Ryanodine Receptor Current Amplitude Controls Ca\textsuperscript{2+} Sparks in Cardiac Muscle

Tao Guo, Dirk Gillespie, Michael Fill

**Rationale:** In cardiac muscle, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) from the sarcoplasmic reticulum (SR) is mediated by ryanodine receptor (RyR) Ca\textsuperscript{2+} release channels. The inherent positive feedback of CICR is normally well-controlled. Understanding this control mechanism is a priority because its malfunction has life-threatening consequences.

**Objective:** We show that CICR local control is governed by SR Ca\textsuperscript{2+} load, largely because load determines the single RyR current amplitude that drives inter-RyR CICR.

**Methods and Results:** We differentially manipulated single RyR Ca\textsuperscript{2+} flux amplitude and SR Ca\textsuperscript{2+} load in permeabilized ventricular myocytes as an endogenous cell biology model of the heart. Large RyR-permeable organic cations were used to interfere with Ca\textsuperscript{2+} conductance through the open RyR pore. Single-channel studies show this attenuates current amplitude without altering other aspects of RyR function. In cells, the same experimental maneuver increased resting SR Ca\textsuperscript{2+} load. Despite the increased load, Ca\textsuperscript{2+} spark (inter-RyR CICR events) frequency decreased and sparks terminated earlier.

**Conclusions:** Spark local control follows single RyR current amplitude, not simply SR Ca\textsuperscript{2+} load. Spark frequency increases with load because spontaneous RyR openings at high loads produce larger currents (ie, a larger CICR trigger signal). Sparks terminate when load falls to the point at which single RyR current amplitude is no longer sufficient to sustain inter-RyR CICR. Thus, RyRs that spontaneously close no longer reopen and local Ca\textsuperscript{2+} release ends. (Circ Res. 2012;111:28-36.)

**Key Words:** calcium-induced calcium release ■ cardiac muscle ■ ryanodine receptor ■ sarcoplasmic reticulum ■ spark

In cardiac muscle, Ca\textsuperscript{2+} influx through surface membrane Ca\textsuperscript{2+} channels activates underlying ryanodine receptor (RyR) channels. This form of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) is the basis of cardiac excitation–contraction coupling. In resting cells, single RyR channels spontaneously open with a very low frequency. The Ca\textsuperscript{2+} released by one of these openings may activate neighboring RyR channels. This inter-RyR CICR occurs within a cluster of RyR at discrete SR Ca\textsuperscript{2+} release sites and generates spontaneous non-propagating Ca\textsuperscript{2+} sparks. The Ca\textsuperscript{2+} released at one release site (if large enough) may activate RyRs at a neighboring release site. This inter-site CICR generates propagating Ca\textsuperscript{2+} waves that can lead to arrhythmia and even sudden death. This study defines the control of localized inter-RyR CICR (sparks) in permeabilized cardiac ventricular myocytes. Permeabilized cells are used as an endogenous cell biology model of the heart to allow precise experimental manipulation of the cytosol and to eliminate Ca\textsuperscript{2+} influx through surface membrane Ca\textsuperscript{2+} channels (ie, excitation–contraction coupling-based CICR).

A long-standing unknown in spark local control is the mechanism that terminates inter-RyR CICR. Intuitively, CICR should operate with “explosive” positive feedback (released Ca\textsuperscript{2+} triggering further release until the sarcoplasmic reticulum [SR] Ca\textsuperscript{2+} store is empty). This does not happen in cells. Instead, local inter-RyR CICR events (sparks) terminate when SR Ca\textsuperscript{2+} load falls to a critical termination threshold, not when the SR is empty. Elevating SR Ca\textsuperscript{2+} load above normal levels dramatically increases spark frequency and the likelihood of arrhythmogenic Ca\textsuperscript{2+} waves. This modulation of sparks by SR Ca\textsuperscript{2+} load is commonly attributed to intra-SR (luminal) Ca\textsuperscript{2+} acting on luminal RyR Ca\textsuperscript{2+} regulatory sites. Single RyR Ca\textsuperscript{2+} current amplitude, however, varies proportionally to SR Ca\textsuperscript{2+} load, and this current is what drives inter-RyR CICR. The regulatory contribution of RyR current amplitude is largely
unexplored because there has been no means to differentially manipulate SR Ca\(^{2+}\) load and single RyR current in cells. Here, we use large RyR-permeable organic cations to decrease single RyR current without reducing resting SR Ca\(^{2+}\) load. Our results indicate that local SR Ca\(^{2+}\) load control of inter-RyR CICR is predominately governed by the current, not only intra-SR Ca\(^{2+}\) acting on intra-SR sites.

**Methods**

Single RyR channel function was recorded in planar lipid bilayers and Ca\(^{2+}\) sparks were monitored in acutely dissociated myocytes. Details of Methods are provided in the Online Supplement.

Heavy SR microsomes were prepared from rat ventricular muscle using established methods. These microsomes were fused into planar lipid bilayers composed of a 5:4:1 mixture of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine, respectively. Once RyR channel activity was observed, solutions in both compartments were changed as specified in the text or in the Figure legends. Many single RyR recordings were made in cell-like salt solutions. The cytosolic cell-like salt solution contained 120 mmol/L K\(^{+}\), 0.1 to 10 mmol/L free Ca\(^{2+}\), 1 mmol/L free Mg\(^{2+}\), and 5 mmol/L total ATP. The luminal cell-like salt solution contained 120 mmol/L K\(^{+}\), 1 mmol/L free Mg\(^{2+}\), and 0.1 to 1 mmol/L free Ca\(^{2+}\). All recordings were performed at room temperature with current sampled at 50 microsecond/point, filtered at 1 kHz, and analyzed using pCLAMP9 software (Molecular Devices, Sunnyvale, CA).

Sparks were recorded in single saponin-permeabilized rabbit ventricular myocytes using a fluorescent cytosolic Ca\(^{2+}\) indicator (Fluo-4). Permeabilized cells were placed in a standard recording solution containing 120 mmol/L K-aspartate, 5 mmol/L MgATP, 0.4 mmol/L EGTA, 10 mmol/L phosphocreatine, 10 mmol/L HEPES, 5 U/mL creatine phosphokinase, and pH 7.25. Cytosolic free Ca\(^{2+}\) and Mg\(^{2+}\) were 150 mmol/L and 1 mmol/L, respectively. Spark studies were performed at room temperature with Fluo-4 excited by 488 nm light and its emission monitored at >515 nm. Sparks were detected and analyzed using the Spark-Master program with a detection threshold of 3.8. SR Ca\(^{2+}\) load was assessed by peak caffeine-evoked Ca\(^{2+}\) release or intra-SR Fluo-5N fluorescence following standard methods. Fluo-5N was excited at 488 nm and its emission was measured at 500 to 530 nm. Summary results are presented as mean±standard error of the mean of several individual determinations. Statistical comparisons were performed using the Student t test (unpaired, P<0.05).

