^{2+}\)]\(_{\text{SR}}\),\(^{24}\) the time period immediately post-VF may represent a particularly vulnerable time for spontaneous SR Ca\(^{2+}\) release. This observation may have important clinical implications for successful defibrillation and the treatment of electrical storm.\(^{38}\)

Our findings may also offer new insights into the interpretation of intracellular Ca\(^{2+}\) signals during VF. Consistent with previous studies, our dual optical mapping data of V\(_{m}\) and [Ca\(^{2+}\)]\(_{\text{i}}\) show peak-to-peak oscillation during VF that is approximately 30% of the amplitude of the normal intracellular Ca\(^{2+}\) transient (Figure 7B, iii). On the other hand, dual imaging of V\(_{m}\) and [Ca\(^{2+}\)]\(_{\text{SR}}\) demonstrates that very minimal SR Ca\(^{2+}\) release occurs during VF (Figure 7A, iii, Figure 8, iii, and Online Figure IX). Because SR Ca\(^{2+}\) release and reuptake accounts for approximately 70% of the total intracellular Ca\(^{2+}\) transient in the normal rabbit heart\(^{24}\) (with the remaining 30% representing trans-sarcolemmal Ca\(^{2+}\) flux through L-type Ca\(^{2+}\) channels and NCX), typical intracellular Ca\(^{2+}\) recordings during VF are likely reflective of trans-sarcolemmal Ca\(^{2+}\) fluxes only. It is possible that I\(_{\text{Ca}}\) may also be reduced during VF due to the depolarized diastolic V\(_{m}\) (Figure 7B, ii) and decreased diastolic interval, which would lead to a secondary reduction in SR Ca\(^{2+}\) release. However, a peak-to-peak amplitude of ~30% of the normal intracellular Ca\(^{2+}\) transient amplitude during VF (Figure 7B, iii) is more consistent with fully available I\(_{\text{Ca}}\) and RyR refractoriness as a mechanism of reduced SR Ca\(^{2+}\) cycling during VF.

**RyR refractoriness as a therapeutic target.**

Given the role of RyR refractoriness in contributing to the onset and progression of alternans and in contributing to SR Ca\(^{2+}\) overload during VF, sensitizing RyR to reduce alternans or improve SR Ca\(^{2+}\) release during VF may represent an enticing therapeutic target. However, RyR sensitization and reduced refractoriness (either via CPVT-linked mutations or enhanced phosphorylation) can significantly enhance arrhythmogenic spontaneous SR Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks and waves), and lead to DADs and focal arrhythmias.\(^{39}\) Therefore, the pro- or anti-arrhythmic consequences of RyR sensitization ultimately depend on the underlying cardiac pathology and arrhythmia mechanism in question.

**Study limitations.**

V\(_{m}\) and [Ca\(^{2+}\)]\(_{\text{SR}}\) were imaged using single-wavelength emission. Unlike myocyte experiments,\(^ {25}\) it was not feasible to calibrate [Ca\(^{2+}\)]\(_{\text{SR}}\) signals in the intact heart (as is true for V\(_{m}\) and [Ca\(^{2+}\)]\(_{\text{i}}\)). However, our [Ca\(^{2+}\)]\(_{\text{SR}}\) measurements agree qualitatively with rabbit myocyte measurements, and thus relative [Ca\(^{2+}\)]\(_{\text{SR}}\) changes are likely valid. The detailed mechanisms of alternans and VF induction in pathological conditions might differ from those of healthy hearts used here. However, future studies using simultaneous optical mapping of V\(_{m}\) and [Ca\(^{2+}\)]\(_{\text{SR}}\) in the intact heart may provide a novel tool for examining SR Ca\(^{2+}\) handling and arrhythmia in pathological conditions and the findings of the present study will provide an important mechanistic foundation.

