Quirky Calcium Release in the Heart

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Rationale: In cardiac myocytes, “Ca\textsuperscript{2+} sparks” represent the stereotyped elemental unit of Ca\textsuperscript{2+} release arising from activation of large arrays of ryanodine receptors (RyRs), whereas “Ca\textsuperscript{2+} blinks” represent the reciprocal Ca\textsuperscript{2+} depletion signal produced in the terminal cisterns of the junctional sarcoplasmic reticulum. Emerging evidence, however, suggests possible substructures in local Ca\textsuperscript{2+} release events.

Objective: With improved detection ability and sensitivity provided by simultaneous spark–blink pair measurements, we investigated possible release events that are smaller than sparks and their interplay with regular sparks.

Methods and Results: We directly visualized small solitary release events amid noise: spontaneous Ca\textsuperscript{2+} quark-like or “quirky” Ca\textsuperscript{2+} release (QCR) events in rabbit ventricular myocytes. Because the frequency of QCR events in paced myocytes is much higher than the frequency of Ca\textsuperscript{2+} sparks, the total Ca\textsuperscript{2+} leak attributable to the small QCR events is approximately equal to that of the spontaneous Ca\textsuperscript{2+} sparks. Furthermore, the Ca\textsuperscript{2+} release underlying a spark consists of an initial high-flux stereotypical release component and a low-flux highly variable QCR component. The QCR part of the spark, but not the initial release, is sensitive to cytosolic Ca\textsuperscript{2+} buffering by EGTA, suggesting that the QCR component is attributable to a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism. Experimental evidence, together with modeling, suggests that QCR events may depend on the opening of rogue RyR2s (or small cluster of RyR2s).

Conclusions: QCR events play an important role in shaping elemental Ca\textsuperscript{2+} release characteristics and the nonspark QCR events contribute to “invisible” Ca\textsuperscript{2+} leak in health and disease. (Circ Res. 2011;108:210-218.)

Key Words: quarky Ca\textsuperscript{2+} release ■ Ca\textsuperscript{2+} spark ■ Ca\textsuperscript{2+} blink ■ ryanodine receptor ■ Ca\textsuperscript{2+} leak

The Ca\textsuperscript{2+} ion is the most versatile intracellular messenger involved in vital cellular processes that include contraction, secretion, apoptosis, and gene expression regulation underlying cell proliferation and differentiation.\textsuperscript{1} In cardiac cells, Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels in the plasma membrane during an action potential triggers intracellular Ca\textsuperscript{2+} release through the type 2 ryanodine receptor (RyR2) Ca\textsuperscript{2+} release channels in the sarcoplasmic reticulum (SR), a process known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR). Depending on species and developmental stage, the SR amplification of the Ca\textsuperscript{2+} influx varies from almost 0 (early development) up to 16 (adult rat or mouse heart).\textsuperscript{2,3}

As one continuous organelle,\textsuperscript{4} the SR takes the form of an elaborated nanoscopic tubular and cisternal network that extends throughout the entire cytoplasm but occupies only \(\approx 4\%\) of the “cytoplasmic” volume in rabbit ventricular myocytes.\textsuperscript{5} It is also connected to both the endoplasmic reticulum and the nuclear envelope Ca\textsuperscript{2+} stores that are implicated in noncontractile Ca\textsuperscript{2+} signaling.\textsuperscript{4} Ca\textsuperscript{2+} release from the cardiac SR is controlled almost exclusively by RyR2 Ca\textsuperscript{2+} release channels. The SR membrane harbors \(\approx 10\textsuperscript{6}\) RyR2s per myocyte, and \(\approx 30\) to 300 RyR2s form a 2D paracrystalline array or Ca\textsuperscript{2+} release unit (CRU). A 3D constellation of \(\approx 10\textsuperscript{4}\) CRUs are spangled regularly throughout the cell.\textsuperscript{6,7} Most recent data suggest that some RyR2s within the regular organization of an array could be missing,\textsuperscript{8,9} suggesting that clusters of RyR2s of different sizes (including rogue RyR2s\textsuperscript{10}) could share the same junctional SR (jSR).

The prevalent view is that activation of a single CRU gives rise to a local, discrete Ca\textsuperscript{2+} release event: a Ca\textsuperscript{2+} spark.\textsuperscript{11} Summation of thousands of Ca\textsuperscript{2+} sparks that are activated during each heart beat gives rise to a whole-cell [Ca\textsuperscript{2+}], transient during cardiac excitation–contraction coupling.\textsuperscript{12,13} Simply put, this view suggests that there are no other forms of Ca\textsuperscript{2+} release but sparks in cardiac myocytes under physiological conditions. Several lines of observation, however, have challenged this view. Lipp and Niggli initially reported...
that CICR-dependent SR Ca\textsuperscript{2+} release may occur in a spatially uniform fashion under specific conditions.\textsuperscript{14} They ascribed the unresolved fundamental Ca\textsuperscript{2+} release events to the subresolution entity dubbed “Ca\textsuperscript{2+} quarks.” Later, they supported this hypothesis by showing that 2-photon photolysis of Ca\textsuperscript{2+}-caged compounds can trigger local Ca\textsuperscript{2+} release events that appears to be smaller than sparks.\textsuperscript{15} Using loose-seal patch-clamp and confocal imaging techniques, Cheng and colleagues demonstrated that individual release events evoked from the same CRU differ significantly by virtue of amplitude, suggesting polymorphism of Ca\textsuperscript{2+} sparks.\textsuperscript{16} Moreover, Sobie et al hypothesized that rogue RyR2s may act to produce SR Ca\textsuperscript{2+} efflux at a lower level than seen with the stereotyped CRU activation that produces Ca\textsuperscript{2+} sparks.\textsuperscript{10} Collectively, these reports question whether or not Ca\textsuperscript{2+} sparks are the sole “elementary” event underlying SR Ca\textsuperscript{2+} release. Alternatively, Ca\textsuperscript{2+} sparks may possess intriguing characteristics that are still unknown to us despite a 15-year intensive investigation.