**Results**

The RyR is regulated by cytosolic Ca\(^{2+}\), Mg\(^{2+}\), and ATP, as well as luminal Ca\(^{2+}\) in cells. Single RyR channel function is defined here with all these important agents present at cell-relevant concentrations. In these cell-like salt solutions, the RyRs cytosolic Ca\(^{2+}\) EC\(_{50}\) is approximately 10 μmol/L, and sample RyR recordings with 10 μmol/L cytosolic free Ca\(^{2+}\) (1 mmol/L luminal Ca\(^{2+}\)) are shown in Figure 1A. Single RyR channels repeatedly open (upward) and close. The open probability (Po) and mean open time were not membrane potential-dependent (Online Figure IA), and thus this likely reflects RyR function at 0 mV (the resting SR potential in cells). Sample RyR recordings after application of cytosolic 60 mmol/L tris(hydroxymethyl)aminomethane (Tris\(^{+}\)) are also shown in Figure 1A. With Tris\(^{+}\) present, event frequency and mean open time were the same (Online Figure 1A, B) but current amplitude was clearly smaller. Figure 1B shows all-points histograms showing that Tris\(^{+}\) clearly shifts the open current peak (labeled) but does not substantially change its area. Figure 1C shows RyR Po before and after Tris\(^{+}\) application when 10 μmol/L cytosolic free Ca\(^{2+}\) is present. The Po was not significantly different with 0, 30, 60, or 120 mmol/L cytosolic Tris\(^{+}\) present. Figure 1C also shows Po after luminal Ca\(^{2+}\) was reduced from 1 to 0.1 mmol/L (open square) and after caffeine application (open circle). Reduced luminal Ca\(^{2+}\) did not significantly change RyR Po, whereas caffeine potentiated it significantly. Online Table II compares RyR Po and mean open time before and after Tris\(^{+}\) application with 1 or 0.1 μmol/L cytosolic free Ca\(^{2+}\). All these data show that Tris\(^{+}\) reduces current amplitude without altering RyR gating in these cell-like salt solutions.

The attenuation of single RyR conductance by increasing cytosolic Tris\(^{+}\) concentration is shown in Figure 1D. Conductance was significantly reduced when cytosolic Tris\(^{+}\) was >30 mmol/L. Figure 1D (inset) plots average single RyR current amplitude (n=9) as a function of voltage in the cell-like salt solutions. In the absence of Tris\(^{+}\) (open squares), the slope conductance is 188 pS and the reversal potential is −2.4 mV. In cells, the SR membrane potential (V_m) is thought to be held near 0 mV by the high resting K\(^{+}\) permeability of SR. Calcium is the only RyR-permeable ion present with a trans-SR electrochemical driving force at 0 mV (the resting SR potential in cells). Thus, the 0.35 pA at 0 mV is entirely Ca\(^{2+}\) current and very similar to the RyR unit Ca\(^{2+}\) current predicted in similar cell-like conditions elsewhere. Conductance is decreased to 145 and 100 pS after 30 or 120 mmol/L cytosolic Tris\(^{+}\) is added (Figure 1D, inset). Because the SR potential rarely will stay far from 0 mV in cells, only currents between +20 mV and −20 mV are shown and these vary linearly with voltage within this narrow range. Cytosolic or luminal Tris\(^{+}\) had similar action (Figure 1D, filled and open circles), indicating this Tris\(^{+}\) action on current is not sided. The conductance of these large monovalent cations is very small (≤20 pS) compared with K\(^{+}\) (>450 pS). If multiple RyR-permeable cations are present, then they will...

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CaSpF</td>
<td>Ca(^{2+}) spark frequency</td>
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<tr>
<td>CPVT</td>
<td>catecholaminergic polymorphic ventricular tachycardia</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca(^{2+})-induced Ca(^{2+}) release</td>
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<tr>
<td>FDHM</td>
<td>full duration at half maximum</td>
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<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
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<tr>
<td>MOT</td>
<td>mean open time</td>
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<tr>
<td>Po</td>
<td>open probability</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Tris(^{+})</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<td>TTP</td>
<td>time to peak</td>
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competes for occupancy of the RyR pore and influences the permeation of each other.\(^{15,19}\) In cell-like salt solutions, Ca\(^{2+}\) primarily competes with Mg\(^{2+}\) and K\(^{+}\) for occupancy of the pore.\(^{15-17}\) A robust model of multi-ion RyR permeation\(^{15,19}\) was used to predict single RyR Ca\(^{2+}\) flux in cell-like salt solutions (no Tris\(^{-}\)) as a function of SR Ca\(^{2+}\) load (Online Figure IC). The lumen-to-cytosol Ca\(^{2+}\) flux decreases from approximately 0.35 pA to 0.19 pA when load decreases from 1 to 0.5 mmol/L (as it likely does during a spark).\(^{13}\) The predicted 46% decrease in Ca\(^{2+}\) flux during a spark is approximately the magnitude of the RyR conductance decrease caused by 120 mmol/L Tris\(^{-}\) (Figure 1D). Although the precise mechanism is unclear at this point, the significant point here is that Tris\(^{-}\) attenuates RyR ion permeation without affecting RyR Po or mean open time.

Single RyR mean open time in cell-like salt solutions is indicated on the plot of open and closed dwell times shown in Figure 1E (arrow). The dwell times shown were measured from five different channels without (symbols) and with Tris\(^{-}\) (60 mmol/L; solid lines) present. The dashed line represents open times in our cell-like salt solution with 1 \(\mu\)mol/L instead of 10 \(\mu\)mol/L cytosolic Ca\(^{2+}\). Average spark time-to-peak (TTP) in our permeabilized cells is indicated by the white arrow. With 10 \(\mu\)mol/L cytosolic Ca\(^{2+}\) present (similar to that around RyRs during a spark), >96% of measured single RyR openings are shorter than spark TTP. This implies that an individual RyR likely opens and closes repeatedly during the rise time of a spark. If so, then spark termination could be attributable to a mechanism that limits RyR reopening, not necessarily only one that drives RyR to close.

