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Cytosolic Ca^{2+} buffering determines the intra-SR Ca^{2+} concentration at which cardiac Ca^{2+} sparks terminate

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Abstract

Single ryanodine receptor (RyR) Ca^{2+} flux amplitude ($i_{\text{Ca-RyR}}$) decreases as intra- sarcoplasmic reticulum (SR) Ca^{2+} levels fall during a cardiac Ca^{2+} spark. Since $i_{\text{Ca-RyR}}$ drives the inter-RyR Ca^{2+} -induced Ca^{2+} release (CICR) that underlies the spark, decreasing $i_{\text{Ca-RyR}}$ may contribute to spark termination because RyRs that spontaneously close may stay closed. To test this possibility, we simultaneously measured local cytosolic and intra-SR ($[\text{Ca}^{2+}]_{\text{cyto}}$ & $[\text{Ca}^{2+}]_{\text{SR}}$) during Ca^{2+} sparks in permeabilized rabbit ventricular myocytes. Local cytosolic or intra-SR Ca^{2+} dynamics were manipulated using Ca^{2+} buffers. Buffer manipulations applied in cells had no effect on individual RyR channels reconstituted in planar lipid bilayers. Presence of a fast cytosolic Ca^{2+} buffer (BAPTA) significantly suppressed Ca^{2+} spark activity and sparks terminated earlier at a higher than usual $[\text{Ca}^{2+}]_{\text{SR}}$ level (~80% vs. ~62%). When cytosolic Ca^{2+} buffer power was reduced (i.e. cytosolic EGTA level decreased), sparks terminated later and at a lower than usual $[\text{Ca}^{2+}]_{\text{SR}}$ level (~45% vs. ~62%). When intra-SR Ca^{2+} buffer power was increased, sparks also terminated later and at a lower than usual $[\text{Ca}^{2+}]_{\text{SR}}$ (~48% vs. ~62%). These results suggest that cytosolic local control of inter-RyR CICR by $i_{\text{Ca-RyR}}$ plays a substantial role during the spark termination process. Thus, alterations in local cytosolic Ca^{2+} handling dynamics in the dyadic cleft (Ca^{2+} buffering, extrusion, etc.) likely influence Ca^{2+} spark termination.

Graphical Abstract

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6. AUTHOR CONTRIBUTIONS

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8. CONFLICT OF INTEREST STATEMENT

None.

1. **Pernicious Attrition** $\Rightarrow$ CICR failure
2. **Resident Site** $\Rightarrow$ RyR2-Residue E4872
3. **Calsequestrin (Casq)-Based** $\Rightarrow$ RyR-Casq Complex

Heart; Ca^{2+} spark; ryanodine receptor; Ca^{2+} -induced Ca^{2+} release; confocal microscopy

In adult ventricular myocytes, the majority of Ca^{2+} that drives contraction is released from the sarcoplasmic reticulum (SR) via ryanodine receptor (RyR) Ca^{2+} release channels. Most RyRs release Ca^{2+} into subcellular microdomains called dyads. At the dyad, RyRs on the SR are arranged in organized clusters [1-3]. L-type Ca^{2+} channels (LTCCs) on the T-tubule membrane are closely associated with some of the RyRs in the cluster [2]. During an action potential (AP), a small LTCC-mediated Ca^{2+} influx activates one or more RyRs in the cluster via the process of Ca^{2+} -induced Ca^{2+} release (CICR) [4]. Once a RyR opens, the Ca^{2+} released into the dyadic cleft drives inter-RyR CICR, which amplifies the AP-initiated Ca^{2+} release event. Individual RyRs can also spontaneously open during diastole. These stochastic events may also drive inter-RyR CICR and generate a local non-propagating release event, called a Ca^{2+} spark [5].

CICR is an inherently self-regenerating process. Once initiated it is expected to continue until intra-SR Ca^{2+} ($[\text{Ca}^{2+}]_{\text{SR}}$) is fully exhausted. However, CICR ends when $[\text{Ca}^{2+}]_{\text{SR}}$ is only partially depleted [6-8]. This indicates that the inherent positive feedback of CICR is countered by some sort of negative control or termination process. Several CICR termination mechanisms have been proposed (including stochastic attrition, Ca^{2+} -dependent inactivation, adaptation [9-12]), but all of these have either been ruled out or deemed insufficient.