As a Ca\textsuperscript{2+} spark appears, a local SR Ca\textsuperscript{2+} depletion signal, called a Ca\textsuperscript{2+} blink, can be observed.\textsuperscript{9} Blinks and sparks are 2 manifestations of the same elementary Ca\textsuperscript{2+} release event, viewed from the SR lumen and the cytosolic space, respectively. The SR consists of special regions that include the jSR and the free SR (fSR), and each component is distinct with varying concentrations of luminal Ca\textsuperscript{2+} buffers (eg, calquestrin, calreticulin), membrane-linked channels and transporters (eg, RyR2s, SR Ca\textsuperscript{2+} ATPase [SERCA]2a) and regulatory proteins (eg, triadin, junctin, phospholamban). The SR lumen does not appear to include cytosolic Ca\textsuperscript{2+} regulatory and buffering proteins (eg, troponin, calmodulin). As such, characterization of Ca\textsuperscript{2+} blinks, particularly when done in parallel with Ca\textsuperscript{2+} sparks, should be able to provide unique insight into SR Ca\textsuperscript{2+} release mechanisms and, more globally, into intracellular Ca\textsuperscript{2+} signaling.

In the present study, we exploit Ca\textsuperscript{2+} spark–blinker pairs to investigate local Ca\textsuperscript{2+} release events with improved detection ability and sensitivity. Our results reveal that low-flux quarky Ca\textsuperscript{2+} release (QCR) events coexist and interplay with regular Ca\textsuperscript{2+} sparks to provide rich Ca\textsuperscript{2+} signaling substructure and complexity (eg, dictating the decay of the sparks and the restoration of the blinks). This QCR component appears to be coupled to the high-flux initial release in a spark via the CICR mechanism and may have arisen from activation of rogue RyR2s or complex regulation of RyR2s within an array. Our results also show that the Ca\textsuperscript{2+} leak mediated through the QCR mechanism appears to be at least as important as that through the Ca\textsuperscript{2+} spark mechanism.

Methods

To simultaneously visualize Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} blinks, New Zealand White rabbit ventricular myocytes were first incubated for 2 hours at 37°C with the low-affinity Ca\textsuperscript{2+} indicator fluo-5N-AM (20 \textmu M/L, Invitrogen) and then for 15 minutes at room temperature with the high-affinity Ca\textsuperscript{2+} indicator rhod-2-AM (5 \textmu M/L, Invitrogen).\textsuperscript{3} With the diastolic spark frequency being very low in rabbit ventricular myocytes, the SR Ca\textsuperscript{2+} load was increased by replacing extracellular Na\textsuperscript{+} (NaCl) by equimolar Li\textsuperscript{+} (LiCl) in the bath solution, unless otherwise noted (see Figures 3 and 5).

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Non-standard Abbreviations and Acronyms

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<th>Abbreviation</th>
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<tr>
<td>CICR</td>
<td>Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release</td>
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<td>CRU</td>
<td>Ca\textsuperscript{2+} release unit</td>
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<tr>
<td>fSR</td>
<td>free sarcoplasmic reticulum</td>
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<td>jSR</td>
<td>junctional sarcoplasmic reticulum</td>
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<td>SERCA</td>
<td>sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase</td>
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Results

Spark–Blink Pair Analysis Increased Detection Ability of In-Focus Ca\textsuperscript{2+} Release Events

Figure 1A showed a representative Ca\textsuperscript{2+} spark–blinker pair in line-scan images from a rabbit ventricular myocyte. Spatial profiles (Figure 1B) revealed that the blink aligned well with the jSR (J) and was sharply confined (full-width at half maximum [FWHM]=1.01±0.05 \mu m; n=51), reflecting the nanometer-sized jSR ultrastructure and restricted intra-SR Ca\textsuperscript{2+} diffusion between the jSR and the fSR.\textsuperscript{3} In contrast, the companion spark, which reflected the local Ca\textsuperscript{2+} release, Ca\textsuperscript{2+} diffusion, and buffering in the cytosolic space, spanned the entire sarcomere and even spread into the neighboring junctions (J−1, J+1), displaying a FWHM of 2.27±0.12 \mu m (n=51) and a volume 10 times greater than for a blink. Because of the sharp spatial delineation of Ca\textsuperscript{2+} blinks, we have exploited blinks as the guide to select in-focus release events, ie, those sparks associated with discernible blinks. Under the present experimental conditions, ≈60% of Ca\textsuperscript{2+} sparks were rejected as out-of-focus events for the lack of companion blinks.