Spontaneous sparks were measured in saponin-permeabilized myocytes. The resting cytosolic salt composition was similar to that used in our single RyR studies. The exception is that it contained 150 mmol/L free Ca\(^{2+}\), which supported a resting spark frequency of 12.6±1.1 sparks (100 \(\mu\)m)\(^{-1}\)s\(^{-1}\). Figure 2A shows representative line scan images of sparks before (control) and after the application of 60 mmol/L cytosolic Tris\(^{-}\). The Tris\(^{-}\) dramatically reduced spark brightness (amplitude) and frequency. This action was readily reversible. It was present within 1 minute of Tris\(^{-}\) application and was gone within 1 minute of its removal (Online Figure IIIA). The addition of an alternative K\(^{+}\) permeability pathway (valinomycin) to the SR had no effect on spark frequency with or without Tris\(^{-}\) present (Online Figure IE), indicating the action of Tris\(^{-}\) on sparks is not attributable to a shift in SR membrane potential. Figure 2B shows that other large organic cations (L-lysine or triethanolamine) had a similar action as Tris\(^{-}\) on spark frequency and amplitude. Sparks were not altered when large uncharged organic molecules (eg, glutamine) were applied. Sample line scan images with these large organic cations present are shown in the Supplemental Materials (Online Figure II). Thus, the spark alteration was not Tris\(^{-}\)-specific, but instead occurred when any large poorly RyR-permeable cation was added.

Numerous (>150) in-focus control or Tris-modified sparks were collected and temporally aligned (to their peak) to...
generate the average spark waveforms shown in Figure 2C (left). Average spark amplitude is approximately half its normal value with 60 mmol/L Tris⁺ present. The action of Tris⁺ on spark TTP and decay time constant is presented in Figure 2C (right). Average spark decay rate was not Tris⁺ sensitive. This is expected because spark decay (local Ca²⁺ removal) is largely attributable to diffusion. Average spark TTP was significantly shorter at high Tris⁺ levels. The Tris⁺-evoked change in spark TTP also is evident in the probability histograms presented in Figure 2D. The spark peak is the moment local Ca²⁺ release no longer outpaces local Ca²⁺ removal (diffusion). Thus, the shorter TTP implies local Ca²⁺ release ends (or terminates) sooner when Tris⁺ is present. Spark termination occurs when local SR Ca²⁺ load declines to a critical level,¹ ¹² and this is thought to be attributable to intra-SR Ca²⁺ acting at intra-SR RyR Ca²⁺ regulatory sites. With Tris⁺ present, however, local intra-SR Ca²⁺ load should decline less because Tris⁺ limits single RyR current. This is evident by the decline in spark mass at high cytosolic Tris⁺ levels (Online Figure IIID). Spark mass is approximately proportional to the decrease in local load during a spark. If termination were solely driven by Ca²⁺ acting on intra-SR RyR regulatory sites, then a smaller decline in local load would drive termination less efficiently (ie, lengthen, not shorten, TTP). Thus, the shorter TTP in Tris⁺ suggests that another mechanism explains the luminal Ca²⁺ sensitivity of spark termination.

The cell-wide resting SR Ca²⁺ load is established by the balance between SR Ca²⁺ uptake and resting SR Ca²⁺ leak. Any form of RyR block should reduce resting leak and thus elevate load. We evaluated the action of Tris⁺ on cell-wide SR Ca²⁺ load three different ways. First, SR Ca²⁺ load was directly measured using intra-SR Fluo-5N. Figure 2E shows that acute cytosolic Tris⁺ application significantly increased Fluo-5N fluorescence (load) by 6%. Second, peak caffeine-evoked Ca²⁺ release peaks was used to assess resting SR load (Online Figure IIIIB). Five minutes after Tris⁺ application, peak caffeine-evoked release also was significantly increased, indicating an elevation in resting load. Third, SR Ca²⁺ release waves were used as an indicator of SR Ca²⁺ overload.², ²⁰ Cytosolic Ca²⁺ levels >250 nmol/L normally result in substantial SR Ca²⁺ overload and frequent Ca²⁺ waves. Figure 3A shows that no waves are observed at increasing cytosolic free Ca²⁺ levels (images 1–4) up to 500 nmol/L if 120 mmol/L Tris⁺ is present. Figure 3B illustrates how load (ie, Fluo-5N fluorescence) varies with cytosolic free Ca²⁺ levels. Note that the Fluo-5N signal is nearly saturated at 150 mmol/L cytosolic Ca²⁺, and thus the small 6% increase in load observed in Figure 2E could be limited by indicator saturation. In any event, no waves were observed at cytosolic Ca²⁺ levels >250 nmol/L if Tris⁺ is present (Figure 3A). However, waves immediately occurred when the Tris⁺ was removed (Figure 3A, image 5), indicating that resting load was high when the Tris⁺ was present. This Tris⁺ block/unblock process was reversible. These data indicate that wave initiation required more than just SR Ca²⁺ overload. In other words, waves are not solely governed by intra-SR Ca²⁺ acting at intra-SR RyR Ca²⁺ regulatory sites as commonly thought. We propose that a different mechanism, one associated with RyR current amplitude, is likely involved.

Cell-wide resting SR Ca²⁺ load also can be altered by blocking the SR Ca²⁺ pump with thapsigargin. Figure 3C
The time constant of this decline was 11.1 minutes. Gray circles (n = 6) indicate load decline without Tris+ present (fit by single exponential; time constant = 11.1 minutes). Gray circles (n = 6) indicate load decay time constant = 11.1 minutes). Gray circles (n = 6) indicate load decline with 120 mmol/L Tris+ (time constant = 17.1 minutes). Loads at 10 and 20 minutes with and without Tris+ were statistically different (*P < 0.05; **P < 0.005).

shows that after thapsigargin application (at 0 minutes), waves occurred immediately after Tris+ removal and simultaneous return to 150 nmol/L cytosolic Ca2+. (image 5). Waves were detected by Rhod-2 fluorescence (excited 543 nm; emitted light measured at >600 nm). After a few minutes (image 6), SR load normalized and waves were no longer observed. B, Fluo-5N fluorescence (mean ± standard deviation; n = 5 experiments) plotted as a function of cytosolic free Ca2+. Solid line is Hill equation fit (EC50 = 42.6 mmol/L). Dashed lines are 95% confidence levels. Fluo-5N is 93% saturated at 150 nmol/L cytosolic Ca2+. suggesting the small 6% increase in Fluo-5N signal in Figure 2E was limited by saturation. C, Decline of SR load (Fluo-5N fluorescence) attributable to SR Ca2+ leak after 10 μmol/L thapsigargin application. Black circles (n = 6) indicate load decline without Tris+ present (fit by single exponential; time constant = 11.1 minutes). Gray circles (n = 6) indicate load decline with 120 mmol/L Tris+ (time constant = 17.1 minutes). Loads at 10 and 20 minutes with and without Tris+ were statistically different (*P < 0.05; **P < 0.005).

