A novel mechanism of tandem activation of ryanodine receptors by cytosolic and SR luminal Ca\(^{2+}\) during excitation–contraction coupling in atrial myocytes

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Key points

- In atrial myocytes excitation–contraction coupling is strikingly different from ventricle because atrial myocytes lack a transverse tubule membrane system: Ca\(^{2+}\) release starts in the cell periphery and propagates towards the cell centre by Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) Ca\(^{2+}\) store.
- The cytosolic Ca\(^{2+}\) sensitivity of the ryanodine receptor (RyRs) Ca\(^{2+}\) release channel is low and it is unclear how Ca\(^{2+}\) release can be activated in the interior of atrial cells.
- Simultaneous confocal imaging of cytosolic and intra-SR calcium revealed a transient elevation of store Ca\(^{2+}\) that we termed ‘Ca\(^{2+}\) sensitization signal’.
- We propose a novel paradigm of atrial ECC that is based on tandem activation of the RyRs by cytosolic and luminal Ca\(^{2+}\) through a ‘fire—diffuse—uptake—fire’ (or FDUF) mechanism: Ca\(^{2+}\) uptake by SR Ca\(^{2+}\) pumps at the propagation front elevates Ca\(^{2+}\) inside the SR locally, leading to luminal RyR sensitization and lowering of the cytosolic Ca\(^{2+}\) activation threshold.

Abstract

In atrial myocytes Ca\(^{2+}\) release during excitation–contraction coupling (ECC) is strikingly different from ventricular myocytes. In many species atrial myocytes lack a transverse tubule system, dividing the sarcoplasmic reticulum (SR) Ca\(^{2+}\) store into the peripheral subsarcolemnal junctional (j-SR) and the much more abundant central non-junctional (nj-SR) SR. Action potential (AP)-induced Ca\(^{2+}\) entry activates Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from j-SR ryanodine receptor (RyR) Ca\(^{2+}\) release channels. Peripheral elevation of \([\text{Ca}^{2+}]_i\) initiates CICR from nj-SR and sustains propagation of CICR to the cell centre. Simultaneous confocal measurements of cytosolic \([\text{Ca}^{2+}]_i\); with the fluorescent Ca\(^{2+}\) indicator rhod-2) and intra-SR \([\text{Ca}^{2+}]_{\text{SR}}\); fluo-5N) Ca\(^{2+}\) in rabbit atrial myocytes revealed that Ca\(^{2+}\) release from j-SR resulted in a cytosolic Ca\(^{2+}\) transient of higher amplitude compared to release from nj-SR; however, the degree of depletion of j-SR \([\text{Ca}^{2+}]_{\text{SR}}\) was smaller than nj-SR \([\text{Ca}^{2+}]_{\text{SR}}\). Similarly, Ca\(^{2+}\) signals from individual release sites of the j-SR showed a larger cytosolic amplitude (Ca\(^{2+}\) sparks) but smaller depletion (Ca\(^{2+}\) blinks) than release from nj-SR. During AP-induced Ca\(^{2+}\) release the rise of \([\text{Ca}^{2+}]_i\); detected at individual release sites of the nj-SR preceded the depletion of \([\text{Ca}^{2+}]_{\text{SR}}\), and during this latency period a transient elevation of \([\text{Ca}^{2+}]_{\text{SR}}\) occurred. We propose that Ca\(^{2+}\) release from nj-SR is activated by cytosolic and luminal Ca\(^{2+}\) (tandem RyR activation) via a novel ‘fire—diffuse—uptake—fire’ (FDUF) mechanism. This novel paradigm of atrial ECC predicts that Ca\(^{2+}\) uptake by sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) at the propagation front elevates local \([\text{Ca}^{2+}]_{\text{SR}}\), leading to luminal RyR sensitization and lowering of the activation threshold for cytosolic CICR.

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Introduction

In atrial myocytes the mechanism of excitation–contraction coupling (ECC) and action potential (AP) induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) reveals unique features that are strikingly different from ventricular myocytes. Atrial myocytes lack or have only a sparsely developed transverse (t) tubular system (Huser et al. 1996; Cordeiro et al. 2001; Mackenzie et al. 2001; Bootman et al. 2006; Smyrnias et al. 2010). This divides the SR Ca\(^{2+}\) store into two types of SR based on their location relative to the surface membrane: junctional (j-SR) and the much more abundant non-junctional (nj-SR) SR. Both j-SR and nj-SR possess ryanodine receptors (RyRs) Ca\(^{2+}\) release channels (Carl et al. 1995; Kockskamper et al. 2001; Mackenzie et al. 2001; Woo et al. 2003; Mackenzie et al. 2004; Bootman et al. 2006; Smyrnias et al. 2010), which are organized in a 3-dimensional array of RyR clusters, and contribute to ECC by active SR Ca\(^{2+}\) release (Huser et al. 1996; Kockskamper et al. 2001; Sheehan & Blatter, 2003; Shkryl & Blatter, 2013). The j-SR forms peripheral couplings with the adjacent sarcolemmal membrane hosting voltage-gated L-type Ca\(^{2+}\) channels (LCCs) that are facing the clusters of RyRs in the SR membrane across a narrow inter-membrane cleft, reminiscent of dyads in ventricular myocytes (McNutt & Fawcett, 1969; Kockskamper et al. 2001). Thus, the Ca\(^{2+}\) release units (Franzini-Armstrong & Jorgensen, 1994) (CRUs) of the j-SR are functionally organized like a ‘classical’ couplon (Stern et al. 1999; Scriven et al. 2013). Ca\(^{2+}\) entry through LCCs raises [Ca\(^{2+}\)]\(_i\) in the cleft fast enough and to sufficiently high levels to activate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from j-SR RyRs in this subsarcolemmal (SS) space. In contrast, the quantitatively much more abundant nj-SR is located in central (CT) regions of the atrial myocyte and does not associate with the surface membrane. As a consequence of these ultrastructural arrangements, AP induced Ca\(^{2+}\) release is spatially inhomogeneous (Huser et al. 1996; Blatter et al. 2003; Bootman et al. 2011; Shkryl & Blatter, 2013). The subsarcolemmal (SS) elevation of cytosolic [Ca\(^{2+}\)]\(_i\) generates Ca\(^{2+}\) gradients that allow for centripetal Ca\(^{2+}\) diffusion and activation of CICR from RyRs of the nj-SR. CICR propagates from the periphery to the centre in a Ca\(^{2+}\) wave-like fashion by a diffusion-reaction process or a ‘fire–diffuse–fire’ mechanism (Keizer et al. 1998; Shkryl & Blatter, 2013), with the inherent consequences of complex [Ca\(^{2+}\)]\(_i\) inhomogeneities and subcellular Ca\(^{2+}\) gradients during ECC.