Simultaneous measurement of Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} blinks provided us with the unprecedented ability to identify small local Ca\textsuperscript{2+} release fluxes amid background noise. Using Gaussian noise–simulated traces, we showed that dual channel simultaneous detection suppresses false-positive events by ≈150-fold as compared with single-channel detection (Online Figure I), allowing the detection of a population of QCR events (see below).

Quarky Ca\textsuperscript{2+} Release: Novel Type of Local Ca\textsuperscript{2+} Signaling

Figure 2A and 2B shows an example of a succession of spark–blinker pairs and QCR–quarky Ca\textsuperscript{2+} depletion (QCD) pairs on the same jSR. The kinetics of these events were very short (QCR: \textit{t}_{\text{peak}}=19.1±1.0 ms, \textit{t}_{\text{decay}}=20.1±1.1 ms; QCD: \textit{t}_{\text{peak}}=19.2±1.3 ms, \textit{t}_{\text{decay}}=20.8±1.9 ms; n=42; Figure 2C). The amplitude of these reciprocal QCR-QCD events were only one-tenth to one-third of the regular spark–blinker events (QCR: \Delta F/F_0=0.069±0.006; QCD: \Delta F/F_0=0.025±0.002; n=42; Figure 2D), suggesting that the QCR events may arise from a mode of Ca\textsuperscript{2+} release that is different from that which underlies the regular spark–blinker pairs. This idea was substantiated by the amplitude histogram that suggests the
existence of 2 distinct populations of Ca^{2+} release events (Figure 2E; n=176). Nevertheless, the properties of QCR events still appear larger than the isolated opening of a single RyR2 (ie, the true Ca^{2+} “quarks” hypothesized by Lipp and Niggli\textsuperscript{14}) because the Ca^{2+} flux of a single RyR2 Ca^{2+} quark is expected to be even smaller with a duration much shorter because of rapid closing by stochastic attrition.\textsuperscript{17}

To evaluate the significance of these QCR events in more physiological conditions, we studied QCR when rabbit ventricular myocytes were electrically stimulated (0.5 Hz) in a regular HEPES buffer (Figure 3A). We found that QCR events were 10 times more frequent than spontaneous Ca^{2+} sparks (2.77 ± 0.31 versus 0.24 ± 0.06 \([100 \text{ mm}^{-1}\text{sec}^{-1}]\); Figure 3B), whereas sparks displayed a 10 times greater signal mass (7.47 ± 1.55 versus 0.62 ± 0.13; Figure 3C). Consequently, the summed SR Ca^{2+} leak attributable to sparks was similar to that mediated by QCR events (1.48 ± 0.37 versus 1.49 ± 0.34). Taking into account that numerous QCR events

Figure 1. Spark–blink pairs. A, Line-scan images of simultaneous measurement of a Ca^{2+} spark (rhod-2) (left) and its companion Ca^{2+} blink (fluo-5N) (middle) in an intact rabbit ventricular myocyte are shown after background subtraction. Unsubtracted fluo-5N image (right) shows the enrichment for the fluo-5N dye in the jSR at the Z-disk. The spark–blink pair is centered on the jSR band which is labeled as “J.” B, Spatial profiles of the spark–blink pair. Arrows mark jSR locations (J, J-1, J-2, J+1, and J+2). C, Time courses of the spark–blink pair.

Figure 2. Quarky Ca^{2+} Release. A, Succession of Ca^{2+} sparks and QCR events on the same jSR (top), the corresponding Ca^{2+} binks and quarky SR Ca^{2+} depletion (QCD) events (middle), and their time courses (bottom). Ticks to the left of the bottom image mark locations of the Z-disks. Arrows denote QCR and QCD on the images and time course plots. B, Enlarged view of the last succession of QCR and spark (top left) and the corresponding QCD and blink (top right) from A, the automated detection of QCD/blink (middle right), and the corresponding time courses (bottom). Arrows denote QCR and QCD on the images and time course plot. C and D, Kinetics (C) and amplitude (D) of QCR and QCD. *p<0.05. E, Histogram distribution of amplitude of Ca^{2+} release events (QCR and spark).
may have gone undetected, we conclude that QCR represent an important, heretofore underappreciated SR Ca\(^{2+}\) leak mechanism (see below).