The $[Ca^{2+}]_{SR}$ at which sparks terminate ($[Ca^{2+}]_{SR}$ termination threshold) has been defined by simultaneous recording of sparks and their corresponding blinks (i.e. local drop in local $[Ca^{2+}]_{SR}$) [6;8;13]. It is commonly thought that local CICR terminates at the $[Ca^{2+}]_{SR}$ termination threshold because Ca^{2+} unbinds from intra-SR RyR regulatory site(s) and promotes RyR closing [13;14]. This possibility is consistent with a large body of experimental evidence [15-20]. However, falling $[Ca^{2+}]_{SR}$ also reduces the trans-SR Ca^{2+}

driving force and consequently unitary RyR Ca^{2+} flux ($i_{\text{Ca-RyR}}$). Since $i_{\text{Ca-RyR}}$ drives the inter-RyR CICR within a cluster underlying the spark, the likelihood of inter-RyR CICR decreases as $[\text{Ca}^{2+}]_{\text{SR}}$ and $i_{\text{Ca-RyR}}$ falls. At some point, $i_{\text{Ca-RyR}}$ could become insufficient to drive inter-RyR CICR, RyRs that spontaneously close will remain closed, and this could also contribute to spark termination. Although this mechanism has been embedded into several CICR termination models [21-26], the contribution of falling $i_{\text{Ca-RyR}}$ in CICR termination has been largely unexplored experimentally. The main limitation is the inability to properly manipulate $i_{\text{Ca-RyR}}$ and $[\text{Ca}^{2+}]_{\text{SR}}$ in the cell environment.

Guo et al [27] recently used large RyR permeable cations to differentially manipulate $i_{\text{Ca-RyR}}$ independent of $[\text{Ca}^{2+}]_{\text{SR}}$. They reported that $i_{\text{Ca-RyR}}$ has a substantial role in Ca^{2+} spark initiation and termination. Subsequently, the $i_{\text{Ca-RyR}}$ based termination process has been termed pernicious attrition [28] or alternatively induction decay [29]. It is important to note that existence of $i_{\text{Ca-RyR}}$ based termination does not mean that other termination mechanisms do not exist. Instead, $i_{\text{Ca-RyR}}$ based termination is likely one of several termination mechanisms that operate in concert.

Here, we examined how experimentally manipulating $i_{\text{Ca-RyR}}$, by applying various Ca^{2+} buffers influences the $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold in permeabilized rabbit ventricular myocytes. The results provide new experimental evidence that $i_{\text{Ca-RyR}}$ based termination process plays a substantial role in Ca^{2+} spark local control.

2. METHODS

2.1. Myocyte Isolation

Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Loyola University which comply with national regulations. Briefly, ventricular myocytes were isolated from hearts of anaesthetized (sodium pentobarbital; 50 mg/kg I.V.) New Zealand white rabbits (2 kg) following a procedure described previously [30]. Chemicals and reagents were purchased from “Sigma-Aldrich” (St. Louis, MO) unless otherwise stated and experimentation done at room temperature (20-24°C).

2.2. Intracellular Ca^{2+} Imaging in Permeabilized Myocytes

Sparks (local $[\text{Ca}^{2+}]_{\text{cyto}}$ changes) were measured using either Fluo-4 or Rhod-2 (Molecular Probes/Invitrogen, Carlsbad, CA) as high affinity Ca^{2+} indicators. Blinks (local $[\text{Ca}^{2+}]_{\text{SR}}$ changes) were monitored using the low affinity Ca^{2+} indicator Fluo-5N (Molecular Probes/Invitrogen). The sarcolemma of acutely dissociated ventricular myocytes was permeabilized with saponin as described previously [31]. For blink studies, cardiomyocytes were incubated with Fluo-5N/AM before permeabilization in conditions that promote dye accumulation within the SR [8;30]. Permeabilized myocytes were bathed in an internal solution composed of (in mM): K aspartate 100; KCl 15; KH_2PO_4 5; MgATP 5; EGTA 0.35; CaCl_2 0.12; MgCl_2 0.75; phosphocreatine 10; HEPES 10; Rhod-2 tripotassium salt 0.04 (or Fluo-4 pentapotassium salt 0.04 mM; when Ca^{2+} sparks were measured alone); creatine phosphokinase 5 U/mL; dextran (MW: 40,000) 8%, and pH 7.2 (KOH). Free $[\text{Ca}^{2+}]$ and

[Mg²⁺] in this solution were always 150 mM and 1 mM (respectively). To manipulate cytosolic Ca²⁺ buffering, either BAPTA was added to this internal solution or the internal solution's [EGTA] was reduced. To alter intra-SR Ca²⁺ buffering, permeabilized cells were incubated for 10 minutes with 5 mM of acetamidoiminodiacetic acid (ADA; K_d for Ca²⁺ ~ 0.2 mM). After ADA incubation, cytosolic ADA was washed out with ADA-free internal solution. The slow diffusion of ADA out of the SR provided a 10-15 min time period during which sparks/blinks could be measured at greater than normal intra-SR Ca²⁺ buffer capacity [31;32].