While this concept of atrial ECC is in principle accepted, one fundamental and baffling question has remained unanswered: the cardiac-specific isofrom of the RyR (RyR2) has, under physiological conditions (particularly with respect to Mg\(^{2+}\) and ATP), a low Ca\(^{2+}\)-sensitivity (Meissner & Henderson, 1987; Cannell & Soeller, 1997; Zima et al. 2003; Qin et al. 2009; Chen et al. 2013). Since the vastly more abundant CRUs of the nj-SR do not face LCCs that generate the rapid and high enough Ca\(^{2+}\) signal required for RyR activation, it has remained an unresolved issue how CICR from the nj-SR even can be activated, considering that bulk cytosolic Ca\(^{2+}\) transient amplitude barely exceeds 1 \(\mu\text{M}\).

In ventricular myocytes we made the novel observation that cell-wide propagation of spontaneous Ca\(^{2+}\) waves, which occur under Ca\(^{2+}\) overload conditions, depends on an intra-SR Ca\(^{2+}\) ‘sensitization’ wave: the elevation of [Ca\(^{2+}\)]\(_i\) at the wave front leads to local uptake by sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), which results in a local increase of [Ca\(^{2+}\)]\(_{SR}\) that sensitizes the RyR to cytosolic CICR via its luminal Ca\(^{2+}\) dependence (Gyorke & Gyorke, 1998; Fill & Copello, 2002). A mechanism of wave propagation involving RyR control by luminal Ca\(^{2+}\) has been proposed based on indirect experimental conclusions (Keller et al. 2007) and theoretical considerations (Ramay et al. 2010), but we were the first to demonstrate the existence of such a mechanism by direct measurements of [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_{SR}\) (Maxwell & Blatter, 2012). Since the centripetal propagation of CICR during physiological ECC in atrial cells is reminiscent of a propagating Ca\(^{2+}\) wave, in the current study we tested the hypothesis of an intra-SR Ca\(^{2+}\) sensitization signal in atrial ECC as a pivotal and obligatory component of physiological atrial ECC. Simultaneous measurements of [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_{SR}\) from individual atrial CRUs revealed two important new features of atrial Ca\(^{2+}\) release and ECC. First, a transient elevation of [Ca\(^{2+}\)]\(_{SR}\) in the nj-SR was observed before the onset of
depletion that served as a luminal sensitization signal for the RyR and supports a novel paradigm of atrial ECC that is based on the simultaneous RyR activation by cytosolic and luminal Ca\(^{2+}\) (tandem RyR activation mechanism). Second, during AP-induced Ca\(^{2+}\) release [Ca\(^{2+}\)]\(_{\text{SR}}\) depleted to a lower level in the nj-SR, suggesting a more efficient mechanism of release that contributes to cytosolic [Ca\(^{2+}\)]; levels in subcellular regions occupied by the nj-SR that are sufficiently high to sustain propagating CICR.

**Methods**

**Ethical approval**

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Rush University Chicago, and comply with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and UK regulations on animal experimentation and the guidelines of *The Journal of Physiology* (Drummond, 2009; Grundy, 2015).

**Solutions and experimental conditions**

All chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless noted otherwise. Tyrode solution contained (in mM): 130 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 d-glucose, 5 Heps; pH 7.4 with NaOH. Isoproterenol (ISO) was dissolved in water and diluted to 1 \(\mu\)M in Tyrode solution. Cyclopiazonic acid (CPA) was dissolved in dimethyl sulfoxide (DMSO) and diluted to 3 \(\mu\)M in Tyrode solution. For spark–blink pair measurements, cells were permeabilized with 0.01% saponin and bathed in an internal solution containing (in mM): 100 potassium aspartate, 15 KCl, 5 KH\(_2\)PO\(_4\), 5 MgATP, 0.35 EGTA, 0.12 Ca\(^{2+}\)Cl\(_2\), 0.75 MgCl\(_2\), 10 phosphocreatine, 10 Heps, 0.04 rhod-2 tripotassium salt. Free [Ca\(^{2+}\)] of the internal solution was 150 \(\mu\)M. To prevent movement of cells during recording, the muscle contraction uncoupler 2,3-butanedione monoxime (10 \(\mu\)M) was included in the Tyrode and internal solutions during experiments. All experiments were performed at room temperature (20–24°C).