Spark–Blink Kinetics: Evidence for Low-Flux Continued Ca\(^{2+}\) Release

Careful examination of the kinetics of Ca\(^{2+}\) spark–blink pairs revealed substantial variation with respect to the decay of the sparks and the restoration of the blinks (Figure 4). Specifically, the spark decay time (t\(_{67}\)) exhibited a broad distribution with a mode at 45 ms, ranging from 25 ms (at 5% cutoff of the lower limit) to 95 ms (at 95% cutoff of the upper limit; Figure 4A). Such a large variation was unexpected (see Discussion) but could not be dismissed as an artifact of confocal detection because we observed a similar large variation in blink recovery time (t\(_{67}\)) (Figure 4A), the latter being essentially undistorted by confocal sampling.\(^5\)

To investigate whether this variability could simply be attributed to regional differences in Ca\(^{2+}\) diffusion or buffering, we examined the relationship between Ca\(^{2+}\) sparks and their corresponding Ca\(^{2+}\) blinks, the latter reflecting local jSR depletion and refilling. Because Ca\(^{2+}\) diffusion and
buffering differ markedly in the SR lumen versus the cytosol, we had expected little correlation between the recovery of blinks and the decay of their companion sparks. However, both Ca$^{2+}$ sparks and blinks exhibited similar average durations ($t_{\text{peak}}$ of sparks: $30.4 \pm 1.3$ ms and $t_{67}$ of sparks: $54.3 \pm 2.8$ ms for sparks versus $t_{\text{peak}}$ of blinks: $29.6 \pm 1.4$ ms and $t_{67}$ of blinks: $55.5 \pm 2.6$ ms for blinks, $n=51$ from 31 cells). In addition, scatter plots showed a strong linear correlation between the $t_{67}$ of spark decay and the $t_{67}$ of blink recovery, with a slope of 0.88 and $r^2=0.91$ (Figure 4B).

To further study the kinetics of the spark–blink pairs, we grouped spark–blink pairs based on $t_{67}$ of the sparks, which usually display better signal-to-noise ratios than the blinks. For events in the same group ($t_{67}<50$ ms, $n=24$ events; $50$ ms<$t_{67}<70$ ms, $n=19$ events; or $t_{67}>70$ ms, $n=7$ events), we aligned the simultaneously recorded sparks and blinks using the spark peak as the reference point, and resultant averaged traces are shown in Figure 4C. The longer the spark decay, the slower the corresponding blink recovery. More strikingly, once normalized by the amplitude, sparks and blinks showed virtually overlapped time courses in every group (Figure 4C). The tight correlation between spark and blink kinetics indicates that a common mechanism underlies the variability both in the cytosol and the SR lumen. That the average $t_{67}$ value varied by more than 2-fold in the 3 groups suggests that this variability is not readily attributable to a change in local Ca$^{2+}$...
recycling by the SERCA2a, which plays only a minor role in shaping the spark kinetics. Rather, these results indicate the presence of low-flux continued Ca\(^{2+}\) release that dictates the decay-recovery kinetics of the spark–blink pairs.

In contrast, the initial Ca\(^{2+}\) release appears to be stereotyped: there was little variation in the time-to-peak of the spark or time-to-nadir of the blink. The average value (\(\approx 30\) ms) was similar for all different categories of spark–blink pairs (Figure 4D); sparks or blinks displayed similar amplitudes regardless of their duration (Figure 4E). Moreover, there was little correlation between the amplitude and \(t_{\text{nadir}}\) of sparks (\(r^2=0.0082\); Figure 4F). This result indicates that, once triggered, the continued release component is self-sustained, independently of the size of initial release. Collectively, our data support the notion that the spark–blink pair consists of a stereotyped initial release component and a highly variable small flux tailing release component.

**Effects of EGTA on Continued Ca\(^{2+}\) Release**

To test the hypothesis that the trailing small-flux release was triggered by initial high flux release via local CICR, we examined Ca\(^{2+}\) spark–blink kinetics in the presence of 2 EGTA concentrations (0.5 or 2 mmol/L) in chemically permeabilized myocytes (Figure 5A and 5B). As an exogenous Ca\(^{2+}\) chelator, EGTA can effectively reduce the physical size and duration of Ca\(^{2+}\) sparks in the cytosol. However, EGTA was expected to exert no such effect on the companion Ca\(^{2+}\) blinks as long as Ca\(^{2+}\) release process remained unaltered. Should EGTA enter the SR, it should not materially affect the dynamics, either, because its high affinity for Ca\(^{2+}\) (\(K_a=150\) mmol/L at pH 7.2) renders it saturated at the high levels of [Ca\(^{2+}\)]\(_{\text{SR}}\) normally observed (in the range of a few hundreds micromolar to a few millimolar).

We found that in the presence of 0.5 mmol/L EGTA, Ca\(^{2+}\) spark and Ca\(^{2+}\) blink \(t_{\text{nadir}}\) values were 40.6±2.2 and 44.7±2.4 ms (n=58), respectively, which were 13.7 and 10.8 ms faster than the spark–blink pairs in intact cells in the absence of EGTA. Furthermore, in 2 mmol/L EGTA, Ca\(^{2+}\) spark and Ca\(^{2+}\) blink \(t_{\text{nadir}}\) values were further shortened to 29.1±2.4 and 31.7±2.5 ms (n=26, \(P<0.005\)), respectively (Figure 5C). Although the EGTA shortening of spark duration has been demonstrated before, the EGTA effect on Ca\(^{2+}\) blinks was totally unexpected for the reasons discussed above. The shortening of Ca\(^{2+}\) blinks suggested that the Ca\(^{2+}\) release process itself has been abbreviated by the inclusion of the Ca\(^{2+}\) buffer in the cytosol. Such EGTA sensitivity provided the first experimental evidence that a CICR-dependent mechanism participates in the release of an ongoing spark.