**Conclusions.**

These findings shed new light on the role of SR Ca\(^{2+}\) in the progression from normal rhythms to cardiac alternans and subsequent arrhythmia. Based on the results of this study, we propose a *continuum of mechanisms* (RyR→SERCA→APD/CV restitution) responsible for the onset and progression of alternans: RyR refractoriness is first encroached upon, which leads to SR Ca\(^{2+}\) release alternans. As heart rate increases, SR Ca\(^{2+}\) release alternans increase, diastolic SR Ca\(^{2+}\) load begins to alternate (impacted by SERCA function), and APD alternans emerge. At even faster rates, dynamical instabilities, such as APD and/or CV restitution may lead to spatially discordant alternans, which can facilitate re-entrant arrhythmia and VF. Importantly, our results demonstrate that SR Ca\(^{2+}\) release is nearly continuously refractory during...
VF despite an increase in diastolic \([Ca^{2+}]_{SR}\) and \([Ca^{2+}]_i\), suggesting that the intracellular Ca\(^{2+}\) transients observed during VF are mainly due to trans-sarcolemmal Ca\(^{2+}\) currents.

**SOURCES OF FUNDING**

This study was supported by the National Institutes of Health R01 HL111600 (C.M.R.), P30 HL101280 (C.M.R. and D.M.B.) and P01 HL80101 (D.M.B.), and the American Heart Association 12SDG9010015 (C.M.R.).

**DISCLOSURES**

None.

**REFERENCES**

3. Diaz ME, O'Neill SC, Eisner DA. Sarcoplasmic reticulum calcium content fluctuation is the key to cardiac alternans. *Circ Res*. 2004;94:650-656

DOI: 10.1161/CIRCRESAHA.114.302505


FIGURE LEGENDS

**Figure 1.** $V_m$-[$Ca^{2+}]_{SR}$ relationship and frequency-dependent [$Ca^{2+}]_{SR}$ response. A. Example traces, activation maps of $V_m$ (i, ii) and [$Ca^{2+}]_{SR}$ (iii, iv) during ventricular pacing at 300 ms. B. Diastolic [$Ca^{2+}]_{SR}$ increases quickly to a new steady state as PCL is reduced. Alternans occur at faster pacing rates (iii). On cessation of rapid pacing, diastolic [$Ca^{2+}]_{SR}$ returns to normal after a pause and a larger release during the first sinus beat (iv). C. Pacing rate dependence of systolic and diastolic [$Ca^{2+}]_{SR}$ (normalized fluorescent signal, n=3). L: larger SR $Ca^{2+}$ transient; S: smaller transient.

**Figure 2.** Frequency dependence of APD and SR $Ca^{2+}$ alternans. A. Maps of APD alternans (spectral magnitude) during progressive increases in pacing rate. B. Corresponding maps of SR $Ca^{2+}$ alternans (spectral magnitude). C. Example $V_m$ traces from the location indicated with a white box in A and B. D. Corresponding example [$Ca^{2+}]_{SR}$ traces (amplitude normalized to large SR $Ca^{2+}$ release to demonstrate alternans progression). $V_m$ and SR $Ca^{2+}$ alternans normally occur in-phase with one another (long APD corresponds to large SR $Ca^{2+}$ transient and vice versa). E. Average spectral alternans magnitude for each PCL showing that SR $Ca^{2+}$ alternans tend to emerge at longer PCLs and have a larger spectral magnitude than APD alternans (n=6). L: longer APD or larger SR $Ca^{2+}$ transient; S: shorter APD or smaller transient.

**Figure 3.** SR $Ca^{2+}$ release alternans vs. diastolic SR $Ca^{2+}$ load alternans. A. White light image of the epicardial surface with locations of traces in (B) marked. B. SR $Ca^{2+}$ release alternans occurring without changes in diastolic SR $Ca^{2+}$ load were routinely observed (i). However, release and diastolic load alternans can occur in same heart at different locations at the same PCL (i-iii). C. Formulae used for quantification of release and load alternans. D. Maps of SR $Ca^{2+}$ release and load alternans at PCL=250ms (i) and PCL=180ms (ii). SR $Ca^{2+}$ release alternans typically occurred before SR $Ca^{2+}$ load alternans (i), with severe load alternans only occurring at shorter PCLs (ii). E. Summary data of SR $Ca^{2+}$ release and load alternans at different PCLs (n=6).