Discussion

There are numerous RyR inhibitors, blockers, and modulators that influence RyR opening/closing9 (ie, RyR gating). The few that alter RyR current amplitude also affect its gating.9 Here, we apply a novel form of current-targeted RyR block to examine CICR local control. Single RyR
current amplitude is attenuated using a large RyR-permeable organic cation (Tris\(^+\)). We used Tris\(^+\) primarily, but other large cations had the same effect. This cation reduces net current amplitude because it interferes/competes for occupancy of the RyR pore with other permeable cations present\(^1\) (ie, Ca\(^{2+}\), Mg\(^{2+}\), and K\(^+\) in cells and in our cell-like salt solutions). This interference with permeation occurs without a change in RyR gating. We used this current-targeted block to differentially manipulate single RyR current amplitude and resting SR Ca\(^{2+}\) load in cells to better-define their roles in CICR local control.

It is well-established that the amplitude, width, and frequency of sparks (local bouts of inter-RyR CICR) vary with resting load.\(^{21}\) Also, sparks terminate after resting load declines to some critical value\(^1\) and SR Ca\(^{2+}\) overload promotes spark activity to the point that propagating Ca\(^{2+}\) waves begin. These phenomena are often explained by intra-SR Ca\(^{2+}\) acting on intra-SR RyR Ca\(^{2+}\) regulatory sites.\(^2,4\) Changes in load clearly act at these sites. Changes in load, however, also will alter single RyR current amplitude, the current that drives local inter-RyR CICR. The role of single RyR current amplitude in CICR local control is poorly understood. One reason is that it has been difficult to independently change resting load and current in cells. Further, it is impossible to directly measure single RyR current in cells. Note that the spark represents the opening of several RyRs within the RyR cluster at a release site. Here, we applied current-targeted RyR block (proven at single-channel level) to address the role of single RyR current amplitude during CICR local control. Our results indicate CICR local control is governed by SR Ca\(^{2+}\) load because load determines single RyR current amplitude.

A CICR local control scheme is presented in Figure 5. Although its applicability to CICR local control in intact cells remains to be established, this scheme provides a context for explaining the potential significance of our results. In this scheme, we assume spontaneous single RyR openings in resting cells result in a spark only when the opening generates a large enough local Ca\(^{2+}\) signal to trigger inter-RyR CICR (ie, large enough to activate an adjacent RyR at the same release site). Logically, the magnitude of the local Ca\(^{2+}\) trigger signal will be determined by the single RyR current amplitude as well as the duration of the RyR opening. Spark frequency will vary with SR load because load determines single RyR current amplitude and, thus, local Ca\(^{2+}\) trigger signal magnitude. Spark frequency varies nonlinearly with load because the local Ca\(^{2+}\) trigger produced by one RyR can act on three to four adjacent RyRs within the RyR cluster. Also, single RyR open times are exponentially distributed (many more short than long). This means that the number of openings that have durations sufficient to trigger inter-RyR (a spark) will...
increase exponentially with load. This is because load and current vary in parallel and when current is larger, shorter openings (exponentially more) will become sufficient to trigger a spark.

We show that RyR mean open time in cell-like salt solutions is substantially shorter than spark time-to-peak (Figure 1, Online Table II). Few single RyR open events (<4% at 10 μmol/L cytosolic Ca2+) have durations that even approach spark time-to-peak. This suggests that individual RyRs may open and close many times during the rise time of a spark. This possibility is supported by our in vitro measurements of inter-RyR CICR after a cluster of RyRs is fused into a bilayer (Online Figure IV). During these in vitro bouts of inter-RyR CICR, the RyRs present repeatedly open and close. Note that this inter-RyR CICR is not FK506-binding protein-based coupled gating23 because it occurs only when Ca2+ is the charge carrier and the RyRs present do not all open and close in a “lock-step” fashion (Online Figure IV).

Assuming individual RyRs open and close many times during a spark, our scheme (Figure 5) suggests open RyRs that spontaneously close will reopen as long as the current carried by any of their open neighbors is sufficient to reactivate them (ie, sufficient to drive inter-RyR CICR). As local load declines, the single RyR current amplitude (at some point) will become too small to drive RyR reopening. At this point, any RyR that spontaneously closes will remain closed, local inter-RyR CICR will cease, and the spark will terminate. This process implies that any RyR manipulation (regulatory, mutagenic, pharmacological, and others) that alters the channel’s cytosolic CICR sensitivity or open time will alter the current level (ie, load level) where inter-RyR CICR begins and ends. For example, the SR load where inter-RyR CICR ends would be smaller if a cardiac RyR mutation (or regulatory event) enhances the RyR CICR sensitivity because the current would need to be smaller for local CICR to cease. Once local inter-RyR CICR ends, the probability of it reoccurring at the same release site will increase over time as the SR Ca2+ pump restores local load (ie, restores single RyR current amplitude).