Analysis of the signal amplitudes showed that the Ca\(^{2+}\) spark amplitude decreased from 0.96±0.06 (n=58) in 0.5 mmol/L EGTA to 0.65±0.06 (n=26, \(P<0.01\)) in 2 mmol/L EGTA, as expected. By contrast, the blink amplitude displayed no significant change (0.22±0.01, n=58 in 0.5 mmol/L EGTA versus 0.20±0.01, n=26 in 2 mmol/L EGTA, \(P>0.05\); Figure 5E), suggesting that the amount of the initial SR Ca\(^{2+}\) release was essentially unchanged. The time-to-nadir of the blink was only marginally shortened (26.6±0.81, n=58, and 23.5±0.95 ms, n=26, in 0.5 and 2 mmol/L EGTA, respectively, \(P<0.01\); Figure 5C), indicating that the initial release, which determined the peak and nadir of the spark–blink pair, was largely EGTA-insensitive. Regardless of EGTA concentration, there was a linear correlation between spark and blink \(t_{\text{nadir}}\) values (Figure 5D), as was the case in intact cells in the absence of EGTA (Figure 4B). Taken together, these results reinforce the idea that elemental SR Ca\(^{2+}\) release events consist of a large EGTA-resistant initial release flux followed by a trailing EGTA-sensitive lower-flux release of variable duration.

**Substructures in Prolonged Spark–Blink Pairs**

The aforementioned data suggested that Ca\(^{2+}\) release continues beyond the peak or the nadir of the spark–blink pair, which confers the variability to the decay-recovery kinetics. We further hypothesized that such continued low-flux Ca\(^{2+}\) release is of the same or similar nature as the solitary QCR events described above. Two questions arise from these observations: (1) How can we directly visualize QCR-QCD substructures in a spark–blink pair? (2) How does the presence of the QCR mechanism fit with robust Ca\(^{2+}\) spark (and Ca\(^{2+}\) blink) termination? In this regard, we noticed a subpopulation of prolonged Ca\(^{2+}\) sparks (\(t_{\text{nadir}}>\text{mean}+4\ SD=135\) ms) occurring spontaneously in intact rabbit ventricular myocytes, examples of which are shown in Figure 6.

Figure 6A shows a spontaneous long-lasting Ca\(^{2+}\) spark that rises to a peak and then develops a low long-lasting plateau before its termination, similar to the prolonged sparks induced by FK506, rapamycin, or low-dose ryanodine in rat ventricular myocytes. The companion blink displays a clear nadir at the peak of the spark and then returns toward a low plateau. The sustained low [Ca\(^{2+}\)]\(_{\text{SR}}\) signal is consistent with continued Ca\(^{2+}\) efflux and reflects a dynamic balance between the efflux and the refilling of the jSR. Presumably, the prolonged or sustained Ca\(^{2+}\) sparks and blinks arise from sustained local CICR at a single jSR attributable to one or more RyR2s remaining active/open, a suggestion consistent with the low-dose ryanodine (or other) treatment. More complex examples of sustained release are shown in Figure 6B and 6C, illustrating repetitive activation of the QCR mechanism during prolonged spark–blink pairs. In both examples, the kinetics of the [Ca\(^{2+}\)]\(_{\text{SR}}\) closely mirrored the kinetics of the sparks. The succession of “bumps” (QCR events) of the prolonged Ca\(^{2+}\) spark are matched by “dips” (QCD events) of the prolonged Ca\(^{2+}\) blink, with an overall negative correlation coefficient \(r=-0.89\) and \(-0.87\), respectively. Interestingly, the long spark–blink pairs were not seen in 2 mmol/L EGTA (Figure 5), consistent with CICR triggering of QCR-QCD events during prolonged Ca\(^{2+}\) sparks.

**Discussion**

Novel features of Ca\(^{2+}\) release from the SR in heart cells have been identified and characterized here by dual imaging of Ca\(^{2+}\) signals in the cytosol and in the lumen of the SR. In addition to Ca\(^{2+}\) sparks, we report on the detection and characterization of small amplitude local Ca\(^{2+}\) release events that are spatially and temporally well-confined (ie, QCR-QCD events). The Ca\(^{2+}\) leak mediated by these QCR events was at least as important as those by Ca\(^{2+}\) sparks. We also show that similar low-flux Ca\(^{2+}\) release events can occur in...
conjunction with a regular Ca\textsuperscript{2+} spark. The latter is vividly manifested by the trailing QCR-QCD events in a prolonged Ca\textsuperscript{2+} spark and may undergo the variability in the decay kinetics of a Ca\textsuperscript{2+} spark. The newly identified QCR and substructure of Ca\textsuperscript{2+} signals in a spark (see Figure 7) provoke a number of questions that are discussed below.