**Figure 4.** Effect of caffeine on RyR refractoriness and SR $Ca^{2+}$ release. A-B. Low concentration caffeine (200 μM) treatment increased the magnitude of SR $Ca^{2+}$ release at coupling intervals of 180 and 200 ms (*p<0.05 vs. baseline, n=6). S1: drive train stimulus; S2: premature stimulus. Caffeine produced a decrease in diastolic [$Ca^{2+}]_{SR}$ after 20 min of treatment (*p<0.05 vs. time=0 min). D. Caffeine had no effect on the time constant ($\tau$) for SR $Ca^{2+}$ recovery.
Figure 5. Effect of caffeine on $V_m$ and SR $Ca^{2+}$ alternans. A. Maps of spectral magnitude of $V_m$ and SR $Ca^{2+}$ alternans at PCL=180 ms under baseline conditions. B. Example traces showing APD and SR $Ca^{2+}$ alternans. C-D. Corresponding SR $Ca^{2+}$ maps and traces following caffeine (200µM) application. Caffeine reduced the magnitude of both $V_m$ and SR $Ca^{2+}$ alternans. E. The magnitude of SR $Ca^{2+}$ release alternans is significantly reduced at PCLs of 200, 180, and 160 ms with caffeine (*p<0.05 vs. baseline, n=6). F. The magnitude of SR $Ca^{2+}$ load alternans is significantly reduced at PCLs of 180 and 160 ms with caffeine (*p<0.05 vs. baseline). G. Caffeine significantly reduced the pacing threshold for emergence SR $Ca^{2+}$ and APD alternans (baseline: 217±10 and 190±13 ms vs. caffeine: 188±15 and 173±12 ms for SR $Ca^{2+}$ and APD, respectively. *p<0.05 vs. baseline).

Figure 6. Effect of caffeine on spatially discordant alternans. A. Maps of spectral magnitude of APD and SR $Ca^{2+}$ alternans at PCL=140 ms showing spatially discordant alternans (nodal lines in black). B. Example $V_m$ and $[Ca^{2+}]_{SR}$ traces from the corresponding locations in A showing spatial discordance on opposite sides of the nodal line. C-D. Corresponding SR $Ca^{2+}$ maps and traces following caffeine (200 µM) application. Spatial discordance still occurs with caffeine. In this example, the magnitude of APD alternans increased with caffeine whereas the magnitude of SR $Ca^{2+}$ alternans decreased. E. Average spectral alternans magnitude at PCL=140 ms for SR $Ca^{2+}$ and APD before and after caffeine (*p<0.05 vs. baseline, n=6). F. Caffeine has no effect on the threshold for induction of spatially discordant alternans for either SR $Ca^{2+}$ or APD. G. Caffeine has no effect on the threshold for induction of VF. L: longer APD or larger SR $Ca^{2+}$ transient; S: shorter APD or smaller transient.

Figure 7. Comparison of $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_{i}$ signals during VF. A. Dual imaging of $V_m$ and $[Ca^{2+}]_{SR}$ during ventricular pacing at PCL=300 ms (left column) and during VF (right column, cycle length≈96 ms). Compared to a pacing-induced SR $Ca^{2+}$ transient, minimal SR $Ca^{2+}$ release occurs during VF (<10% of normal SR $Ca^{2+}$ release amplitude). B. Dual imaging of $V_m$ and $[Ca^{2+}]_{i}$ during ventricular pacing (left column) and VF (right column) shows that $[Ca^{2+}]_{i}$ oscillates at a peak-to-peak amplitude of approximately 30% of pacing-induced intracellular $Ca^{2+}$ transient amplitude during VF.