**Myocyte isolation**

Left atrial and left ventricular myocytes were isolated from male New Zealand White rabbits (16 animals, \~2.5 kg; Myrtle’s Rabbitry, Thompsons Station, TN, USA) (Maxwell & Blatter, 2012; Kanaporis & Blatter, 2015). Rabbits were anaesthetized with intravenous injection of sodium pentobarbital (50 mg kg\(^{-1}\)) and heparin (1000 UI kg\(^{-1}\)). Hearts were excised, mounted on a Langendorff apparatus and retrogradely perfused via the aorta. After an initial 5 min perfusion with oxygenated Ca\(^{2+}\)-free Tyrode solution (in mM: 140 NaCl, 4 KCl, 10 d-glucose, 5 Heps, 1 MgCl\(_2\), 10 BDM; 1000 UI l\(^{-1}\) heparin, pH 7.4 with NaOH), the heart was perfused with Eagle’s minimal essential medium (MEM) solution containing 20 \(\mu\)M Ca\(^{2+}\) and 22.5 \(\mu\)g ml\(^{-1}\) Liberase TH (Roche Diagnostic Corporation, Indianapolis, IN, USA) for 20 min at 37°C. The left atrium was removed from the heart and digested for an additional 5 min in the enzyme solution at 37°C. The left ventricular free wall was removed. Ventricular or atrial tissue was minced, filtered and washed in a MEM solution containing 50 \(\mu\)M Ca\(^{2+}\) and 10 mg ml\(^{-1}\) bovine serum albumin. Isolated cells were washed and kept in MEM solution with 50 \(\mu\)M Ca\(^{2+}\) at room temperature (20–24°C) and Ca\(^{2+}\) measurements were made within 1–8 h after isolation.

**[Ca\(^{2+}\)] measurements**

To directly monitor [Ca\(^{2+}\)]\(_{\text{SR}}\) the SR was loaded with the low-affinity Ca\(^{2+}\) indicator fluo-5N (Molecular Probes/Life Technologies, Grand Island, NY, USA) by incubation of myocytes with 10 \(\mu\)M of the membrane-permeant fluo-5N AM together with 0.25% Pluronic F-127 in nominally Ca\(^{2+}\)-free Tyrode solution for 2.5 h for ventricular myocytes or 2 h for atrial myocytes, followed by a 30 min wash, all at 37°C. To monitor [Ca\(^{2+}\)]\(_{\text{cyt}}\), in intact myocytes, cells preloaded with fluo-5N were subsequently loaded with the spectrally distinct Ca\(^{2+}\) indicator rhod-2 AM (5 \(\mu\)M) for 10 min followed by a 10 min wash. For simultaneous recording of [Ca\(^{2+}\)]\(_{\text{SR}}\) (fluo-5N) and [Ca\(^{2+}\)]\(_{\text{cyt}}\) (rhod-2) by laser scanning confocal microscopy (Nikon A1R, Nikon Corporation, Melville, NY, USA) fluo-5N was excited at 488 nm and emission collected at 515 ± 15 nm, while rhod-2 was excited at 543 and emission collected at wavelengths > 600 nm. Calcium transient and depletion measurements were acquired from intact myocytes during 1 Hz stimulation in line scan mode at 512 lines s\(^{-1}\) using a \(\times\)60 oil-immersion objective lens (NA = 1.49). The scan line was placed along the transverse axis of the cell (pixel dimension 0.02–0.04 \(\mu\)m pixel\(^{-1}\)). For all [Ca\(^{2+}\)] measurements cells were placed on laminin-coated coverslips. APs and global Ca\(^{2+}\) transients (CaTs) were elicited by electrical field stimulation using a pair of platinum electrodes (voltage set at \~50% above the threshold for contraction). For simultaneous Ca\(^{2+}\) spark and blink measurements myocytes preloaded with fluo-5 N were permeabilized with saponin and bathed in the internal solution containing 40 \(\mu\)M rhod-2 tripotassium salt. Spark–blink recordings were made in longitudinal line scan mode (0.12 \(\mu\)m pixel\(^{-1}\)) and sparks were detected and analysed by an automated process (Sparkmaster; Picht *et al.* 2007).
Membrane staining

Sarcolemma and t-tubular membranes were visualized (Huser et al. 1996) with the membrane-bound fluorescent probe di-8-ANEPPS (Molecular Probes/Life Technologies) by 2-dimensional confocal microscopy. Cells were loaded for 15 min with di-8-ANEPPS (5 μM) in Tyrode solution and the indicator was excited at 488 nm, and emission was measured at > 600 nm.

Data presentation

Confocal line scan data and fluorescence traces are presented as individual observations representative of multiple recordings or as the average of multiple recordings. Fluorescence traces were background subtracted and plotted as F/F₀, where F₀ is the basal fluorescence in resting cells at the beginning of a recording or diastolic fluorescence in stimulated cells. Ca²⁺ signal amplitudes are expressed as ΔF/F₀, where ΔF = F − F₀. Summary data are presented as the means ± SEM of n measurements. Statistical comparisons between groups were performed with Student’s t test. Differences were considered statistically significant at P < 0.05.