**Local Ca\textsuperscript{2+} Release During a Spark**

Although polymorphism of Ca\textsuperscript{2+} spark amplitude has been demonstrated previously,\textsuperscript{16,21} here, we show for the first time that the kinetics of in-focus Ca\textsuperscript{2+} sparks display duration variation ascribed to the variability of the declining phase. Surprisingly, there is a tight correlation between kinetics of sparks and their corresponding blinks despite marked differences in calcium diffusion and buffering in the cytosol and the SR lumen. Moreover, increased cytosolic Ca\textsuperscript{2+} buffering by EGTA similarly abbreviates both Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} blinks. These results suggest a number of important features of the spark release function. First, the SR Ca\textsuperscript{2+} release flux that contributes to the Ca\textsuperscript{2+} spark must last longer than initially hypothesized,\textsuperscript{11} i.e., it must continue after the peak of the spark. Second, the release fluxes that underlie Ca\textsuperscript{2+} sparks appear to be a decreasing function of time, as also suggested by derivation of the release function from spark measurement\textsuperscript{22} and in the modeling work of Sobie et al.\textsuperscript{17} Third, the SR Ca\textsuperscript{2+} flux is long enough and strong enough to dominate the effect of cytosolic Ca\textsuperscript{2+} diffusion on spark kinetics, and the effect of jSR refilling on the blink kinetics. These findings in cardiac cells are also in general agreement with reports on “Ca\textsuperscript{2+} embers” under various experimental conditions in skeletal muscles,\textsuperscript{23} although, unlike heart, square-wave release fluxes are thought to be the norm.\textsuperscript{24} It should also be noted that Zima et al\textsuperscript{25} recently reported a very long blink recovery time (≈161 ms on average) that was attributed to a slow intra-SR Ca\textsuperscript{2+} diffusion caused by restrained connection between fSR and some jSR. However, our data suggest that the recovery of blinks can be very fast, as evidenced by the population of blinks with \( t_{67} < 70 \) ms in the absence and presence of millimolar EGTA. In light of the present findings, a more plausible explanation for the findings of Zima et al\textsuperscript{26} is that Ca\textsuperscript{2+} sparks can have variable kinetics and can display enhanced trailing SR Ca\textsuperscript{2+} release, which would confer the unexpected slow recovery of the associated Ca\textsuperscript{2+} blink.

Furthermore, we show that the initial high Ca\textsuperscript{2+} release flux in a Ca\textsuperscript{2+} spark is stereotypical, in contrast to the trailing Ca\textsuperscript{2+} release flux. More importantly, the trailing, but not the initial, Ca\textsuperscript{2+} release flux is sensitive to cytosolic Ca\textsuperscript{2+} buffering by EGTA. Hence, the local SR Ca\textsuperscript{2+} release during a Ca\textsuperscript{2+} spark occurs in 2 modes (one that involves a high-flux Ca\textsuperscript{2+} release that operates in a largely all-or-none fashion; and a second that involves low-flux Ca\textsuperscript{2+} release that is highly variable). The fact that EGTA abbreviates the spark and blink duration by selectively acting on the low-flux release mode is important because it indicates that the trailing release is activated and sustained by a CICR mechanism and may be more susceptible to modulation or perturbation by physiological and pathophysiological factors, as well as changes in experimental conditions.

**Possible Mechanism Underlying Subtler Local Ca\textsuperscript{2+} Release**

Although QCR can occur at the same site as regular sparks (at the optical resolution), QCR events have distinct features including their small size and apparent lack of refractoriness; the latter is evidenced by repetitive activation of QCR events in long sparks. Our working hypothesis (Figure 7) is based on recent publications from the Soeller\textsuperscript{a} and Hoshijima\textsuperscript{a} laboratories. Using optical superresolution technique (PALM)\textsuperscript{8} or electron tomography,\textsuperscript{9} they have shown that large clusters of RyR2s constituting the CRU were incompletely filled with RyR2. This leaves the possibility for some RyR2s to be rogue (single or in a small cluster) and in close proximity to the large cluster of RyR2s. Such rogue RyR2s may display higher CICR sensitivity (at low [Ca\textsuperscript{2+}], levels) than the large cluster of RyR2s.\textsuperscript{10} Using our previous numeric spark model,\textsuperscript{26} the differential effect of EGTA (between 0.5 and 2 mmol/L) on square-wave release fluxes are thought to be the norm.\textsuperscript{24} It should also be noted that Zima et al\textsuperscript{25} recently reported a very long blink recovery time (≈161 ms on average) that was attributed to a slow intra-SR Ca\textsuperscript{2+} diffusion caused by restrained connection between fSR and some jSR. However, our data suggest that the recovery of blinks can be very fast, as evidenced by the population of blinks with \( t_{67} < 70 \) ms in the absence and presence of millimolar EGTA. In light of the present findings, a more plausible explanation for the findings of Zima et al\textsuperscript{26} is that Ca\textsuperscript{2+} sparks can have variable kinetics and can display enhanced trailing SR Ca\textsuperscript{2+} release, which would confer the unexpected slow recovery of the associated Ca\textsuperscript{2+} blink.