Like the early theory of CICR local control,24 our working local control scheme assumes the Ca2+ current carried by an open RyR in cells acts only on neighboring channels, not on itself.5 Single RyRs are activated by cytosolic Ca2+, and thus it might be reasonable to expect that the current may feedback and prolong the opening of the RyR carrying it. Accordingly, smaller currents would reduce mean open time because there would be less self-RyR cytosolic Ca2+ activation. This could contribute to the reduction in spark amplitude, width, and time-to-peak we observed when single RyR current was decreased. However, we have shown elsewhere that single RyR open times do not change significantly in the presence or absence of a lumen-to-cytosol Ca2+ current.5,25 This is consistent with the voltage-independent RyR mean open time we show here (Online Figure IA, D). Single RyRs in cells appear to be largely “immune” to their own Ca2+ current, even though that current is more than sufficient to activate a nearby neighboring RyR.5 This appears to be contradictory to some previous reports of RyR Ca2+ feedback regulation.8,26,27 However, these previous studies were performed using superfysiological Ca2+ fluxes, highly potentiated cytosolic Ca2+ sensitivity, and/or pharmacologically altered channels to promote feedback. For example, Sitsapesan and Williams26 observed RyR Ca2+ feedback activation only in sulmazole-activated channels. Xu and Meissner27 reported that significant RyR Ca2+ feedback activation at physiological current levels (<0.5 pA) only when caffeine was present. Laver8 defined Ca2+ feedback of ATP-activated RyRs (no Mg2+ present), which have greatly potentiated cytosolic Ca2+ sensitivity.25

Our spark local control scheme (Figure 5) implies that some spontaneous openings will be too brief to trigger sparks (CICR). This is consistent with recent reports of nonspark RyR-mediated SR Ca2+ leak.21,22 It is also consistent with our previous low-dose caffeine (≤0.15 mmol/L) study that also concluded that only relatively long RyR openings trigger sparks.28 Caffeine is well-known to potentiate RyR cytosolic Ca2+ sensitivity.9 Caffeine (0.25 mmol/L) also has been shown to reduce the luminal Ca2+ level at which sparks terminate.13 This is also consistent with our scheme. Because caffeine-sensitized RyRs would be more responsive to a Ca2+ trigger, local load would need to decline further before the RyR current becomes insufficient to sustain inter-RyR CICR. Another rather elegant spark study has shown that cardiac sparks peak before the underlying local SR Ca2+ release stops.4 In other words, the active RyRs generating the spark close asynchronously over a period of time that extends past the spark peak (ie, not all at once). Our scheme suggests open RyRs spontaneously close (asynchronously) and local CICR ends because those channels fail to reopen. Thus, some quite disparate spark phenomena are consistent with our spark current control scheme. This form of spark local control, however, needs to be tested in intact cells. This will likely entail perfusion of patch-clamped myocytes with large organic cations and/or expressing of RyRs that have modified permeation properties.

Some form of negative control must exist to counter the inherent positive feedback of CICR.29 Many possible mechanisms have been proposed, but none (by themselves) is sufficient to explain the termination of local SR Ca2+ release.9 Consequently, there is a growing consensus that the negative control may arise from a composite of factors/processes. One of these factors/processes is luminal Ca2+ acting on intra-SR RyR Ca2+ regulatory mechanisms.4,20,30 There is a large body of data (including our own) indicating that RyR gating is modulated by intra-SR Ca2+ sensing sites.4,20,30 Our results here show that spark termination also occurs when luminal Ca2+ declines because at some point single RyR Ca2+ current becomes insufficient to sustain inter-RyR CICR. These factors/processes likely are not mutually exclusive because the magnitude of the local cytosolic Ca2+ trigger signal that governs inter-RyR CICR will depend on unit Ca2+ current amplitude and single RyR open duration (Po or mean open time). Any mutation, genetic manipulation, drug, or dis-
ease that alters RyR gating thus would influence the efficacy of a set unit Ca\(^{2+}\) current level to drive inter-RyR CICR. For example, the higher likelihood of inter-RyR CICR during catecholaminergic polymorphic ventricular tachycardia might be reduced by manipulations that either reduce unit current or shorten RyR open time. The RyR-targeted actions of flecainide and carvedilol reduce RyR unit current or shorten RyR open time. The RyR-tachycardia might be reduced by manipulations that either reduce CICR during catecholaminergic polymorphic ventricular tachycardia.34 These observations are quite consistent with our spark local control scheme (Figure 5).

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Disclosures

None.

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**Novelty and Significance**

**What Is Known?**

- Sparks are normal sarcoplasmic reticulum (SR) Ca\(^{2+}\) release events that are mediated by the opening of a cluster of ryanodine receptor (RyR) channels.
- Intra-SR Ca\(^{2+}\) overload increases spark frequency and sparks terminate when intra-SR Ca\(^{2+}\) declines to a critical threshold level.
- The intra-SR Ca\(^{2+}\) dependence of sparks often is explained by intra-SR RyR regulatory sites sensing changes in intra-SR Ca\(^{2+}\) concentration.

**What New Information Does This Article Contribute?**

- The amplitude of the Ca\(^{2+}\) current carried by individual open RyR channels determines the likelihood of the inter-RyR Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) that generates the spark, not only the direct sensing intra-SR Ca\(^{2+}\) concentration.
- Arrhythmogenic sparks and Ca\(^{2+}\) waves occured only when the individual RyR Ca\(^{2+}\) current was large, not when intra-SR Ca\(^{2+}\) concentration was high and the Ca\(^{2+}\) current was small.
- Sparks terminate when intra-SR Ca\(^{2+}\) declines to a point at which the single RyR Ca\(^{2+}\) current becomes insufficient to support ongoing inter-RyR CICR (ie, it becomes insufficient to trigger RyR reopening after local RyRs have spontaneously closed).

Abnormal local control of CICR can be arrhythmogenic and/or contribute to heart failure. How CICR is controlled has been debated for decades. However, it is clear that local control of CICR is governed by intra-SR Ca\(^{2+}\) concentration. This intra-SR Ca\(^{2+}\) control is generally attributed to intra-SR Ca\(^{2+}\) acting at intra-SR sites. However, changes in intra-SR Ca\(^{2+}\) also vary the size of the Ca\(^{2+}\) current carried by the individual RyR channel, the current that drives the inter-RyR CICR (sparks). We devised a means to differentially manipulate current amplitude and intra-SR Ca\(^{2+}\) concentration. We report that spark local control largely follows changes in current amplitude, not simply intra-SR Ca\(^{2+}\) concentration. We propose that spark control by single RyR Ca\(^{2+}\) current generates the intra-SR Ca\(^{2+}\) dependency, whereas intra-SR Ca\(^{2+}\) acting at intra-SR sites likely modulates it. Both of these mechanisms control spontaneous sparks and waves, making them clinically relevant as potential sites for pathological failure and/or therapeutic intervention.
Ryanodine Receptor Current Amplitude Controls Ca\(^{2+}\) Sparks in Cardiac Muscle
Tao Guo, Dirk Gillespie and Michael Fill