Figure 8. Elevated $[Ca^{2+}]_{SR}$ during VF. In this example, spontaneous self-termination of VF (cycle length≈106 ms) was recorded. Although absolute $[Ca^{2+}]_{SR}$ concentration cannot be quantified with this method, a comparison of the $[Ca^{2+}]_{SR}$ fluorescence during VF and following spontaneous cardioversion to sinus rhythm shows that, during VF, $[Ca^{2+}]_{SR}$ is approximately 30% higher than normal diastolic levels.
Novelty and Significance

What Is Known?

- T-wave alternans (a non-invasive marker of increased risk for arrhythmia and sudden cardiac death) can be caused by beat-to-beat alternation in amplitude of the intracellular Ca$^{2+}$ transient.

- Several mechanisms may contribute to Ca$^{2+}$ transient alternans, including alternating sarcoplasmic reticulum (SR) Ca$^{2+}$ load and alternating refractoriness of ryanodine receptors (RyR: the SR Ca$^{2+}$ release channel).

- Previous studies on detailed mechanisms of Ca$^{2+}$ alternans have mostly been performed in isolated cardiac myocytes, where direct measurement of [Ca$^{2+}$]$_{SR}$ is possible.

What New Information Does This Article Contribute?

- Using a novel imaging approach to directly monitor [Ca$^{2+}$]$_{SR}$ and transmembrane potential (V$_{m}$) in the intact heart, we found that as heart rate increases, RyR refractoriness is the first mechanism encroached upon and leads to initial development of SR Ca$^{2+}$ release alternans.

- At faster heart rates, SR Ca$^{2+}$ load also begins to alternate, which further increases the magnitude of SR Ca$^{2+}$ release alternans and consequent repolarization alternans.

- At extremely fast activation rates during ventricular fibrillation (VF), SR Ca$^{2+}$ load is high, but RyRs remain nearly continuously refractory, resulting in minimal SR Ca$^{2+}$ release.

For the first time, we performed dual optical mapping of [Ca$^{2+}$]$_{SR}$ and V$_{m}$ to determine the role of SR Ca$^{2+}$ handling during cardiac alternans and VF. Previous studies on the detailed mechanisms of SR Ca$^{2+}$ handling during alternans have mostly been performed in isolated cardiac myocytes. However, cardiac arrhythmias (including alternans and VF) are spatio-temporally complex, emergent phenomena that must be studied in intact tissue. This study revealed that, in the intact heart, RyR refractoriness is the mechanism that is first encroached upon as heart rate increases and contributes to the initial development of SR Ca$^{2+}$ alternans. As heart rate increases further, the SR Ca$^{2+}$-ATPase (SERCA) can no longer sequester all the Ca$^{2+}$ released on the prior beat before the onset of the next action potential. Therefore, SR Ca$^{2+}$ load also begins to alternate, which further increases the magnitude of SR Ca$^{2+}$ release alternans and consequent repolarization alternans. During VF, ventricular activation rates are extremely fast, leading to near-continuous RyR refractoriness and thus, minimal SR Ca$^{2+}$ release, despite an increase in SR Ca$^{2+}$ load. These data are the first to define the role of SR Ca$^{2+}$ handling in the intact heart during cardiac alternans and VF.
A Ventricular Pacing

(i) $V_m$

(iii) $Ca_{SR}^{2+}$

150 ms

(ii) $V_m$ Activation

(iv) $Ca_{SR}^{2+}$ Activation

Figure 1

B Frequency-dependent $Ca_{SR}^{2+}$ Changes

<table>
<thead>
<tr>
<th>PCL (ms)</th>
<th>500</th>
<th>350</th>
<th>250</th>
<th>Sinus</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ii)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iii)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iv)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C $Ca_{SR}^{2+}$ (AU)