Changes in [Ca²⁺]_{cyto} and [Ca²⁺]_{SR} were measured simultaneously using a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) equipped with a 40× oil-immersion objective (N.A.=1.3). Fluo-5N was excited at 488 nm and its emitted fluorescence recorded at 515±15 nm. Rhod-2 was excited at 543 nm and its emitted fluorescence recorded at wavelengths >600 nm. Images were acquired in linescan mode (3 ms per scan; pixel size 0.12 μm). Sparks were detected and analyzed using SparkMaster [33]. Maximum SR Ca²⁺ release flux during a spark was calculated from the peak of the first derivative of the cytosolic fluorescence intensity and expressed as $d(F/F_0)/ms$ [31]. Sparks and blink parameters were defined as previously described [8]. The Fluo-5N signal was corrected for its Ca²⁺-independent component as assessed by completely depleting SR of Ca²⁺ using 10 mM caffeine [34].

2.3. Single RyR channel recordings

Regulation of cardiac intracellular Ca²⁺ homeostasis is species-specific [35], but single mammalian cardiac RyR function is not [36]. As single cardiac RyR function in bilayers is species-independent, heavy SR microsomes were prepared from rat ventricular muscle using a standard cellular sub-fractionation process [37]. Planar lipid bilayers were formed across an 100 μm diameter hole in a 12 μm thick Teflon partition. This partition separated two 1 ml compartments. One (*cis*) was virtually grounded and initially filled with a HEPES-Tris solution (250 mM HEPES, 120 mM Tris, pH 7.4). This chamber was always facing the cytosolic side of the channel. The other (*trans*) chamber was initially filled with HEPES-Ca solution (250 mM HEPES, 53 mM Ca(OH)₂, pH 7.4) and faced the luminal side of the channel. Exposure to this high luminal [Ca] assured that no calsequestrin (Casq) was associated with the channels tested here [38].

Planar bilayers were formed from a 5:4:1 mixture (50 mg/ml total lipids in decane) of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. Then, 500 mM CsCl and heavy SR microsomes were added to the *cis* chamber. Once ion channels were incorporated into the bilayer, solutions in the *cis* and *trans* chambers were replaced by cell-like recording solutions. The *trans* (luminal) cell-like recording solution contained 1 mM free Ca²⁺, 200 mM Cs-Methansulfonate and 10 mM Hepes (pH 7.4). The *cis* (cytosolic) cell-like recording solution contained 10 μM free Ca²⁺, 0.5 mM EGTA, 10 mM HEPES (pH 7.4), 200 mM Cs-Methansulfonate and MgATP (1 mM free Mg²⁺, 5 mM total ATP). For some studies, BAPTA was added to the *cis* recording solution as well as sufficient CaCl₂ to keep the free Ca²⁺ concentration constant. Free [Ca²⁺] was verified using a Ca-selective electrode. Single RyR channel recordings were sampled at 100 μs/pt and filtered (8-pole

Bessel) at 1 kHz. Single channel analysis was done using pCLAMP9 software (Axon Instruments/Molecular Devices, Sunnyvale, CA). Single channel recordings were idealized using the half-amplitude threshold method. Unit current, mean open time, mean closed times and open probability (P_o) were determined from idealized traces.

2.4. Statistics

Data are presented as mean $\pm$ SEM of n measured cells, Ca^{2+} release events or channels. Statistical comparisons between groups were performed by the Student's t test. Differences were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1. Action of Fast Cytosolic Ca^{2+} Buffering

Figure 1A shows representative line-scan Fluo-4 images of sparks recorded in permeabilized myocytes before (control) and after cytosolic BAPTA (0.7 mM) was added. In both conditions, cytosolic EGTA (0.35 mM) was present and the free $[\text{Ca}^{2+}]_{\text{cyto}}$ was 150 nM. In the control condition, sparks occurred at a stable frequency of $12.0 \pm 2.1 \text{ sparks} \times \text{s}^{-1} \times (100 \mu\text{m})^{-1}$ (Fig. 1B; $n=8$ cells). With the cytosolic BAPTA present, spark frequency was significantly lower (>7 fold) at $1.6 \pm 1.2 \text{ sparks} \times \text{s}^{-1} \times (100 \mu\text{m})^{-1}$. The added cytosolic BAPTA also significantly reduced spark amplitude, width and duration (Fig. 1B). Moreover, BAPTA decreased the Time-to-Peak of Ca^{2+} sparks by 68%: from $36.3 \pm 0.6 \text{ ms}$ ($n=208$ events) in control conditions to $11.6 \pm 0.9 \text{ ms}$ ($P < 0.05$; $n=52$ events) in the presence of BAPTA. These changes in spark properties persisted as long as the cytosolic BAPTA was present (~ 5 min) and were fully reversed when the cytosolic BAPTA was removed. The substantial spark attenuation by 0.7 mM cytosolic BAPTA may impose a methodological limitation, the possible failure to detect the small sparks. Thus, subsequent studies were done using a lower cytosolic BAPTA concentration.