Furthermore, we show that the initial high Ca\textsuperscript{2+} release flux in a Ca\textsuperscript{2+} spark is stereotypical, in contrast to the trailing Ca\textsuperscript{2+} release flux. More importantly, the trailing, but not the initial, Ca\textsuperscript{2+} release flux is sensitive to cytosolic Ca\textsuperscript{2+} buffering by EGTA. Hence, the local SR Ca\textsuperscript{2+} release during a Ca\textsuperscript{2+} spark occurs in 2 modes (one that involves a high-flux Ca\textsuperscript{2+} release that operates in a largely all-or-none fashion; and a second that involves low-flux Ca\textsuperscript{2+} release that is highly variable). The fact that EGTA abbreviates the spark and blink duration by selectively acting on the low-flux release mode is important because it indicates that the trailing release is activated and sustained by a CICR mechanism and may be more susceptible to modulation or perturbation by physiological and pathophysiological factors, as well as changes in experimental conditions.

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In our Ca²⁺ spark and QCR model, solitary QCR-QCD events arise from spontaneous activation of rogue RyR2s (Figure 7B), whereas the spark-commingled QCR events are activated by preceding CRU release via the CICR mechanism (Figure 7A). An important distinction of CRU and rogue release is that the former has a steeper [Ca²⁺]i dependence attributable to the cooperativity of interconnected RyR2s.¹⁰ This logic suggests 2 additional features. First, QCR-QCD events from rogue RyR2s would rarely trigger their nearby “master” CRU (in a retrograde manner). Second, repetitive openings of rogue RyR2 U (or CICR interplay among a few rogue RyR2 U) explain the high variability and the EGTA sensitivity of the trailing release. As a cautionary note, we have assumed that isolated QCR and the straggling QCR on the tail of long sparks are of common mechanism, as judged by their phenotypic similarities. Nevertheless, the similarities may be only apparent, and they may arise from distinct populations of rogue RyR2s. Future investigations are warranted to discriminate these possibilities.

**Implications in SR Ca²⁺ Leak**

All means by which Ca²⁺ exits the SR is “Ca²⁺ leak.” The most common measurement of Ca²⁺ leak is Ca²⁺ spark. Larger SR Ca²⁺ releases like macrosparks, long sparks, or Ca²⁺ waves are also easy to detect. However, so far, the small SR Ca²⁺ release events have been elusive until now, with the new results described here. By using spark–blink pairs as a guide to identify events amid noise, we have been able to show that QCR is a new form of active Ca²⁺ leak that otherwise would have been “invisible” or dismissed as nonsignificant. Because the real frequency of QCR events is difficult to estimate, we can only suggest that the real contribution of these small release events to Ca²⁺ leak is at least as important as Ca²⁺ leak caused by sparks. In addition, when a QCR is occurring, the nearby jSR could be more likely to have a spontaneous Ca²⁺ spark or participate in a conducted wave. Overall, this component of Ca²⁺ leak may be part of an invisible Ca²⁺ leak that might become aggravated in disease. The existence of this invisible leak may also be of importance in setting the integrated “pump–leak balance” of the SR and thereby in the fine tuning of local and global Ca²⁺ signaling.

In summary, we have demonstrated substructures in elementary SR Ca²⁺ release events and identified a new population of subtler release events, the QCR, which can be seen when the viewing is guided by “spark–blink pair” measurements. The cumulative Ca²⁺ leak associated with QCR appears to be as large as those associated with spontaneous Ca²⁺ sparks. Evidence suggests that the QCR may depend on the opening of rogue RyR2s or complex CRU regulation of RyR2s. Taken together, the understanding of coexistence and interplay of quarky and spark Ca²⁺ release events and their distinct regulation leads us to novel and important insight into cardiac Ca²⁺ signaling. If supported by additional independent evidence, our new understanding of QCR may lead to novel strategies for the control of SR Ca²⁺ leak and for the treatment of heart failure and Ca²⁺-dependent arrhythmias.

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**Disclosures**

None.

**References**

Using this method, we detected low amplitude, solitary Ca$^{2+}$ sparks evoked from in-focus Ca$^{2+}$ release units in cardiac myocytes. Biophys J. 2004;86:182–190.

Clusters of type 2 ryanodine receptors (RyR2s) can be different sizes but could share the same jSR.

What New Information Does This Article Contribute?

- In paced myocytes, QCR events were so frequent that the SR Ca$^{2+}$ leak from these events could be equal to that through Ca$^{2+}$ sparks.
- QCR events not associated Ca$^{2+}$ sparks could contribute to “invisible” Ca$^{2+}$ leak in health and disease.

Ca$^{2+}$ sparks represent the elemental unit of Ca$^{2+}$ release. They result from the activation of large arrays of RyR2s. Recently, it has been shown that clusters of RyR2s of different sizes could share the same jSR, suggesting possible substructures in local Ca$^{2+}$ release events. We tested this hypothesis by visualizing QCR events, which probably depend on the opening of small clusters of RyR2s (or rogue RyR2s). QCR events might also be responsible for the variability of the declining phase of a spark. Because the frequency of QCR events in paced myocytes is much higher than the frequency of Ca$^{2+}$ sparks, the total Ca$^{2+}$ leak caused by small QCR events is approximately equal to that of the spontaneous Ca$^{2+}$ sparks. This new mode of Ca$^{2+}$ release may be the mechanism behind the so-called “invisible” Ca$^{2+}$ leak. These findings suggest new approaches for the treatment of heart diseases characterized by exaggerated SR Ca$^{2+}$ leak, such as heart failure and catecholaminergic polymorphic ventricular tachycardia.
Quarky Calcium Release in the Heart
Didier X.P. Brochet, Wenjun Xie, Dongmei Yang, Heping Cheng and W. Jonathan Lederer

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Supplemental Material

Detailed Methods

Cells.

For measurement of blinks in the sarcoplasmic reticulum (SR), enzymatically isolated ventricular myocytes from adult New Zealand White rabbits were incubated with the low affinity Ca\textsuperscript{2+} indicator fluo-5N-AM (20 µmol/L, Invitrogen) at 37°C for 2 hr\textsuperscript{1-3}. In order to simultaneously measure sparks in the cytosol, loading of the cells with fluo-5N-AM was then followed by incubation with the high affinity Ca\textsuperscript{2+} indicator rhod-2-AM (5 µmol/L, Invitrogen) at room temperature for 15 min\textsuperscript{3}. During electrical stimulation at 0.5 Hz, intact myocytes were superfused by a HEPES solution at 1 ml/min containing (in mmol/L): NaCl 137, KCl 4.9, MgCl\textsubscript{2} 1.2, NaH\textsubscript{2}PO\textsubscript{4} 1.2, CaCl\textsubscript{2} 1, glucose 15, NaHCO\textsubscript{3} 5 and HEPES 20 (pH 7.4). In order to inhibit Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange and therefore maintain adequate SR Ca\textsuperscript{2+} load for the study of spontaneous Ca\textsuperscript{2+} sparks and blinks, extracellular Na\textsuperscript{+} (NaCl) was replaced by equimolar Li\textsuperscript{+} (LiCl). In a subset of experiment, cells were permeabilized by adding for the first 30 s, 50 µg/ml saponin to the superfusion solution containing (in mmol/L) KOH 100, aspartic acid 100, KCl 20, MgCl\textsubscript{2} 0.81, MgATP 3, phosphocreatine ditris 5, creatine phosphokinase 5, HEPES 20, EGTA 0.5 or 2, free Ca\textsuperscript{2+} 100 nmol/L and dextran 8 %, pH = 7.2\textsuperscript{4}. All experiments were done at room temperature.

Confocal Ca\textsuperscript{2+} Imaging.

Linescan images were acquired using a confocal microscope (LSM510, Zeiss) equipped with a 63x1.4NA oil immersion objective, at sampling rates of 1.5 ms/line and 50 nm/pixel. The scan line was placed along the longitudinal axis of the cell. Fluo-5N and rhod-2 were excited alternatively by 488 and 543 nm laser lines respectively (at 667 Hz) and fluorescence emission was collected at 500-530 and >560 nm, respectively.

Data Analysis and Statistics.

Computer programs were coded in Interactive Data Language (IDL, Research Systems Inc., Boulder, CO). The computer algorithm used for automated blink detection was the same as previously described\textsuperscript{3}. “Quarky” Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} spark mass were calculated by using the formula: mass = (ΔF/F\textsubscript{0}) × FWHM\textsuperscript{3} × 1.206 \textsuperscript{5}. Data were expressed as mean ± S.E.M. The significance of difference between means was determined, when appropriate, using a student t-test. A p<0.05 was considered statistically significant.
Online Figure I Dual Channel Imaging Enhances the Detection Ability by Suppressing False Positive Events.

A. Modeling of dual channel imaging in the presence of Gaussian noise. Gaussian noise corresponding to the standard deviation ($\sigma$) of real confocal images (0.2 $F_0$ for rhod-2 images (upper panel) and 0.1 $F_0$ for fluo-5N images (bottom panel)), was added to the simulated x-t linescan images and smoothed by a 5×5 filter. The dashed lines on the images indicate the positions for the traces plotted to the right. B. Amplitude distributions of false positive events from single-channel and dual-channel recordings from 1000 randomly generated Gaussian-noise image pairs. Single-channel false events were defined as those upward (light blue colored segments in the upper trace in A) or downward deflections (dark red colored segments in the bottom trace in A) that last longer than 15 ms (10 scanning lines) with no constraint on the amplitude. A dual channel false event was considered when two false events from the two channels overlapped by at least 70% (see the example between the dashed vertical lines of the traces in A). Note that dual channel detection rejects 99.3% of false events occurring in single-channel detection, thus enhancing the ability to detect true small events.
Online Figure II Effect of EGTA on the spatial [Ca\textsuperscript{2+}] distribution

A. Spatial [Ca\textsuperscript{2+}] distribution 10 ms after the peak of a spark for different [EGTA] (0.5 and 2 mmol/L). B. Difference of effect of EGTA (0.5 versus 2 mmol/L) on the spatial [Ca\textsuperscript{2+}] distribution 10 ms after the peak of a spark. C. Fractional buffering of EGTA (0.5 versus 2 mmol/L) on the spatial [Ca\textsuperscript{2+}] distribution 10 ms after the peak of a spark.
Supplemental References