Diastolic

Systolic

1 s
Figure 2

A. APD Alternans (Spectral Magnitude)

B. Ca^{2+}_{SR} Alternans (Spectral Magnitude)

C. APD Alternans

D. Ca^{2+}_{SR} Alternans

E. Average Spectral Alternans Magnitude
Alternans Amplitude

\[
\text{Release Alternans} = 1 - \frac{S}{L}
\]

\[
\text{Load Alternans} = \frac{D}{L}
\]

(i) No load alternans:

(ii) Moderate load alternans:

(iii) Large load alternans:

Figure 3
Figure 4

A. SR Ca\(^{2+}\) tracings with baseline and caffeine interventions. S1 = 300 ms, S2 = 200 ms, Sinus rate of 250 ms.

B. Restitution of SR Ca\(^{2+}\) Release

C. Time Constant for SR Ca\(^{2+}\) Recovery

D. Diastolic Fluorescence (AU) vs. Time (min)
**Alternans Magnitude Before and After Caffeine**

**Baseline**

- **A** APD Alternans (spectral magnitude)
- **B** SR Ca$^{2+}$ Alternans (spectral magnitude)

**Caffeine**

- **C** APD Alternans (spectral magnitude)
- **D** SR Ca$^{2+}$ Alternans (spectral magnitude)

**Figures**

- **E** SR Ca$^{2+}$ Release Alt
- **F** SR Ca$^{2+}$ Load Alt
- **G** Alternans Threshold

-- Baseline
-- Caffeine

* indicates significant difference.
Discordant Alternans Before and After Caffeine (CL=140 ms)

Baseline

APD Alternans (spectral magnitude)

SR Ca\(^{2+}\) Alternans (spectral magnitude)

Caffeine

APD Alternans (spectral magnitude)

SR Ca\(^{2+}\) Alternans (spectral magnitude)

E

Spectral Magnitude (AU)

Baseline

Caffeine

F

Discordant Alternans Induction Threshold

PCL (ms)

Baseline

Caff

Baseline

Caff

G

VF Induction Threshold

PCL (ms)

Baseline

Caff
A  Dual Imaging of $V_m$ and $[\text{Ca}^{2+}]_{\text{SR}}$

Ventricular Pacing  Ventricular Fibrillation

(i)  ECG  ||  ECG

(ii)  $V_m$

(iii)  $[\text{Ca}^{2+}]_{\text{SR}}$

B  Dual Imaging of $V_m$ and $[\text{Ca}^{2+}]_i$

Ventricular Pacing  Ventricular Fibrillation

(i)  ECG  ||  ECG

(ii)  $V_m$

(iii)  $[\text{Ca}^{2+}]_i$

\[ \text{250 ms} \]
Sinus Rhythm

ECG

Ventricular Fibrillation

Sinus Rhythm

$\text{[Ca}^{2+}]_{SR}$
Optical Mapping of Sarcoplasmic Reticulum Ca\textsuperscript{2+} in the Intact Heart: Ryanodine Receptor Refractoriness During Alternans and Fibrillation
Lianguo Wang, Rachel C. Myles, Nicole M De Jesus, Alex K Ohlendorf, Donald M Bers and Crystal M Ripplinger

Circ Res. published online February 25, 2014;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2014/02/25/CIRCRESAHA.114.302505

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/02/25/CIRCRESAHA.114.302505.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Optical Mapping of Sarcoplasmic Reticulum Ca\(^{2+}\) in the Intact Heart: Ryanodine Receptor Refractoriness during Alternans and Fibrillation

SUPPLEMENTAL MATERIAL

Lianguo Wang\(^1\), M.D., Rachel C. Myles\(^2\), MBBS, Ph.D., Nicole M. De Jesus\(^1\), B.S., Alex K.P. Ohlendorf\(^1\), B.S., Donald M. Bers\(^1\), Ph.D., and Crystal M. Ripplinger\(^1\), Ph.D.*

\(^1\)Department of Pharmacology, School of Medicine, University of California, Davis

\(^2\)Institute of Cardiovascular & Medical Sciences, University of Glasgow, Glasgow, UK