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EXPANDED METHODS

Channel Measurements. Cardiac intracellular Ca\(^{2+}\) homeostasis regulation is species specific\(^1\) but single mammalian cardiac RyR function is not\(^2\). Thus, the results would be the same no matter what mammalian species our single RyR2 channels are isolated from. We used rat channels because we are most experienced isolating RyR2s from rat. Heavy SR microsomes here were prepared from rat ventricular muscle using the method described by Chamberlain et al\(^3\). Planar lipid bilayers were composed of a 5:4:1 mixture (50 mg/ml in decane) of bovine brain phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine. Bilayers were formed across a 100 µm diameter hole in a Teflon partition separating two compartments. One compartment (cis) was virtually grounded and filled with a HEPES-Tris solution (250 mM HEPES, 120 mM Tris, pH 7.4). The other compartment (trans) was filled with HEPES-Ca solution (250 mM HEPES, 50 mM Ca(OH)\(_2\), pH 7.4). Then, 500 mM CsCl, 2 mM CaCl and 5-15 µg heavy SR microsomes were added to the cis chamber. All the channels were exposed to 50 mM luminal Ca\(^{2+}\) for at least 10 minutes and thus any calsequestrin present likely has dissociated\(^4\). Channel incorporation always resulted in the cytosolic side of the RyR2 channel facing the cis compartment\(^4\)–\(^6\). Thus, the cis compartment is referred to as cytosolic and the trans as luminal. Immediately upon observing single-channel activity, the cytosolic solution was replaced to establish the test conditions as described in the text. Calcium buffer solutions used were planned using WinMAXC program (Stanford University, Palo Alto, CA). Channel Po was determined using the half-amplitude threshold method ignoring current fluctuations <0.75 ms. Open and closed dwell time histograms were fit by 2 exponentials. The number of exponential components used for this fitting was determined by goodness of fit (i.e. minimization of the Chi\(^2\) value). One component provided a pore fit (Chi\(^2\) >4.5). The fit was substantially improved using 2 components (Chi\(^2\) <0.3) but improved only slightly when more components were used. This fitting suggests there are at least 2 classes of open times with no particular RyR gating scheme assumed.

Spark Measurements. Spontaneous Ca\(^{2+}\) sparks were measured in saponin-permeabilized cardiac ventricular myocytes\(^7,8\) that were acutely dissociated from adult male rabbit hearts as approved by the Institutional Animal Care and Use Committee. Sparks were collected from >250 different cells isolated from >80 different animals. Our measurements were made at room temperature (~22°C). Although sparks at this temperature occur more frequently and peak faster than at physiological temperature\(^9,10\), they are a good model of events that occur in the living heart\(^9\). Thus, our results are likely representative of events at physiological temperatures.

Removing the surface membrane (permeabilization) allows soluble cytosolic agents that may regulate SR Ca\(^{2+}\) handling proteins to be lost. Permeabilization may also cause small changes in the underlying membrane microdomains. Consequently, sparks in permeabilized and intact cells have slightly different properties (i.e. more frequent, larger amplitude) and this is the case here. The use of permeabilized cells, however, provides direct control of the cytosol which is key in this study. Avoids uncertainties associated with dialysis of large cations into intact cells (i.e. dialysis efficiency, time course, retention of cellular constituents) and eliminates complexities associated with control of membrane potential and sarcolemma Ca\(^{2+}\) handling processes. Although clearly not physiologically ideal, this study of large cation action on sparks is well-served by the use of permeabilized cells.
After permeabilization, cells were placed in the cytosolic solution listed in the text. The free Ca\(^{2+}\) concentration was 150 nM as calculated using WinMAXC program (see above). Cytosolic free Ca\(^{2+}\) was measured using a laser scanning confocal microscope and a 60x water-immersion objective (N.A.=1.3). Images were acquired in line scan mode (2 ms per line; pixel size 0.082 µm). The F\(_0\) was taken as the resting fluorescence in steady-state conditions (no sparks present) and \(\Delta F = F - F_0\). The spark detection was done using a threshold-based algorithm. The algorithm identifies sparks based on their deviation from the background noise. Specifically, images are low-pass filtered using a 5 x 5-pixel median filter and a 4 x 4-pixel smoothing filter. The mean and standard deviation (SD) are determined and regions exceeding 1.5 times SD are excised to create a baseline to normalize the whole image. Sparks were detected in this normalized image as deviations exceeding 3.8 times SD above the mean. Spark frequency (sparks × (100 µm\(^{-1}\) × s\(^{-1}\)), amplitude (\(\Delta F/F_0\)), full duration at half-maximal amplitude (FDHM; ms) and full width at half-maximal amplitude (FWHM; µm) were determined in each experimental condition.

Solution Comparison. The cytosol in cells is a highly complex solution containing innumerable substances and many of these regulate RyR function. No in vitro experimental solution can ever completely reproduce the cytosol. The free K\(^+\), free Mg\(^{2+}\) and total ATP levels in our cell-like salt solution used for our in vitro single-channel studies were the same as in our spark recording solutions. The free Ca\(^{2+}\) levels were different (10 µM vs. 150 nM, respectively). The higher Ca\(^{2+}\) level is near the RyR channel’s cytosolic Ca\(^{2+}\) EC\(_{50}\) assuring ample single RyR channel activity\(^{11}\). It is also about the Ca\(^{2+}\) level reached near an open RyR’s cytosolic edge when Ca\(^{2+}\) is being released from its central pore\(^{12}\). Thus, our cell-like salt solution replicates some important RyR regulatory factors but like all in vitro experimental solutions it falls short of matching the cellular reality. Online Table I compares the salient solution compositions in the solution used for our single channel and spark studies.

Online Table I: Single Channel and Spark Study Solution Comparison

<table>
<thead>
<tr>
<th>Cytosolic Solution</th>
<th>Free [Ca]</th>
<th>Free [Mg]</th>
<th>Total [ATP]</th>
<th>[K]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single RyR Studies</td>
<td>10 µM</td>
<td>1 mM</td>
<td>5 mM</td>
<td>120 mM</td>
</tr>
<tr>
<td>Ca Spark Studies</td>
<td>0.15 µM</td>
<td>1 mM</td>
<td>5 mM</td>
<td>120 mM</td>
</tr>
<tr>
<td>Luminal Solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single RyR Studies</td>
<td>1 mM</td>
<td>1 mM</td>
<td>---</td>
<td>120 mM</td>
</tr>
<tr>
<td>Ca Spark Studies</td>
<td>1 mM(^*)</td>
<td>1 mM(^#)</td>
<td>---</td>
<td>120 mM(^#)</td>
</tr>
</tbody>
</table>

Cells contain many additional components not listed here.
*Luminal SR as measured in cells elsewhere.
*SR has no active transport of these substances.