Results

Subcellular heterogeneity of CaTs and SR depletion signals in atrial myocytes

To determine the dynamics of both [Ca²⁺]i and [Ca²⁺]SR during AP-induced Ca²⁺ release these two parameters were measured simultaneously using spectrally distinct fluorescent Ca²⁺ reporter dyes. [Ca²⁺]i was measured using rhod-2, while SR Ca²⁺ was monitored simultaneously with the low-affinity Ca²⁺ indicator fluo-5N entrapped inside the SR. Figure 1A shows a series of AP-induced cytosolic CaTs together with the corresponding SR depletion signal recorded simultaneously from a subsarcolemmal (SS) and a central (CT) region of an atrial myocyte, thus representing Ca²⁺ release from j-SR and nj-SR, respectively. Consistent with earlier results (Sheehan & Blatter, 2003) CT CaT amplitudes are smaller than SS CaTs. Surprising, however, was the consistent observation of significantly larger [Ca²⁺]SR depletion amplitudes in regions of the nj-SR, despite a smaller amplitude of the cytosolic signal. Figure 1B shows average cytosolic CaT (ΔF/F₀: 0.92 ± 0.07 vs. 0.74 ± 0.06; P < 0.001) and [Ca²⁺]SR depletion (ΔF/F₀: 0.20 ± 0.03 vs. 0.31 ± 0.04; P < 0.001) amplitudes.

Figure 1C shows the cytosolic CaT and [Ca²⁺]SR depletion amplitudes at individual Ca²⁺ release units (CRUs) of the SR during propagation of activation from the periphery to the cell centre. The largest cytosolic CaT amplitude was found in the SS space reflecting the initial AP-induced release of Ca²⁺ from the j-SR. Activation then propagates through the approximately 1 μm wide transition zone (TZ) between j-SR and the first row of nj-SR CRUs with no decrement in amplitude. However, as activation reaches the first CT nj-SR CRU (CT₁), the cytosolic CaT amplitude decreased to less than half, with further small progressive decline along the centripetal direction of propagation. In contrast, the depletion signal was smallest in the cell periphery (j-SR), showed little change in the TZ (reflecting SR depletion in non-CRU regions of the SR (Zima et al. 2008; Picht et al. 2011)), but was significantly larger at nj-SR CRUs and constant along the direction of propagation.

We also investigated the properties of spontaneous elementary cytosolic Ca²⁺ release (Ca²⁺ sparks) and corresponding Ca²⁺ depletion (Ca²⁺ blinks) events...
measured simultaneously from individual CRUs. Figure 1D shows averaged confocal line scan images of SS and CT Ca$^{2+}$ sparks and blinks. Figure 1E shows $F/F_0$ profiles for averaged SS and CT Ca$^{2+}$ sparks and blinks taken from the line scan images in panel D. SS Ca$^{2+}$ sparks have a larger amplitude than CT Ca$^{2+}$ sparks ($ΔF/F_0$: 1.18 vs. 1.05), consistent with earlier findings ((Sheehan et al. 2006); however, the nadir of CT/nj-SR Ca$^{2+}$ blinks reached a lower $[\text{Ca}^{2+}]_{\text{SR}}$ level than SS/j-SR blinks and the depletion amplitude was larger ($ΔF/F_0$: 0.23 vs. 0.15). Thus, spontaneous elementary Ca$^{2+}$ release and depletion events from individual CRUs with respect to their relative amplitudes reflect the properties of AP-induced CaTs originating from j-SR and nj-SR (Fig. 1A).

**CRU Ca$^{2+}$ signals during ECC: evidence for an intra-SR Ca$^{2+}$ sensitization signal in atrial myocytes**

To further determine the dynamics of both $[\text{Ca}^{2+}]$, and $[\text{Ca}^{2+}]_{\text{SR}}$ during AP-induced Ca$^{2+}$ release, we monitored $[\text{Ca}^{2+}]$ and $[\text{Ca}^{2+}]_{\text{SR}}$ simultaneously from individual CRUs. In a first set of experiments we compared subcellular $[\text{Ca}^{2+}]$, and $[\text{Ca}^{2+}]_{\text{SR}}$ signals between ventricular and atrial myocytes. Rabbit ventricular cells have a fully developed t-tubular system (Fig. 2A) as revealed by membrane staining with the fluorescent probe di-8-ANEPPS, whereas in rabbit atrial myocytes t-tubules are absent (Fig. 2B).

Cytosolic CaTs and $[\text{Ca}^{2+}]_{\text{SR}}$ depletion signals were recorded with transverse confocal line scan imaging of ventricular (Fig. 2C) and atrial (Fig. 2D) myocytes during AP-induced Ca$^{2+}$ release elicited by electrical field stimulation. Local fluorescence intensity profiles ($F/F_0$) representing subcellular SR regions encompassing an individual CRU were recorded together with the corresponding cytosolic signal from 0.6 μm wide regions of interest. In ventricular myocytes, the presence of t-tubules assures the simultaneous activation of essentially all CRUs. Consequently, the onset of the cytosolic CaT was identical in the cell periphery (Fig. 2Ca) and in the cell centre (Fig. 2Cb). Furthermore, the onset of the cytosolic CaT coincided strictly with the onset of the decline of $[\text{Ca}^{2+}]_{\text{SR}}$, i.e. rise of $[\text{Ca}^{2+}]$, and depletion of $[\text{Ca}^{2+}]_{\text{SR}}$ are tightly coupled and spatially homogeneous across the cell. Rabbit atrial myocytes, however, due to their lack of t-tubules, exhibited spatially inhomogeneous cytosolic and SR Ca$^{2+}$ signals. CICR from j-SR was activated first upon electrical stimulation, resulting in a simultaneous rise of $[\text{Ca}^{2+}]_i$, and decline of $[\text{Ca}^{2+}]_{\text{SR}}$ (Fig. 2Da). However, release from central regions (Fig. 2Db) is delayed due to the time required for activation to reach the centre of the cell. As shown in Fig. 2Da the rise of $[\text{Ca}^{2+}]$, is delayed, the rate of rise of $[\text{Ca}^{2+}]_i$ in central regions of the cell is slower and peaks at a lower level compared to the cell periphery.