Running title – Wang, sarcoplasmic reticulum Ca\(^{2+}\) and cardiac alternans

Detailed Methods

**Langendorff perfusion**

All procedures involving animals were approved by the Animal Care and Use Committee of the University of California, Davis and adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Male New Zealand White rabbits (n=27) weighing 3-3.5 kg were anesthetized with a single intravenous injection of pentobarbital sodium (50 mg/kg) containing 1000 IU heparin. Hearts were rapidly removed and perfused as described previously.\(^1\) Briefly, following cannulation of the aorta, Langendorff perfusion was initiated with oxygenated (95% O\(_2\), 5% CO\(_2\)) modified Tyrode’s solution of the following composition (in mmol/L): NaCl 128.2, CaCl\(_2\) 1.3, KCl 4.7, MgCl\(_2\) 1.05, NaH\(_2\)PO\(_4\) 1.19, NaHCO\(_3\) 20 and glucose 11.1 (pH 7.4±0.05). Flow rate (25-35 mL/min) was adjusted to maintain a perfusion pressure of 60-70 mmHg. Two Ag/AgCl disc electrodes were positioned in the bath to record an electrocardiogram (ECG) analogous to a lead I configuration. A bipolar pacing electrode was positioned on the base of the left ventricular (LV) epicardium for pacing, which was performed at a pacing cycle length (PCL) of 300 ms using a 2 ms pulse at twice the diastolic threshold.
Dual optical mapping of $[\text{Ca}^{2+}]_{\text{SR}}$ and $V_m$

To optically monitor intra-SR free $[\text{Ca}^{2+}]$, hearts were loaded with the low-affinity $\text{Ca}^{2+}$ indicator fluo-5N AM (Invitrogen, Carlsbad, CA). After stabilization of perfusion (~10 min), blebbistatin (Tocris Bioscience, Ellisville, MO; 10-20 µM) was added to the perfusate to reduce energy demands of the heart during dye loading and to eliminate motion artifact during optical recordings. Hearts were then switched to a recirculating perfusate (200 mL) containing 5 µM Fluo-5N AM (initially dissolved in 0.25 mL dimethyl sulfoxide [DMSO] and 0.25 mL 20% pluronic acid for a final concentration of 0.25% and 0.025%, respectively during the dye loading) for 60 min at room temperature, followed by 15 min washout at 37°C. A lead I ECG was continuously monitored throughout the loading procedure (Online Figure II). Hearts were subsequently stained with the voltage-sensitive dye RH237 (Invitrogen, Carlsbad, CA; 50 µl of 1 mg/ml in DMSO). All experiments were performed at 37°C.

Based on previous studies of dual imaging of Fluo-3 or Fluo-4 and RH237, we developed an optical setup using the voltage-sensitive indicator RH237 and the low-affinity $\text{Ca}^{2+}$ indicator Fluo-5N AM (Online Figure I). The anterior epicardial surface was excited using LED light sources centered at 470 nm (Mightex, Pleasanton, CA) and bandpass filtered from 475-495 nm (Semrock, Rochester, NY). The emitted fluorescence was collected through a 50 mm objective (Nikon, Japan) and split with a dichroic mirror at 545 nm (Omega, Brattleboro, VT). The longer wavelength moiety, containing the $V_m$ signal, was longpass filtered at 700 nm and the shorter wavelength moiety, containing the $[\text{Ca}^{2+}]_{\text{SR}}$ signal, was bandpass filtered with a 32 nm filter centered at 518 nm (Omega, Brattleboro, VT). The emitted fluorescence signals were recorded using two CMOS cameras (MiCam Ultima-L, SciMedia, Costa Mesa, CA) with a sampling rate of 0.5-1 kHz and 100x100 pixels with a 20x20 mm field of view. In a separate set of hearts (n=3), dual optical mapping of $V_m$ with RH237 (50 µl of 1 mg/ml in DMSO) and intracellular $\text{Ca}^{2+}$ with Rhod-2 AM (Invitrogen, Carlsbad, CA: 0.5 ml of 1 mg/ml in DMSO) was performed as previously described.