Organic Cation Manipulation. The large organic cations were applied to single RyR channels in bilayers and the cytosol of permeabilized myocytes two ways: 1) “cation exchange” where the large cation replaced an equal molar amount of K\(^+\) or 2) “direct addition” where the large cation was added in addition to all other solution components present. The large cation attenuated single RyR current and altered sparks no matter how it was added. However, most studies in cells were done by cation exchange to minimize changes in ionic strength and osmolarity. Single-channel studies were done both ways but only direct addition data is shown in Figure 1. Direct addition maintains the symmetric K\(^+\) across the bilayer. The symmetrical K\(^+\) and Mg\(^{2+}\) hold the reversal potential near 0 mV. In this situation, the large cation reduced slope conductance between ±20 mV. The current-voltage relationship became non-linear outside this voltage range.

Similar large cation actions were seen with Tris\(^+\), triethanolamine and L-lysin. Many single RyR channels studies have been done using Tris\(^+\) as a cation substitute. The presence of Tris\(^+\) likely attenuated single RyR current in those studies. However, the Tris\(^+\) concentration (and its action) was constant and largely overcome by the presence of a very large concentration of another highly RyR permeable ion. Single RyR current attenuation by Tris\(^+\) was limited and inconsequential in those studies. Here, the cell-like salt solution contain 3 highly RyR permeable charge carriers (Ca\(^{2+}\), Mg\(^{2+}\),
and $K^+$), but with the divalent at 10-50 times lower concentration. This more cellular situation allows large organic RyR permeable cations (e.g. $\text{Tris}^{+}$) to compete more effectively for occupancy of the pore and attenuate the net current.

**Chemical and Drugs.** Fluo-4 and Fluo-5N were purchased from Molecular Probes/Invitrogen (Carlsbad, CA). $\text{CaCl}_2$ standard for calibration was purchased from World Precision Instruments Inc. (Sarasota, FL). Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO) and were reagent grade.

**Online Table II**

<table>
<thead>
<tr>
<th>[Ca] $\mu$M</th>
<th>[Tris] mM</th>
<th>[K] mM</th>
<th>[Caff] $\mu$M</th>
<th>RyR Po</th>
<th>RyR MOT, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>120</td>
<td>0</td>
<td>0.49 ± 0.05</td>
<td>8.2 ± 1.0</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>0</td>
<td>0</td>
<td>0.47 ± 0.04</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>120</td>
<td>0</td>
<td>0.020 ± 0.006</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>1.0</td>
<td>120</td>
<td>0</td>
<td>0</td>
<td>0.023 ± 0.014</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>120</td>
<td>0</td>
<td>0.00015 ± 0.00019</td>
<td>1.97 ± 0.26</td>
</tr>
<tr>
<td>0.1</td>
<td>120</td>
<td>0</td>
<td>0</td>
<td>0.00016 ± 0.00027</td>
<td>1.92 ± 0.19</td>
</tr>
<tr>
<td>0.1</td>
<td>120</td>
<td>0</td>
<td>0.25</td>
<td>0.00064 ± 0.00048</td>
<td>6.65 ± 0.79</td>
</tr>
<tr>
<td>0.1</td>
<td>120</td>
<td>0</td>
<td>0.50</td>
<td>0.00092 ± 0.00083</td>
<td>20.8 ± 2.7</td>
</tr>
</tbody>
</table>

[Ca], [Tris], [K] and [Caff] is cytosolic $\text{Ca}^{2+}$, $\text{Tris}^{+}$, $K^+$ and Caffeine concentration. Cytosolic solution also contained 1 mM free $\text{Mg}^{2+}$ and 5 mM total ATP. Luminal solution contained 1 (clear) or 50 mM (shaded) free $\text{Ca}^{2+}$. Data represent at least 4 different determinations.
Online Figure I: Single cardiac RyR channel function. A. Single RyR channel summary data collected our cell-like salt solutions with 0 (filled circles), 40 (open circles) and 80 mM (triangles) cytosolic Tris+ present. Opening frequency and mean open time results were collected at +20 mV. Data are presented as means ± SEM (n>8). An unpaired T-test was used to compare control (no Tris+) and Tris+ results (n.s. indicates no significant difference). B. Summary data (at +20 mV) with 0 and 80 mM luminal Tris+ present. Data are presented as means ± SEM (n>5). There was no significant difference (n.s.) between control and Tris+ results. C. Predicted cellular single RyR Ca2+ current plotted as function of luminal Ca2+ concentration. This plot was generated using a combination of our PNP/DFT permeation model and SR equivalent circuit analysis as presented elsewhere. Note that the SR reversal potential is calculated at each luminal Ca2+ level (all are within a few millivolts of zero) and the Ca2+ current (shown) is at that potential. The logic of this approach is also described elsewhere. D. Summary mean open time data collected in our cell-like salt solution (at +20 mV) but with 0.1 and 1 mM luminal Ca2+ present with corresponding Ca2+ fluxes of 0.07 and 0.61 pA, respectively. Data are presented as means ± SEM (n>8). There was no significant difference (n.s.) between control and Tris+ results. E. Tris+ action on Ca2+ spark frequency in permeabilized ventricular myocytes in the absence and presence of valinomycin (a K+ ionophore). The * indicates that Tris+ significantly (p<0.05) reduced spark frequency with or without the valinomycin present.
Online Figure II

Large Cation Action on Sparks

A. 120 mM Cytosolic K
B. 120 mM Cytosolic Lysine

C. 120 mM Cytosolic Triethanolamine
D. 120 mM Cytosolic Glutamine

Online Figure II : Sample line-scan images acquired with different large cytosolic molecules present. Lysine and triethanolamine are large organic cations. Glutamine is a neutral amino acid. A. Control image acquired with only cytosolic K\(^+\) present. Several sparks are evident. B. Image acquired after cytosolic lysine was added. C. Image taken after cytosolic triethanolamine was added. D. Image taken after cytosolic glutamine was added and several sparks are evident.
Online Figure III: Tris⁺ action reversibility and slowing of Ca²⁺ release in permeabilized ventricular myocytes. A. Time course of Ca²⁺ spark frequency changes in response to repeated cytosolic Tris⁺ applications. Spark frequency rapidly (<1 min) decreases and increases upon Tris⁺ application and washout, indicating the Tris⁺ action is reversible and not due to a long-term time dependent process. Spark frequency remained low as long as Tris⁺ was present. This is interesting because Tris⁺ limits SR Ca²⁺ leak and thus SR Ca²⁺ load would be expected to increase over time. Increasing load typically increases spark frequency. B. Evaluation of SR Ca²⁺ load by 10 mM caffeine stimulation with 0 and 120 mM cytosolic Tris⁺. The Tris⁺ was present for about 5 minutes before the caffeine stimulation. Peak caffeine evoked Ca²⁺ release (load) was significantly greater (~30%) following Tris⁺ application (p<0.05; control n=13, Tris⁺ n=3). This suggests that the observed 6% load elevation observed using Fluo-5N (see Fig. 2E) was limited by indicator saturation (see Fig. 3B). The right panel plots the rate of the caffeine-evoked release with and without Tris⁺ present. The presence of Tris⁺ increased resting SR Ca²⁺ load (left) and tended to slow the rate of SR Ca²⁺ release. C. The cumulative histograms (%) of spark maximum release flux with 0 (black), 60 (red) or 96 mM (blue) cytosolic Tris⁺ present. Histograms represent 3574 (94), 154 (54) and 46 (18) individual sparks (cells), respectively. Lines are Hill equation fits having 50% values of 27.6, 20.3 and 14.8, respectively. These fit values were all significantly different (F-test; p<0.0001). Inset shows average spark maximum release flux (open circles) as a function of cytosolic Tris⁺ concentration. Color coded small filled circles represent the 50% values obtained from the Hill fits of the cumulative histograms. D. Cytosolic Tris⁺ dependence of spark mass (SM). SM was defined as 1.206 × ΔF/F₀ × FWHM³ and is roughly proportional to how much Ca²⁺ is locally released during a spark¹⁴. Relative SM was used to predict local SR Ca²⁺ depletion (red dashed line) as a function of cytosolic Tris⁺.
Online Figure IV: Inter-RyR CICR between neighboring single RyR channels. Clusters of RyR channels were incorporated into bilayers by fusion of crude cardiac SR vesicles. Openings are upward with net current in lumen-to-cytosol direction. Numbered hatch marks (right) indicate full open RyR current levels. **A.** Synchronized opening of 2 clustered RyR channels. All data are from the same cluster incorporation. Cytosolic contained 1 mM free Mg$^{2+}$, 5 mM total ATP, Tris-HEPES (120/250 mM), 5 or 0.1 µM free Ca$^{2+}$ and 1 mM EGTA. Luminal solution had 50 mM Ca$^{2+}$. Dashed line (top recording) indicates mean single RyR current of a single (lone) RyR (line thickness roughly equals its SEM; n=8). Corresponding all-points histograms are also shown (from 6 minutes of recording). Inset shows open current peaks on an expanded scale. Relative areas of Gaussian fits indicates the probability of both RyRs being simultaneously open is 0.14 at 5 µM and 0.01 at 0.1 µM cytosolic Ca$^{2+}$. We applied the Kenyon and Bauer formulation and determined their parameter D, which was negative (with r>0) for both of these histograms. This indicates that gating of the 2 channels present here is positively coupled. Single duration is single channel open dwell time. Cluster duration is the time between when channels in the cluster open to when they all close. **B.** Single (lone) RyR and clustered RyR function is significantly different. The 0.1 µM Ca$^{2+}$ solutions above were used here. These data (mean±SEM; ** indicate p<0.001) represent 8 different single (lone) RyR incorporations and 3 different cluster incorporation (each having 2 RyRs). Lone RyR values are from our previous work$^{16}$. Mean cluster current is less than 2x lone current because the channels present fluctuate between the 1 and 2 RyR current levels during the coordinated event. **C.** Sample recordings from a
different 2-RyR cluster before and after cytosolic Mg$^{2+}$ application (no ATP present). Bar graph at right shows that cytosolic Mg$^{2+}$ significantly (p<0.001) reduced the duration of coordinated RyR events (n=209 events). This is consistent with inter-RyR CICR coordinating the intra-event RyR function. D. Sample recordings (with cytosolic Mg$^{2+}$ present) from a 3-RyR channel cluster before and after application of 1 mM caffeine. Bar graph at right shows that caffeine significantly (p<0.01) increased the duration of coordinated RyR events (n=263 events). Again, this is consistent with inter-RyR CICR coordinating the intra-event RyR function. E. Clustered RyR function depends on charge carrier identity. Sample recordings (top) from another 2-RyR channel cluster showing function as charge carrier is sequentially changed (see numbers 1 to 4). All data are from the same cluster incorporation. Luminal solution contained 50 mM Ca$^{2+}$ or Ba$^{2+}$ and caffeine (1 mM) was applied as indicated. Bottom shows plots of current as a function of open dwell time. Blue or red points represent events where 1 or 2 RyRs are open, respectively. Coordinated openings were present only when Ca$^{2+}$ was the charge carrier, which is again consistent with inter-RyR CICR.

Online Figure V

A. Spark amplitude distributions (EC$_{50}$’s 0.40, 0.29 & 0.24; Hc’s 3.2, 5.3 & 10.9, respectively). B. Spark full width at half maximum (FWHM) distributions (EC$_{50}$’s 1.8, 1.3 & 0.75; Hc’s 4.9, 4.1 & 3.9, respectively). C. Spark full duration at half maximum (FDHM) distributions (EC$_{50}$’s 22.4, 20.9 & 16.6; Hc’s 5.4, 4.7 & 5.1, respectively). D. Spark decay time constant (Tau) distributions (EC$_{50}$’s 20.5, 20.3 & 17.5; Hc’s 4.0, 3.5 & 3.7, respectively).

Online Figure V: Cumulative histograms (%) of various spark measurements with 0 (black), 60 (red) or 96 mM (blue) cytosolic Tris$^+$ present. Insets show the raw distributions of the spark measurement (normalized top peak) at 0 or 60 mM cytosolic Tris$^+$. The 0, 60 and 96 mM Tris$^+$ results represent 3574 (94), 154 (54) and 46 (18) individual sparks (cells), respectively. Lines are Hill equation fits with EC$_{50}$’s and Hill coefficients (Hc) given below. Fit values were all significantly different (F-test; p<0.0001) except for the spark decay measurements.
Online Supplemental Material References