The simultaneous recording of $[\text{Ca}^{2+}]_{\text{SR}}$ allowed the observation of novel features of atrial Ca$^{2+}$ release. First, in the cell periphery the rise of SS $[\text{Ca}^{2+}]$, and the begin of the decline of $[\text{Ca}^{2+}]_{\text{SR}}$ was simultaneous (line 1 in Fig. 2D) and strongly resembled the situation in ventricular myocytes. This is consistent with the notion that the j-SR is organized in peripheral couplings where LCCs face RyR clusters across a narrow cleft, similar to ventricular couplings. Here, CICR was activated rapidly upon Ca$^{2+}$ entry with no measurable delay between $[\text{Ca}^{2+}]$, increase and $[\text{Ca}^{2+}]_{\text{SR}}$ decrease. In contrast, in the cell centre there was a temporal dispersion between onset of the cytosolic CaT and the decline of $[\text{Ca}^{2+}]_{\text{SR}}$. The time interval between rise of SS $[\text{Ca}^{2+}]$, and begin of decline of CT $[\text{Ca}^{2+}]_{\text{SR}}$ was defined here as latency ($Δt$ between dashed vertical lines 1 and 3 in Fig. 2D). Along the transverse axis the latency steadily increased with increasing distance from the cell periphery and in the cell centre the average latency was 27 ± 2 ms. Second, the $[\text{Ca}^{2+}]_{\text{SR}}$ signal during the latency period revealed a surprising feature. Instead of the expected decline a rise of $[\text{Ca}^{2+}]_{\text{SR}}$ was observed that peaked immediately before $[\text{Ca}^{2+}]_{\text{SR}}$ began to decrease. Based on our earlier observation of an intra-SR Ca$^{2+}$ sensitization wave (Maxwell & Blatter, 2012) that drives spontaneous Ca$^{2+}$ wave propagation in ventricular myocytes we hypothesize that this rise of $[\text{Ca}^{2+}]_{\text{SR}}$ during the latency period serves as an intra-SR Ca$^{2+}$ sensitization signal that via luminal action lowers the activation threshold of the RyR to cytosolic CICR. Under control conditions the average amplitude of the sensitization signal was $ΔF/F_0 = 0.15 ± 0.01$ (Fig. 3B).

To further characterize the Ca$^{2+}$ sensitization signal in the nj-SR, we monitored $[\text{Ca}^{2+}]$, and $[\text{Ca}^{2+}]_{\text{SR}}$ from individual j-SR and nj-SR CRUs over a period of consecutive AP-induced CaTs (Fig. 2E). Cells were field stimulated at 1 Hz. Figure 2E shows local $[\text{Ca}^{2+}]$, and $[\text{Ca}^{2+}]_{\text{SR}}$ profiles of five consecutive beats. In both subcellular locations, cell periphery (top) and cell centre (bottom), $[\text{Ca}^{2+}]_i$, and $[\text{Ca}^{2+}]_{\text{SR}}$ signals revealed a high degree of beat-to-beat reproducibility. As expected the intra-SR Ca$^{2+}$ sensitization signal as well as the latency period were absent in the cell periphery. In the cell centre the latency period and the intra-SR Ca$^{2+}$ sensitization signal were highly preserved in magnitude and kinetics from one beat to the next.

**Intra-SR Ca$^{2+}$ sensitization signal and latency are regulated**

Since we had found a central role of SERCA activity for the generation of the intra-SR sensitization signal for spontaneous Ca$^{2+}$ wave propagation in ventricular myocytes (Maxwell & Blatter, 2012), we tested the hypothesis that in atrial myocyte the intra-SR Ca$^{2+}$ sensitization...
Figure 2. Intra-SR Ca$^{2+}$ sensitization signal during atrial ECC
Membrane staining with the fluorescent probe Di-8-ANEPPS reveals the regular structure of the t-tubule system in ventricular myocytes (A) and the absence of t-tubules in atrial cells (B). C, [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_{SR}$ signals (F/F$_0$) elicited by AP depolarization and recorded from individual peripheral (a) and central (b) release sites in a ventricular myocyte. F/F$_0$ signals are averaged over 0.6 $\mu$m wide regions of interest. The dashed vertical line marks the simultaneous onset of the cytosolic CaT and SR depletion in both subcellular regions. D, [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_{SR}$ signals (F/F$_0$) elicited by AP depolarization in an atrial cell. In the cell periphery (a) the rise of [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_{SR}$ decline is simultaneous (vertical dashed line 1). In the cell centre (b) the rise of [Ca$^{2+}$]$_i$ is delayed and [Ca$^{2+}$]$_{SR}$ depletion lags behind the rise of [Ca$^{2+}$]. The decline of [Ca$^{2+}$]$_{SR}$ is preceded by the Ca$^{2+}$ sensitization signal, a transient elevation of [Ca$^{2+}$]$_{SR}$ (arrow). Vertical dashed lines 2 and 3 mark the duration of the Ca$^{2+}$ sensitization signal and the time interval 1–3 is defined as latency.