Experimental protocol

Baseline electrophysiological parameters were determined during LV epicardial pacing at a PCL of 300 ms. To induce alternans and ventricular arrhythmia, the PCL was decremented in 10 ms steps until loss of 1:1 capture or induction of VF (Online Figure III). In a subset of animals, either low-concentration caffeine (200 µM, n=6) to sensitize RyR to isoproterenol (ISO, 100 nM, n=3) to stimulate β-adrenergic receptors, was added to the perfusate.

Data analysis and statistics

Data analysis was performed using two commercially available analysis programs (BV_Analyze, Brainvision, Tokyo, Japan; and Optiq, Cairn, UK). $V_m$ and $[\text{Ca}^{2+}]_{\text{SR}}$ datasets were spatially
aligned and processed with a Gaussian spatial filter (radius 3 pixels). For both action potentials (APs) and SR Ca\textsuperscript{2+} transients, activation time was determined at 50% of the maximal amplitude. For APs, repolarization time at 90% return to baseline was used to calculate action potential duration (APD\textsubscript{90}). Conduction velocity (CV) was calculated using a polynomial fitting method as previously described.\textsuperscript{8} SR recovery was quantified using the time constant (\(\tau\)) of a single exponential fit to the recovery portion of the SR Ca\textsuperscript{2+} trace (from 5-90% recovery).

The spectral method, which has been used clinically for detecting micro-volt T-wave alternans,\textsuperscript{9} was used to detect the presence of significant APD and SR Ca\textsuperscript{2+} alternans as previously described.\textsuperscript{10} The spectral method was chosen due to its high sensitivity and relative immunity to noise. This approach allowed us to determine if an area within the mapping field of view was experiencing significant APD or SR Ca\textsuperscript{2+} alternans (greater than the background noise levels) as well as the spatial extent of significant alternans. A spectral magnitude of \(\geq 2\) was used as the minimum threshold for significant APD or SR Ca\textsuperscript{2+} alternans, corresponding to a beat-to-beat change in APD\textsubscript{90} \(\geq 5\) ms or beat-to-beat change in SR Ca\textsuperscript{2+} release amplitude \(\geq 5\%\), respectively. To more precisely differentiate between the onset of diastolic SR Ca\textsuperscript{2+} load alternans and SR Ca\textsuperscript{2+} release alternans, detailed quantification of the SR Ca\textsuperscript{2+} transient was also performed. The amplitude of SR Ca\textsuperscript{2+} release alternans was calculated as 1 minus the ratio of the average small beat (S) release amplitude to the average large beat (L) release amplitude (1-S/L) during a 1-2 sec recording. The amplitude of diastolic SR Ca\textsuperscript{2+} load alternans was calculated as the average difference between diastolic levels (D) of S and L beats divided by the average L amplitude (D/L) during a 1-2 sec recording (Figure 3B). Data are expressed as mean\(\pm\)standard deviation (SD) and were compared using Student's t-tests, paired where appropriate. \(P<0.05\) was considered statistically significant.
Online Figures
Online Figure I

**Online Figure I.** Optical setup for dual $V_m$ and $[Ca^{2+}]_{SR}$ imaging. A. Schematic diagram of the optical system for dual mapping of $V_m$ and $[Ca^{2+}]_{SR}$. B. Imaging of hearts loaded with either (i) Fluo-5N only or (ii) RH237 only confirmed that there was complete spectral separation between the $V_m$ and $[Ca^{2+}]_{SR}$ fluorescent signals.
Online Figure II. Example ECG recording. A. Before the SR Ca\(^{2+}\) indicator Fluo-5N AM was added to the perfusate. B. During loading of the heart with Fluo-5N AM at room temperature (~23\(^o\)C) for 60 min. C. After 15 min of recovery at 37\(^o\)C and dye washout.
Online Figure III