Measurement of spark-blink pairs provides information about the local rise in $[\text{Ca}^{2+}]_{\text{cyto}}$ and the corresponding local decline in intra-SR $[\text{Ca}^{2+}]_{\text{SR}}$, respectively. The latter does not depend on the fate of the released Ca^{2+} (e.g. diffuse out of the permeabilized cell, bind to cytosolic proteins, bind to cytosolic buffers, etc). Figure 2A shows simultaneously measured Rhod-2 (top) and Fluo-5N (bottom) line-scan images of spark-blink pairs before (control) and after addition of cytosolic BAPTA (0.36 mM). Cytosolic free $[\text{Ca}^{2+}]$ and [EGTA] was the same (150 nM & 0.35 mM, respectively) in both test conditions. With the cytosolic BAPTA present, spark frequency was $5.9 \pm 1.1 \text{ sparks} \times \text{s}^{-1} \times (100 \mu\text{m})^{-1}$ ($n=10$ cells). Also, the blink nadir occurred at a higher $[\text{Ca}^{2+}]_{\text{SR}}$ level (compared to control). Figure 2B compares the pre-spark $[\text{Ca}^{2+}]_{\text{SR}}$ levels and blink nadirs before and after addition of the cytosolic BAPTA. In control conditions, the blink nadir occurred when the $[\text{Ca}^{2+}]_{\text{SR}}$ reached $61.3 \pm 4.6\%$ ($n=19$ events) of its initial level. With cytosolic BAPTA present, the blink nadir occurred when the $[\text{Ca}^{2+}]_{\text{SR}}$ reached just $79.5 \pm 7.1\%$ ($P < 0.05$; $n=9$ events). The added cytosolic BAPTA did not change the initial $[\text{Ca}^{2+}]_{\text{SR}}$ level, which is proportional to resting SR Ca^{2+} load.

Individual cardiac RyR channels were reconstituted in lipid bilayers and their function was defined in cell-like recording solutions with and without cytosolic BAPTA (0.36 mM) present. Cytosolic EGTA (0.35 mM) was present in both cases. Single RyR P_o was very low at submicromolar diastole-like levels of $[Ca^{2+}]_{cyto}$ (Supplemental Fig. S1A). Therefore, single RyR function was defined with 10 μ M cytosolic free Ca^{2+} present. This level was chosen because local $[Ca^{2+}]_{cyto}$ levels within the dyad are likely near 10 μ M when a spark terminates [21]. Figure 3A shows sample single RyR recordings before (control) and after the cytosolic BAPTA was added. The cytosolic BAPTA did not alter the RyR's unitary current (or slope conductance; Supplemental Fig S1B). Figure 3B compares single RyR open probability, mean open time and mean closed time before and after addition of the cytosolic BAPTA. There was no significant change in any of these parameters. Thus, addition of 0.36 mM cytosolic BAPTA did not alter single RyR function.

3.2. Action of Low Cytosolic Ca^{2+} Buffer Capacity

Figure 4A shows representative Fluo-4 line-scan images of Ca^{2+} sparks recorded with 0.36 mM (control) and 0.12 mM cytosolic EGTA present (low EGTA). The free $[Ca^{2+}]_{cyto}$ in both cases was 150 nM. Figure 4B compares spark frequency, amplitude, width and duration at the two cytosolic EGTA levels. Average spark frequency, width and duration were significantly greater at the lower [EGTA]. The low [EGTA] also increased the Time-to-Peak of sparks by 31%: from 34.9 ± 0.7 ms ($n=177$ events) in control conditions to 45.7 ± 0.7 ms ($P<0.05$; $n=212$ events) in the presence of low [EGTA]. There was also a small elevation in basal Fluo-4 fluorescence that was likely the consequence of the increased spark activity (i.e. the in- and out-of-focus events). Figure 5A shows spark-blink pairs in control and at the lower cytosolic [EGTA]. Recurring sparks at the same release site (Fig 5A) were more frequently observed at the lower cytosolic Ca^{2+} buffer capacity. Consecutive sparks at the same release site consistently generated blinks whose nadir's occurred at the same $[Ca^{2+}]_{SR}$ level. The reduction in cytosolic buffer power (from 0.36 to 0.12 mM EGTA) altered the $[Ca^{2+}]_{SR}$ level at which the blink nadir occurred. Figure 5B compares initial and blink nadir $[Ca^{2+}]_{SR}$ levels at the two cytosolic [EGTA]. Both initial and blink nadir $[Ca^{2+}]_{SR}$ were significantly decreased at the lower cytosolic [EGTA]. The initial $[Ca^{2+}]_{SR}$ decreased by $20 \pm 6\%$ ($P<0.05$; $n=6$). The $[Ca^{2+}]_{SR}$ at which the blink nadir decreased from $62.4 \pm 5.1\%$ ($n=12$ events) to $46.4 \pm 7.1\%$ ($n=16$ events, $P<0.05$). This indicates that at the lower cytosolic Ca^{2+} buffer capacity, spark termination occurs after $[Ca^{2+}]_{SR}$ drops to a significantly lower level. Interestingly, the low cytosolic EGTA results are nearly identical (see supplemental Fig. S2) to the initial and blink nadir $[Ca^{2+}]_{SR}$ level changes we have previously reported in a pressure/volume overload rabbit heart failure (HF) model [30].

3.3. Action of Higher Intra-SR Ca^{2+} Buffer Capacity

To increase the intra-SR Ca^{2+} buffer capacity, permeabilized myocytes were incubated in a cytosolic solution containing the low affinity Ca^{2+} buffer, N-(2-acetamido)iminodiacetic acid (ADA; 5 mM), which accumulates in the SR over time. We showed in a previous study [31] that incubation of permeabilized cells in 5 mM ADA increases total SR Ca^{2+} content by ~40%. Figure 6A shows representative spark-blink pairs after ADA loading. The cytosolic solution contained 0.36 mM EGTA (150 nM free Ca^{2+}). Spark frequency was not altered by the presence of ADA in the SR. However, it did significantly increase the spark amplitude

(0.83 ± 0.09 to 1.22 ± 0.11 F/F_0 ; $P < 0.05$), Ca^{2+} release flux (0.052 ± 0.007 to 0.103 ± 0.012 $d(F/F_0)/dt$; $P < 0.05$) and spark duration (38.3 ± 7.9 to 112.2 ± 35.6 ms; $P < 0.05$). Figure 6B compares the time-to-nadir (TTN) of blinks with and without ADA in the SR. With ADA present, TTN increased more than three times (69 ± 8 vs. 236 ± 1 ms; $P < 0.05$). Figure 6C compares the average time constant of local SR Ca^{2+} refilling following a spark. With ADA present, the time constant of SR refilling was increased from 161 ± 19 to 856 ± 122 ms ($P < 0.05$). Figure 6D compares the initial and blink nadir $[\text{Ca}^{2+}]_{\text{SR}}$ levels without and with ADA in the SR. With ADA present, the $[\text{Ca}^{2+}]_{\text{SR}}$ at which the blink nadir occurs decreased from $63 \pm 7\%$ ($n=21$ events) to $48 \pm 7\%$ ($n=10$ events, $P < 0.05$).

4. DISCUSSION

Diastolic Ca^{2+} sparks are spontaneous bouts of localized inter-RyR CICR that are likely triggered by a rare stochastic opening of a single RyR channel. A spark occurs if the $i_{\text{Ca-RyR}}$ mediated by that rare opening of the channel is sufficient to drive inter-RyR CICR. During a spark, local $[\text{Ca}^{2+}]_{\text{SR}}$ falls and inter-RyR CICR terminates when $[\text{Ca}^{2+}]_{\text{SR}}$ reaches a critical $[\text{Ca}^{2+}]_{\text{SR}}$ level, known as the termination threshold [8]. Here, we report that the spark $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold depends on cytosolic and intra-SR Ca^{2+} buffering. In control conditions, sparks terminated when $[\text{Ca}^{2+}]_{\text{SR}}$ fell to $\sim 62\%$ of its initial value. When a fast cytosolic Ca^{2+} buffer was present, sparks terminated when $[\text{Ca}^{2+}]_{\text{SR}}$ fell to $\sim 80\%$ of its initial value. At the reduced Ca^{2+} buffer power, sparks terminated when $[\text{Ca}^{2+}]_{\text{SR}}$ fell to $\sim 45\%$ of its initial value. Increasing intra-SR Ca^{2+} buffer power delayed termination which occurred when $[\text{Ca}^{2+}]_{\text{SR}}$ fell to $\sim 50\%$ of its initial value. Pre-spark $[\text{Ca}^{2+}]_{\text{SR}}$ was not significantly different than control (except in the reduced cytosolic Ca^{2+} buffer power case). The action of Ca^{2+} buffers on Ca^{2+} spark-blink pairs are difficult to explain if local CICR termination was solely due to Ca^{2+} unbinding from luminal-SR RyR regulatory sites as previously suggested [13;39].

4.1. Intra-SR Ca^{2+} Control of Spark Termination

Figure 7 (see also Graphical Abstract) depicts three ways by which falling $[\text{Ca}^{2+}]_{\text{SR}}$ could promote spark termination. The first involves Ca^{2+} -dependent intra-SR calsequestrin (Casq) polymerization [40]. As $[\text{Ca}^{2+}]_{\text{SR}}$ falls, more Casq monomers become available to associate with the RyR-junctin-triadin complex and this association inhibits RyR activity [38;41]. Recently, Chen et al. [42] identified an intra-SR Ca^{2+} sensing domain on the RyR itself. As $[\text{Ca}^{2+}]_{\text{SR}}$ falls, Ca^{2+} occupation at this intra-SR RyR-resident site decreases and this could also inhibit RyR activity. Guo et al [27] provided experimental evidence indicating pernicious attrition [28;29] as a contributor to spark termination as well. Pernicious attrition (or induction decay [29]) describes the failure of local inter-RyR CICR as unitary $i_{\text{Ca-RyR}}$ amplitude decreases during a spark. The $i_{\text{Ca-RyR}}$ amplitude decreases because the trans-SR Ca^{2+} driving force decreases during local $[\text{Ca}^{2+}]_{\text{SR}}$ depletion (or a blink). The idea is that at some point $i_{\text{Ca-RyR}}$ will become insufficient to sustain inter-RyR CICR. At that point, RyRs that stochastically close will remain closed and thus contribute to termination. Note that pernicious attrition is not stochastic attrition [9], which describes the possibility that all open RyRs could simultaneously close. In pernicious attrition, RyR closing is innately asynchronous. It is described as pernicious because declining $i_{\text{Ca-RyR}}$ halts inter-RyR CICR

in a way that is not easily seen or noticed. We propose that the concerted operation of the three mechanisms depicted in Figure 7 is what terminates Ca^{2+} sparks and produces the experimentally observed $[\text{Ca}^{2+}]_{\text{SR}}$ spark termination threshold.

4.2. Cytosolic Ca^{2+} Buffering and the $[\text{Ca}^{2+}]_{\text{SR}}$ Spark Termination Threshold

If spark termination was solely due to intra-SR Ca^{2+} acting on intra-SR sites (Casq-based or RyR-resident site), the spark $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold should be independent of the cytosolic fate of released Ca^{2+} . In other words, the Ca^{2+} interaction with the salient intra-SR Ca^{2+} sites would not depend on whether the released Ca^{2+} binds to a cytosolic buffer or simply diffuses away. However, the contribution of pernicious attrition to termination should be sensitive to cytosolic buffering, which determines the cytosolic spatiotemporal Ca^{2+} signal generated by an open RyR. In our study, the spark $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold changed predictably when cytosolic buffering changed. Fast cytosolic Ca^{2+} buffers increased the $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold while reduced cytosolic Ca^{2+} buffer capacity decreased it. Since the fast buffer narrows the cytosolic spatiotemporal spread of $i_{\text{Ca-RyR}}$, one would predict that a smaller drop in $[\text{Ca}^{2+}]_{\text{SR}}$ (i.e. $i_{\text{Ca-RyR}}$) would be needed for pernicious attrition to occur. Since lower cytosolic buffer widens the cytosolic spatiotemporal spread of $i_{\text{Ca-RyR}}$, a larger drop in $[\text{Ca}^{2+}]_{\text{SR}}$ would be needed for pernicious attrition to occur. Thus, the $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold sensitivity to cytosolic Ca^{2+} buffering is consistent with pernicious attrition contributing to termination.

4.3. Intra-SR Ca^{2+} Buffer Capacity and Spark Termination

In agreement with previous studies [43;44], we found that higher SR Ca^{2+} buffer capacity significantly increased Ca^{2+} spark amplitude, time to blink nadir and time constant of local SR Ca^{2+} refilling. The higher buffer capacity slows the fall in local $[\text{Ca}^{2+}]_{\text{SR}}$ and $i_{\text{Ca-RyR}}$ amplitude, delaying termination. Prolonged local release explains the larger sparks and time to blink nadir. Refilling takes longer, because the SR has a larger Ca^{2+} buffer capacity to be replenished. Interestingly, higher SR Ca^{2+} buffer capacity significantly lowered the $[\text{Ca}^{2+}]_{\text{SR}}$ at which sparks terminate (~45%). This suggests that pernicious attrition is not the only factor responsible for termination because intra-SR Ca buffering should not change the effectiveness of the unitary $i_{\text{Ca-RyR}}$ to drive inter-RyR CICR. Thus, higher SR buffer capacity should not change the $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold if termination were only due to pernicious attrition. One possible explanation of the lower $[\text{Ca}^{2+}]_{\text{SR}}$ termination threshold at the higher SR buffer capacity is that intra-SR ADA somehow promotes RyR opening either directly or by altering the intra-SR Ca^{2+} interaction with the Casq-based and/or RyR-resident mechanisms. Another possibility is that the ADA may alter intra-SR Mg^{2+} levels or dynamics. Since Ca^{2+} and Mg^{2+} compete for occupancy of the open RyR pore, intra-SR Mg^{2+} changes would alter unitary $i_{\text{Ca-RyR}}$ amplitude [45]. If so, then this could also explain the lower $[\text{Ca}^{2+}]_{\text{SR}}$ spark termination threshold at the higher SR buffer capacity. Yet, another possibility is that the prolonged Ca^{2+} release at the higher SR buffer capacity results in a larger than normal number of locally active RyRs. It would be inherently more difficult to terminate release from a larger group of active channels.

4.4. Physiological Implications

This study revealed that the $[Ca^{2+}]_{SR}$ spark termination threshold in permeabilized ventricular cardiomyocytes depends on cytosolic Ca^{2+} buffer speed and capacity. This indicates that that pernicious attrition likely contributes to CICR termination. Dependence of termination on local cytosolic Ca^{2+} dynamics has some interesting physiological ramifications. Shorter RyR open time would make unitary i_{Ca-RyR} less effective at driving inter-RyR CICR, promoting pernicious attrition and consequently preventing Ca^{2+} waves. Ischemia is associated with cellular acidification which could alter local cytosolic Ca^{2+} buffering and thus local CICR termination. The similar actions of HF and low Ca^{2+} buffer capacity on the $[Ca^{2+}]_{SR}$ spark termination threshold (see supplemental Fig. S2) suggests that HF alters pernicious attrition in some way. One possibility is that RyR's cytosolic Ca^{2+} sensitivity is heightened in HF so that smaller than normal unitary i_{Ca-RyR} can drive inter-RyR CICR, explaining the lower $[Ca^{2+}]_{SR}$ termination threshold observed in HF [17;30]. It is also possible that any diastolic Ca^{2+} handling dysfunction in the dyad (e.g. abnormal Na^+ - Ca^{2+} exchange; NCX) could influence local cytosolic Ca^{2+} dynamics and thus termination. Indeed, we recently found that in healthy myocytes local Ca^{2+} extrusion from the dyadic cleft by NCX limited inter-RyR CICR, suppressing Ca^{2+} sparks during diastole [46]. In HF, this mechanism can be impaired due to a mismatch in the organization of the t-tubular system and the SR network [47]. This may explain why HF myocytes are prone to arrhythmogenic SR Ca^{2+} release events during stress. Dependence of CICR termination on local cytosolic Ca^{2+} dynamics in the dyadic cleft could thus have some interesting pathogenic ramifications in many disease phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Cardiac Ca sparks terminate when $[Ca]_{SR}$ falls to a certain critical level
2. Local $[Ca]_{SR}$ depletion can terminate a spark by reducing the unitary RyR current, decreasing cytosolic $[Ca]$ around a RyR cluster and thus breaking the positive feedback of local CICR
3. We conclude that multiple $[Ca]_{SR}$ -dependent termination mechanisms likely co-exist to ensure a stability of cardiac Ca cycling

Figure 1. Fast cytosolic buffering attenuates sparks in permeabilized ventricular myocytes
 Sparks were detected as local changes in cytosolic Fluo-4 fluorescence. **A.** Representative sparks in the control and BAPTA-containing cytosolic solutions. The control cytosolic solution had 0.36 mM EGTA and a free $[\text{Ca}^{2+}]$ of 150 nM. The BAPTA cytosolic solution had 0.36 mM EGTA, 0.7 mM BAPTA and a free $[\text{Ca}^{2+}]$ of 150 nM. Line scan images are shown at top. Shown at the bottom is fluo-4 fluorescence profile at the point marked on the line scan image (see left margin). **B.** Summary results of various measured spark parameters in the two tested conditions.

Figure 2. Action of fast cytosolic buffering on spark-blink pairs in permeabilized ventricular myocytes

Sparks were detected as changes in cytosolic Rhod-2 fluorescence. Blinks were detected as changes in intra-SR Fluo-5N fluorescence. **A.** Representative spark-blink pairs in the control and BAPTA-containing cytosolic recording solutions. The control cytosolic solution had 0.36 mM EGTA (150 nM free $[Ca^{2+}]$). The BAPTA cytosolic solution here had 0.36 mM EGTA, 0.36 mM BAPTA (150 nM free $[Ca^{2+}]$). Line scan images are shown at top (spark) and bottom (blink). The fluorescence profiles at the marked point on the line scan image are shown in the center. **B.** Summary $[Ca^{2+}]_{SR}$ results in the 2 test conditions. Grey bars reflect the average initial $[Ca^{2+}]_{SR}$ level before the spark. Black bars reflect the average $[Ca^{2+}]_{SR}$ level at which the blink nadir occurred.

Figure 3. Fast cytosolic buffer action on single cardiac RyR function

Single channel recordings were done at +40 mV in cell-like solutions. **A.** Representative single RyR recordings in control and BAPTA-containing cytosolic solutions. The control cytosolic solution had 0.36 mM EGTA (10 μ M free $[Ca^{2+}]$). The BAPTA cytosolic solution here had 0.36 mM EGTA, 0.36 mM BAPTA (10 μ M free $[Ca^{2+}]$). Open events are upward deflections from the marked zero current level. **B.** Summary results (open probability, mean open time and mean closed time) in the 2 test conditions. No significant difference in single RyR function was detected after the cytosolic BAPTA addition.

Figure 4. Low cytosolic buffer power enhances spark activity in permeabilized ventricular myocytes

Sparks were detected as local changes in cytosolic Fluo-4 fluorescence. **A.** Representative sparks in the control and BAPTA-containing cytosolic recording solutions. The control cytosolic solution had 0.36 mM EGTA (150 nM free $[\text{Ca}^{2+}]$). The low EGTA cytosolic solution had 0.12 mM EGTA (150 nM free $[\text{Ca}^{2+}]$). Line scan images are shown at top. At the bottom are fluorescence profiles at the point marked on the line scan images (see left margin). **B.** Summary results of various measured spark parameters in the control and low EGTA test conditions.

Figure 5. Low cytosolic buffer power action on spark-blink pairs in permeabilized ventricular myocytes

Sparks and blinks were measured simultaneously. **A.** Representative spark-blink pairs in the control and low EGTA recording solutions. The control cytosolic solution had 0.36 mM EGTA (150 nM free $[Ca^{2+}]$) and the low EGTA cytosolic solution had 0.12 mM EGTA (150 nM free $[Ca^{2+}]$). Line scan images are shown at top (spark) and bottom (blink). Corresponding fluorescence profiles are shown in the center. Changes in the initial and blink nadir $[Ca^{2+}]_{SR}$ are indicated by arrows. **B.** Summary $[Ca^{2+}]_{SR}$ results in the 2 test conditions. The average initial and blink nadir $[Ca^{2+}]_{SR}$ levels were significantly lower in the low EGTA solution.

Figure 6. High intra-SR buffer power action on spark-blink pairs in permeabilized ventricular myocytes

Sparks and blinks were measured simultaneously after the SR was loaded with the low affinity Ca^{2+} buffer ADA. **A.** Representative spark-blink pair is shown. The cytosolic solution had 0.36 mM EGTA (150 nM free $[\text{Ca}^{2+}]$). Line scan images are shown at top (spark) and bottom (blink). Corresponding fluorescence profiles are shown in the center. **B.** Comparison of blink time-to-nadir (TTN) with and without intra-SR ADA present. **C.** Time constant of $[\text{Ca}^{2+}]_{\text{SR}}$ recovery after a spark is compared with and without intra-SR ADA present. **D.** Comparison of average initial and blink nadir $[\text{Ca}^{2+}]_{\text{SR}}$ levels with and without intra-SR ADA present.

Figure 7. Intra-SR Ca^{2+} dependent termination mechanisms
The pernicious attrition, RyR-resident and Casq-based processes are illustrated (not to scale).