E, reproducibility of the SR Ca$^{2+}$ sensitization signal. Subcellular [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_{SR}$ signals (F/F$_0$) induced by a train of APs (1 Hz). F/F$_0$ profiles are recorded from 0.6 $\mu$m wide subcellular regions of interest in the cell periphery (top) and the cell centre (bottom). Five consecutive AP induced CaTs are shown. [Ca$^{2+}$]$_{SR}$ profiles from CT nj-SR reveal a reproducible Ca$^{2+}$ sensitization signal (arrows). Vertical dashed lines mark latency.

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signal during ECC depends on Ca\textsuperscript{2+} uptake by SERCA at the front of the propagating CICR. The \(\beta\)-adrenergic agonist isoproterenol (ISO) was used to increase SERCA activity which occurs through phosphorylation of the inhibitory protein phospholamban (Inui et al. 1986; Luo et al. 1994). Figure 3A shows the averaged [Ca\textsuperscript{2+}]\textsubscript{SR} profile from individual CRUs located in the cell centre under control conditions (left) and after exposure to isoproterenol (ISO, 1 \(\mu\)M; right). In the presence of isoproterenol the amplitude of the Ca\textsuperscript{2+} sensitization signal increased to \(\Delta F/F_0 = 0.20 \pm 0.01\) (Fig. 3B), together with its duration (grey bars in Fig. 3A). Average duration of the Ca\textsuperscript{2+} sensitization signal was 12 ± 0.3 ms in control, and 25 ± 1.1 ms in ISO. Furthermore, the latency (Fig. 3C) measured in the cell centre increased from 27 ± 2 ms in control to 39 ± 2 ms in ISO \((P < 0.02)\). In the presence of the SERCA blocker CPA no Ca\textsuperscript{2+} sensitization signal was observed. These observations are consistent with the hypothesis that increased SERCA activity enhances SR Ca\textsuperscript{2+} uptake at the front of propagating activation, which increases the Ca\textsuperscript{2+} sensitization signal, but also prolongs the time until a decline of [Ca\textsuperscript{2+}]\textsubscript{SR} and thus SR Ca\textsuperscript{2+} depletion can be observed.

**Discussion**

A fundamental unresolved question in atrial ECC remains how a spatio-temporally effective CICR at the nj-SR arises and centripetal CICR propagation is sustained. This is particularly critical because nj-SR CRUs are much larger in numbers and largely carry the bulk of the Ca\textsuperscript{2+} release burden of atrial contraction. Because of the absence or scarcity of the t-tubular network, nj-SR CRUs are not driven by the LCC-supplied fast local Ca\textsuperscript{2+} trigger. Furthermore, the normal cytosolic Ca\textsuperscript{2+} sensitivity of the cardiac RyR is inherently low and its open probability at cytosolic Ca\textsuperscript{2+} concentrations < 1 \(\mu\)M is extremely low. Nonetheless, during AP stimulation nj-SR RyRs participate in ECC with robust Ca\textsuperscript{2+} release (Huser et al. 1996; Sheehan & Blatter, 2003; Shkryl & Blatter, 2013). In this study we present several novel findings that form the basis for a novel paradigm of atrial ECC: (1) during atrial ECC CaT transients elicited by AP depolarization, Ca\textsuperscript{2+} release from nj-SR results in larger Ca\textsuperscript{2+} depletions in the nj-SR compared to the j-SR; (2) also, Ca\textsuperscript{2+} bleeds from nj-SR, the complementary elementary Ca\textsuperscript{2+} depletion events to spontaneous Ca\textsuperscript{2+} sparks from an individual CRU, deplete to lower [Ca\textsuperscript{2+}]\textsubscript{SR} levels; and (3) during propagation of activation through the nj-SR, the onset of depletion of [Ca\textsuperscript{2+}]\textsubscript{SR} is delayed compared to the rise of cytosolic [Ca\textsuperscript{2+}], (referred to here as ‘latency’, Fig. 2D) and [Ca\textsuperscript{2+}]\textsubscript{SR} shows a characteristic transient elevation that we termed ‘SR Ca\textsuperscript{2+} sensitization signal’.

The lower depletion levels of the nj-SR during physiological ECC and Ca\textsuperscript{2+} release suggest a more effective CICR at nj-SR CRUs, given the fact that the cytosolic CaT is actually smaller in amplitude compared to peripheral CaTs (Fig. 1). This is advantageous for centripetal propagation of CICR because it allows for more robust release by a smaller trigger signal, i.e. fractional Ca\textsuperscript{2+} release, the relationship between magnitude of trigger and amount of released Ca\textsuperscript{2+}, appears to be larger and the efficiency of CICR from nj-SR higher than from j-SR. Several potential mechanisms can be envisioned for the more efficient depletion of nj-SR release sites. Possibilities are that the pool from which Ca\textsuperscript{2+} can be recruited at an individual CRU differs in the nj-SR and j-SR, or j-SR and nj-SR CRUs have different Ca\textsuperscript{2+} release termination mechanisms. Furthermore, intra-SR Ca\textsuperscript{2+} buffering of the j-SR and the nj-SR are different, although under resting steady-state conditions we did not find any difference in [Ca\textsuperscript{2+}]\textsubscript{SR} between j-SR and nj-SR. Ca\textsuperscript{2+} in the SR is heavily

![Figure 3. Regulation of the SR Ca\textsuperscript{2+} sensitization signal by \(\beta\)-adrenergic stimulation](image)

**A**. averaged [Ca\textsuperscript{2+}]\textsubscript{SR} signals \((\Delta F/F_0)\) from CT nj-SR during AP-induced Ca\textsuperscript{2+} release in control conditions and in the presence of isoproterenol (ISO, 1 \(\mu\)M). The Fluo-SN fluorescence signal was normalized to the respective diastolic Fluo-SN fluorescence \(F_0\) measured separately in control and ISO. Therefore, the signal does not reflect the increase in absolute diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} in the presence of ISO. Grey bars indicate duration of Ca\textsuperscript{2+} sensitization signal. **B**, average amplitudes of the SR Ca\textsuperscript{2+} sensitization signal in control (Ctrl) and ISO. **P < 0.01. C**, average latency in control and ISO. \(\ast P < 0.02\).
buffered by the endogenous Ca\(^{2+}\) buffer calsequestrin (CASQ). CASQ has a dual role: Ca\(^{2+}\) load-sensitive Ca\(^{2+}\) buffering, thereby determining SR Ca\(^{2+}\) storage capacity of the SR and the functional size of the Ca\(^{2+}\) store (Terentyev et al. 2003), and luminal regulation of RyR gating (Gyorke & Terentyev, 2008; Gyorke et al. 2009; Knollmann, 2009) by regulating free [Ca\(^{2+}\)]\(_{SR}\) and/or conformational regulation in conjunction with the auxiliary proteins junctin and triadin. High resolution studies have shown that RyR and CASQ show a lower degree of colocalization in the nj-SR (Schulson et al. 2011), suggesting higher excitability for RyR in the interior of the cells and facilitation of the spread of excitation from the periphery to the centre. Furthermore, lower CASQ levels and thus reduced Ca\(^{2+}\) buffering would allow depletion to lower [Ca\(^{2+}\)]\(_{SR}\) levels, consistent with our observations during CaTs (Fig. 1A) and also during spontaneous Ca\(^{2+}\) sparks and blinks (Fig. 1E). Additional factors may contribute to the observed amplitude difference of SS and CT CaTs (Fig. 1A). In the cell periphery the higher cytosolic Ca\(^{2+}\) signal can be the result of a larger amount of Ca\(^{2+}\) release from a j-SR CRU; however, geometrical and structural factors may contribute to the higher signal as well. In the cell periphery Ca\(^{2+}\) is released into the narrow cleft of the j-SR peripheral couplings. [Ca\(^{2+}\)]\(_{c}\) in this confined space reaches high levels rapidly, while the same amount of Ca\(^{2+}\) release from a source that is not confined by surrounding membranes is likely to dissipate more rapidly and will not reach comparable peak levels. We addressed this issue in previous studies. We investigated the spatial organization of spontaneous Ca\(^{2+}\) sparks originating form j-SR and nj-SR.

In the cell periphery sparks were spatially asymmetrical. Full width at half-maximum (FWHM) fluorescence was elongated in longitudinal direction by \(\sim 1.7\) compared to the transverse dimension (Shkryl & Blatter, 2013), whereas Ca\(^{2+}\) sparks from nj-SR were symmetrical. Membrane permeabilization with a detergent (saponin) reduced the asymmetry of j-SR sparks (presumably by disrupting the physical integrity of the narrow cleft of the peripheral couplings), but did not affect the spatial dimensions of nj-SR sparks (Shkryl & Blatter, 2008). Furthermore, membrane permeabilization reduced the amplitude of j-SR Ca\(^{2+}\) sparks, but was without effect on central nj-SR spark amplitude (Sheehan et al. 2006). These earlier experiments indeed indicate that the geometry of the narrow cleft of the peripheral couplings of the j-SR is essential for shaping the local Ca\(^{2+}\) signal and contributes to the larger amplitude of peripheral Ca\(^{2+}\) release.

Furthermore, Ca\(^{2+}\) entry through LCCs contributes to cleft [Ca\(^{2+}\)]\(_c\), a source of Ca\(^{2+}\) that is absent in the cell centre.

RyR gating is controlled by both cytosolic and luminal Ca\(^{2+}\) (Gyorke & Gyorke, 1998; Fill & Copello, 2002). A key finding of our study is the observation that Ca\(^{2+}\) release from nj-SR is delayed (latency) relative to the rise of cytosolic [Ca\(^{2+}\)]\(_c\), and is preceded by a local elevation of [Ca\(^{2+}\)]\(_{SR}\) (Fig. 2). This is in stark contrast to ventricular ECC where the rise of [Ca\(^{2+}\)]\(_c\) is highly synchronous across the cell, occurs without latency and the transient elevation of [Ca\(^{2+}\)]\(_{SR}\) is completely absent. We propose that the transient [Ca\(^{2+}\)]\(_{SR}\) elevation in atrial cells acts as a 'Ca\(^{2+}\) sensitization signal'. The unique atrial nj-SR Ca\(^{2+}\) sensitization signal is due to local Ca\(^{2+}\) uptake by SERCA as the rise of cytosolic [Ca\(^{2+}\)]\(_c\) propagates towards the cell centre (Fig. 4). Enhanced Ca\(^{2+}\) uptake at the propagation front leads to a local elevation of [Ca\(^{2+}\)]\(_{SR}\) that increases RyR cytosolic Ca\(^{2+}\) sensitivity via RyR's luminal Ca\(^{2+}\) regulation sites. The higher luminal [Ca\(^{2+}\)]\(_{SR}\) also lengthens RyR open time (Chen et al. 2013, 2014) and increases RyR unitary Ca\(^{2+}\) flux. Together, these luminal Ca\(^{2+}\) actions promote RyR activation and inter-RyR CICR,

and are favourable to robust propagation of activation by a mechanism that we call 'tandem activation' of the nj-SR RyRs. Furthermore, \(\beta\)-adrenergic stimulation (isoproterenol (ISO)) increases the amplitude and duration of the Ca\(^{2+}\) sensitization signal (Fig. 3), consistent with stimulation of SERCA, whereas the SERCA blocker CPA abolished the Ca\(^{2+}\) sensitization signal. The effects of ISO and CPA clearly demonstrate a central involvement of SERCA. Interestingly, expression levels of the endogenous SERCA inhibitor phospholamban (PLN) are lower in atrial myocytes (Koss et al. 1995; Luss et al. 1999) which would logically favour the generation of the SR Ca\(^{2+}\) sensitization signal.

A critical element of the atrial ECC process is the transition of activation from the peripheral j-SR through the TZ to the first (most peripheral) array of nj-SR CRUs. The underlying mechanism is controversial. In rat atrial myocytes mitochondria located in the TZ have been suggested to curtail Ca\(^{2+}\) propagation by sequestering Ca\(^{2+}\) (‘mitochondrial firewall’ (Mackenzie et al. 2004; Bootman et al. 2011)). In a previous study, however, we found that the TZ between j-SR and first row of nj-SR CRUs is actually devoid of mitochondria in rabbit atrial cells and propagation through the TZ occurs nearly twice as fast as through the central regions occupied by nj-SR (Hohendanner et al. 2015a). Thus, the structural feature of absence of mitochondria in the TZ is critical for the propagation of CICR from j-SR to the nj-SR.

Several limitations of the study are noted. (1) Experiments were conducted at room temperature. [Ca\(^{2+}\)]\(_{SR}\) recordings from individual CRUs consist of fluorescence signal originating from very small volumes with limited number of fluorophores. Fluorescent indicator dyes are known to be transported across membranes rather rapidly at higher temperature, jeopardizing reliable [Ca\(^{2+}\)]\(_{SR}\) measurements from individual CRUs. Activity of various ECC Ca\(^{2+}\) handling proteins is temperature dependent, but it is unlikely that
the key findings of this study (Ca\(^{2+}\) sensitization signal and FDUF mechanism) would be qualitatively different at higher temperatures. (2) We used the myosin ATPase inhibitor BDM to eliminate motion artifacts; however, BDM has reported additional cellular effects, including RyR stimulation (Tripathy et al. 1999) and potentially enhancing SR Ca\(^{2+}\) leak. While the RyR effect of BDM may influence SR Ca\(^{2+}\) load and potentially the magnitude of Ca\(^{2+}\) sensitization signal, the absence of motion artifacts is paramount for the recording of CRU [Ca\(^{2+}\)]\(_{SR}\) signals with high fidelity (Maxwell & Blatter, 2012), and was deemed most important in these experiments. (3) Finally, β-adrenergic stimulation not only affects SERCA, but also alters the activity of the RyR. The effect on RyRs includes phosphorylation of the channels and increased activity; however, the effects are complex. For example, we have shown previously that for a given SR Ca\(^{2+}\) load the [Ca\(^{2+}\)]\(_{SR}\) threshold where spontaneous Ca\(^{2+}\) waves occur is higher in the presence of ISO (Domeier et al. 2012). Since the mechanism of ECC and the propagating Ca\(^{2+}\) release from nj-SR in atrial cells is reminiscent of a propagating Ca\(^{2+}\) wave, it is conceivable that β-adrenergic stimulation might increase the threshold for release. In that case, a larger Ca\(^{2+}\) sensitization signal in the presence of ISO (Fig. 3) would be favourable to assure robust CICR from nj-SR.

In summary we propose a novel paradigm of atrial ECC that we term ‘fire–diffuse–uptake–fire’ or FDUF mechanism (Fig. 4). The sequence of events of atrial ECC are: AP-induced membrane depolarization activates LCCs in the cell membrane that in turn trigger CICR from peripheral couplings of the j-SR. Ca\(^{2+}\) release from peripheral j-SR sets up a robust Ca\(^{2+}\) gradient that initiates Ca\(^{2+}\) movement through the TZ that subsequently triggers CICR from the first array of nj-SR CRUs aided by the FDUF mechanism that leads to the aforementioned tandem activation of nj-SR RyRs. CICR then continues to propagate by the FDUF mechanism through the entire network of the nj-SR until release reaches the most central regions of the cell. The observation that ISO and CPA affect the Ca\(^{2+}\) sensitization signal indicates that the FDUF mechanism is regulated and thus a potential target for therapeutic interventions. We have shown previously that in earlier stages of ventricular failure, Ca\(^{2+}\) transients are enhanced (Hohendanner et al. 2015b, 2016) and improve atrial contractile function, ventricular filling and thus ejection fraction and cardiac output. While it remains to be determined to what extent the FDUF mechanism is remodelled and regulated in heart failure, interventions that manipulate the Ca\(^{2+}\) sensitization signal and the FDUF mechanism are potentially beneficial to preserve cardiac output in failing hearts.
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Additional information

Competing interests
None declared.

Author contributions
J.T.M. and L.A.B. contributed to the conception and design of the experiments, analysis and interpretation of the data, and writing of the article. J.T.M. performed the experimental work. Both authors are accountable for all aspects of the work, qualify for authorship, and have approved the submitted version of the manuscript.

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