Ventricular Pacing Protocol

A Incremental Ventricular Pacing

PCL (ms): 300 / 250 / 240 / 230 / 220 / 210 / 200 ..... -10ms to VF / failure of 1:1 conduction

B (i) 1:1 Conduction (ii) Failure of 1:1 Conduction

PCL=130 ms

ECG

PCL=120 ms

Vm

Online Figure III. Left ventricular pacing protocol for induction of alternans and ventricular fibrillation (VF). A. Pacing cycle length (PCL) was decremented in 10 ms steps until loss of 1:1 capture or induction of VF. B. Example ECG and V_m traces of 1:1 capture (i) and loss of 1:1 capture (ii).
Online Figure IV. Restitution of conduction velocity (CV). A. Restitution curve of CV (n=3) during alternans at various pacing cycle lengths (PCL). Highlighted grey box indicates approximate threshold for discordant alternans. B. $V_m$ activation maps and calculated CV at different PCLs. C. CV vectors overlaid on activation maps at PCL=140 ms. CV vectors show chaotic patterns due to slow and tortuous conduction during discordant alternans.
Online Figure V. Spatial heterogeneity of APD and SR Ca$^{2+}$ alternans. A-D. APD and SR Ca$^{2+}$ alternans magnitude maps and example traces from various locations.
Online Figure VI  

Effect of Isoproterenol on SR Ca\(^{2+}\) Release and Reuptake

A. Sinus Rhythm

B. Example SR Ca\(^{2+}\) traces showing accelerated release and reuptake with ISO.

C. ISO decreased the time to nadir and the time constant (tau) for recovery of SR Ca\(^{2+}\), *p<0.05, n=3.

Online Figure VI. Effect of isoproterenol (ISO, 100 nM) on SR Ca\(^{2+}\) release and reuptake. A. No spontaneous diastolic SR Ca\(^{2+}\) release was observed during either sinus rhythm or ventricular pacing following ISO treatment. B. Example SR Ca\(^{2+}\) traces showing accelerated release and reuptake with ISO. C. ISO decreased the time to nadir and the time constant (tau) for recovery of SR Ca\(^{2+}\), *p<0.05, n=3.
Online Figure VII

Online Figure VII. Effect of isoproterenol (ISO, 100 nM) on APD and SR Ca\(^{2+}\) alternans. A. Maps of spectral magnitude of APD and SR Ca\(^{2+}\) alternans at PCL=180 ms under baseline conditions. B. Example traces showing \(V_m\) and SR Ca\(^{2+}\) alternans at baseline. C-D. Corresponding SR Ca\(^{2+}\) maps and traces following ISO.
Online Figure VIII

Effect of Isoproterenol on Diastolic SR Ca\textsuperscript{2+} Load and Release Alternans

A. Baseline  
B. ISO

C. SR Ca\textsuperscript{2+} Release Alternans

D. SR Ca\textsuperscript{2+} Load Alternans

Online Figure VIII. Effect of isoproterenol (ISO, 100 nM) on SR Ca\textsuperscript{2+} load and release alternans.  A. \(V_m\) and SR Ca\textsuperscript{2+} traces show marked APD and SR Ca\textsuperscript{2+} load and release alternans at PCL=140ms.  B. Example traces following ISO treatment. While ISO significantly reduced SR Ca\textsuperscript{2+} release alternans (C), complete abolishment of diastolic load alternans (D), did not prevent the occurrence of release alternans.
Online Figure IX. $V_m$ and SR Ca$^{2+}$ from different areas of the heart during VF (raw signals corresponding to Figure 7A - longer recording). Example $V_m$ and SR Ca$^{2+}$ traces from four locations (right and left ventricles [RV, LV], apex and base) show small SR Ca$^{2+}$ release events (arrowheads), especially when local activation rate slows to allow for local RyR recovery.
Supplemental References: