Ca\textsuperscript{2+} blinks: Rapid nanoscopic store calcium signaling


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Luminal Ca\textsuperscript{2+} in the endoplasmic and sarcoplasmic reticulum (ER/SR) plays an important role in regulating vital biological processes, including store-operated capacitative Ca\textsuperscript{2+} entry, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, and ER/SR stress-mediated cell death. We report rapid and substantial decreases in luminal [Ca\textsuperscript{2+}], called “Ca\textsuperscript{2+} blinks,” within nanometer-sized stores (the junctional cisternae of the SR) during elementary Ca\textsuperscript{2+} release events in heart cells. Blinks mirror small local increases in cytoplasmic Ca\textsuperscript{2+}, or Ca\textsuperscript{2+} sparks, but changes of [Ca\textsuperscript{2+}] in the connected free SR network were below detection. Store microanatomy suggests that diffusional strictures may account for this paradox. Surprisingly, the nadir of the store depletion trails the peak of the spark by about 10 ms, and the refilling of local store occurs with a rate constant of 35 s\textsuperscript{-1}, which is 6-fold faster than the recovery of local Ca\textsuperscript{2+} release after a spark. These data suggest that both local store depletion and some time-dependent inhibitory mechanism contribute to spark termination and refractoriness. Visualization of local store Ca\textsuperscript{2+} signaling thus broadens our understanding of cardiac store Ca\textsuperscript{2+} regulation and function and opens the possibility for local regulation of diverse store-dependent functions.

Methods

Electron Microscopy. Rabbit hearts were perfused with 6% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at room temperature. The left ventricle trabeculae were dissected out, postfixed in 2% OsO\textsubscript{4} in the same buffer, stained en bloc in uranyl acetate, and embedded in Epon. Sections were stained in uranyl acetate and lead salts and examined in a Philips 410 electron microscope. Linear dimensions of SR profiles were obtained by using the measuring tool of PHOTOSHOP (Adobe Systems, San Jose, CA) and relative volumes of SR compartments, by the point-counting method (23).

Cells. Enzymatically isolated cardiac myocytes from adult New Zealand White rabbits were loaded with 20 µM fluo-5N acetoxyethyl ester (AM) (Molecular Probes) for 2 h at 37°C, as described previously (22, 24). For cytosolic Ca\textsuperscript{2+} imaging, cells were incubated with 5 µM rhod-2 AM for 15 min. To maintain adequate SR Ca\textsuperscript{2+} load for the study of Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} waves, experiments were performed with extracellular Na\textsuperscript{+} replaced by equimolar Li\textsuperscript{+}, an inhibitor of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, in the Hepes solution: 137 mM LiCl/1.2 mM MgCl\textsubscript{2}·6H\textsubscript{2}O, 2.6 mM NaH\textsubscript{2}PO\textsubscript{4}, 15 mM CaCl\textsubscript{2}/15 mM glucose/20 mM Hepes (pH 7.4). Myocytes were perfused at 1 ml/min and field electrical stimulation was delivered at 0.5 Hz.

Confocal Ca\textsuperscript{2+} Imaging. Linescan images were acquired by using a confocal microscope (LSM510, Zeiss) equipped with a ×63, 1.4 numerical aperture, oil-immersion objective, at sampling rates of 0.75–1.5 ms per line and 50 nm per pixel. To measure SR and cytosolic Ca\textsuperscript{2+} simultaneously, fluo-5N and rhod-2 were excited alternatively by 488- and 543-nm laser lines, respectively (at 667 Hz), and fluorescence was measured at 500–530 and >560 nm, respectively. All experiments were performed at room temperature (22–23°C).

Changes in local store free [Ca\textsuperscript{2+}] were determined by the formula Δ[Ca\textsuperscript{2+}]\textsubscript{SR} = K\textsubscript{d}([F\textsubscript{0} + F\textsubscript{min}]/[K\textsubscript{d} – ΔF/[F\textsubscript{0} – F\textsubscript{min}]) – C\textsubscript{0}, where F\textsubscript{0} and F\textsubscript{min} refer to the fluorescence level at rest and after emptying the SR, respectively; the fluo-5N’s Ca\textsuperscript{2+} dissociation constant K\textsubscript{d} = 400 µM, and the diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} C\textsubscript{0} = 1.o mM based on previous in situ calibration (22). Fractional changes in calsequestrin-bound Ca\textsuperscript{2+} store were calculated by K\textsubscript{d,CSQ}[Ca\textsuperscript{2+}]\textsubscript{SR}/[C\textsubscript{0}(K\textsubscript{d,CSQ} + [Ca\textsuperscript{2+}]\textsubscript{SR})], where K\textsubscript{d,CSQ} = 500 µM, referring to the Ca\textsuperscript{2+} dissociation constant of calsequestrin (25).

Cell-Attached Patch Clamp. Cell-attached patch-clamping was established by using an Axopatch 200 B amplifier (Axon Instruments) in loose-seal configuration, as described in ref. 26. Glass patch pipettes of 5–7 MΩ contained the Hepes solution with the L-type Ca\textsuperscript{2+} channel agonist FPL641176 added at 10 µM. Membrane potential was determined by dividing proportionally freely available online through the PNAS open access option. 

Abbreviations: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; jSR, junctional SR; fSR, free SR; RyR, ryanodine receptor; FWHM, full width at half maximum.

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the test voltages between the pipette resistance and the seal resistance (20–50 MΩ).

**Automated Ca^{2+} Blink Detection.** A computer algorithm used for automated spark detection (27) was modified for the detection of blinks. Specifically, the algorithm first identifies islets of connected pixels that are 0.5 standard deviation (σ) below the mean F_0. The signal mass (S, sum of ΔF/ΔF_0) was calculated over N pixels in a box outlining an islet (see Fig. 2 A). The criterion for event detection is z = S/σN^{1/2} > 6.0, which corresponds to <1 false detection in 100 images (512 × 512) with Gaussian noise characteristics.

**Data Analysis and Statistics.** Fitting of blink time courses and spatial profiles used the nonlinear functions described previously for sparks (28, 29). Blink parametric measurement was based on the fitting results. Blinks or sparks were aligned by the onsets to obtain average time courses, and by the nadirs or peaks to obtain average spatial profiles. For spark–blink pairs, the nonlinear fitting was applied to the spark only for superior signal-to-noise properties. Alignment of the spark–blink pairs was then guided by the onsets of sparks. Computer programs were coded in Interactive Data Language, IDL (Research Systems, Boulder, CO). Data were expressed as mean ± SD. The significance of difference between means was determined, when appropriate, by using the nonparametric Kruskal–Wallis test and the Student t test. A P < 0.05 was considered statistically significant.

**Results and Discussions**

**Microanatomy of the SR Network.** The ER/SR is composed of interconnected cisternae and tubules that extend throughout the cytosol (Fig. 1), make signaling contacts with the plasmalemma (30), and are continuous with the nuclear envelope (5). The following description highlights features and morphometrics of cardiac SR in rabbit myocardium that are essential to this work. The SR is divided into two distinct domains: the junctional (jSR) and free (fSR) domains. The jSR forms extended flat cisternae that appear in thin sections as linear profiles, with an average length of 465 ± 200 nm (mean ± SD, n = 147 profiles) and a luminal width of ∼30 nm (Fig. 1 A and B). From this, we estimate an average diameter of 592 nm and a luminal volume of 0.008 fl. The interiors of the cisternae are electron dense (Fig. 1B), because of the Ca^{2+} binding protein calsequestrin. The pancake-shaped cisternae form junctions with the transverse (T) tubules at the level of the sarcomeric Z lines. SR and T tubules are separated by a narrow (∼12 nm) junctional gap, occupied by feet or cytoplasmic domains of RyRs, the Ca^{2+} release channels (Fig. 1E). Each SR cisterna bears one or two groups of RyR feet containing on the average ∼66 feet. The fSR forms a network most abundant over the A band, with occasional continuities across the Z lines, and contains the majority of the Ca^{2+} pumps. The jSR and fSR occupy 2.0% and 2.2%, respectively, of the “cytoplasmic” volume (total volume minus mitochondria, nuclei, and SR volumes) in rabbit ventricular myocytes. Because the jSR occupies a narrow band at the level of the Z lines, whereas the fSR is distributed over most of the sarcomere (Fig. 1F), the jSR volume density at the Z line is higher than that of the fSR elsewhere.

**Ca^{2+} Blinks Inside jSR Cisternae.** Dynamic Ca^{2+} regulation within the SR particularly at the level of the jSR cisternae was visualized with the low-affinity Ca^{2+} indicator fluo-5N, loaded with a protocol favoring SR retention (Fig. 6, which is published as supporting information on the PNAS web site) (22, 31). Under conditions conducive to spontaneous Ca^{2+} sparks in rabbit heart cells (see Methods), we detected small, brief, and spatially limited “darkenings” of the fluo-5N signal or Ca^{2+} blinks (Fig. 2 Ai). Blinks occur at the Z lines, where the jSR cisternae are located, and were better resolved in the normalized images (F/F_0) (Fig. 2 Aii) and fluorescence time-course plots (Fig. 2 Aiii) as clear dips amid background noise. Computer-automated detection of blinks (Fig. 2 Aiii) assisted the unbiased collection of events and showed that their rate of occurrence was 0.61 ± 0.47 s⁻¹ (100 μm)⁻¹ (n = 15 cells). Motion artifacts could be discounted as a cause, because blinks persisted when tension was inhibited by 5–10 mM 2,3-butanedione monoxime (32) but were completely cause, because blinks persisted when tension was inhibited by 5–10 mM 2,3-butanedione monoxime (32) but were completely

**Electrical stimulation-activated sparks summate into global Ca^{2+} transients (33, 34). To determine whether excitation evokes blinks in parallel to sparks, we examined the excitation-evoked Ca^{2+} release in the presence of the L-type Ca^{2+} channel antagonist nifedipine (1 μM, to reduce the density of evoked sparks for resolution of individual events). Linescan image (Fig. 2 Bi), individual records (Fig. 2 Bii), and averaged signals (Fig. 2 Biii) show that depolarization activated synchronized blinks at multiple jSR sites. Such triggered blinks were blocked by the replacement of extracellular Ca^{2+} with equimolar Ba^{2+} (n = 6 cells), indicating that they are activated by L-type Ca^{2+} current.
by the same mechanism as sparks. These observations suggest that the blinks underlie evoked as well as spontaneous sparks, thus representing a direct real-time measure of intraorganelle Ca\(^{2+}\) in a physiological process.

**Blinks Revealing Intra-SR Ca\(^{2+}\) Communication.** Important and surprising findings were uncovered with the spatiotemporal analysis of blinks. First, we found that during a blink, local store \([Ca^{2+}]_i\) falls rapidly, reaching its minimum ("nadir") in 24 \(\pm\) 11 ms (Fig. 3A). This indicates that the blinks occur rapidly and are a direct measure of Ca\(^{2+}\) release.

**Fig. 2.** \(Ca^{2+}\) blink: nanoscopic store \(Ca^{2+}\) depletion. (A) \(Ca^{2+}\) blinks. (i) Raw linescan image of SR \(Ca^{2+}\) with intra-SR indicator fluo-5N. The brief darkening shows a spontaneous jSR \(Ca^{2+}\) release event, a blink. (ii) Raw linescan image (F/F_0 to correct for the nonuniform indicator staining). (iii) Computer-aided automated detection of blinks. (iv) Time course of normalized blink from top. (B) Excitation-activated blinks (arrow). (i) Normalized linescan image showing four blink events. (ii) Time courses of blink events (arrowheads). (iii) Average time course with nonlinear fit (smooth curve; \(\Delta F/F_0 = -0.03, \text{time to nadir } t_{\text{nadir}} = 29 \text{ ms, } \text{t}_\text{recovery} = 31 \text{ ms}\). 1 \(\mu\)M nifedipine. (C) Surface plot of averaged blink (upper) with linescan image (lower) (\(n = 7\)). (D) Temporal characteristics of a blink (averaged: 36 spontaneous and 22 evoked events). Fit curve shows \(\Delta F/F_0 = -0.06, t_{\text{nadir}} = 22 \text{ ms, and time constant } \text{t}_\text{recovery} = 33 \text{ ms}\). (E) Spatial profile of the blink from D shows full width at half maximum (FWHM) 739 nm.

**Fig. 3.** Blinks revealing mechanisms of spark termination. (A) Same-site comparison of jSR depletions in blinks vs. \(Ca^{2+}\) waves. The fractional blink/wave amplitude is 0.61 at the site marked by arrowhead. (B) Statistical analysis of fluo-5N signal (\(\Delta F/F_0\)) for averaged blink from spark–blink pairs (bar 1), individually resolved blinks (bar 2), individual blinks corrected for background contamination (bar 3), full-fledged excitation-evoked release (bar 4), \(Ca^{2+}\) waves (bar 5), and caffeine (10 mM)-elicited \(Ca^{2+}\) release (bar 6). Data are expressed as mean \(\pm\) SD and \(n = 6–86\). Such [Ca\(^{2+}\)] transients correspond to a 65% reduction in the free [Ca\(^{2+}\)] and a 38% diminution of the calsequestrin-bound Ca\(^{2+}\) (see Methods). Arrow and dashed line mark the timing of electrical stimulation. (D) Comparison between jSR scraps and blinks. Traces are normalized to the same amplitude. The traces for scraps at jSR and fSR essentially overlap, and the \(t_{\text{nadir}}\) was 137 \(\pm\) 44 ms, and the half-recovery time was 194 \(\pm\) 92 ms (\(n = 6\)).
in normal Ca\textsuperscript{2+} transients (Fig. 3C). However, the apparent blink amplitude may underestimate the true local Ca\textsuperscript{2+} depletion: our electron microscopy morphometry indicates that a typical jSR cisterna is only 13\% of a diffusion-limited confocal volume element (≈0.06 fl). Thus background fluorescence (F\textsubscript{0}) may be influenced by optically colocalized nonactive cisternae and thereby produce an overestimated F\textsubscript{0} and underestimated ΔF/F\textsubscript{0}. To determine the extent of this possible effect, we carried out serial thin-section electron microscopy to map cisterna organization, and we found that, on average, 1.5 cisternae colocalize to a diffusion-limited confocal volume element (Fig. 7). The corrected blink amplitude in ΔF/F\textsubscript{0} is −0.13 (Fig. 3B), and the blink/wave magnitude ratio is 0.86. Based on in situ calibration of the fluo-5N signal (22), the cisternal Ca\textsuperscript{2+} depletion in a blink corresponds to about 54\% reduction of the free Ca\textsuperscript{2+} and 28\% liberation of the calsequestrin-bound Ca\textsuperscript{2+}, which are nearly as substantial as those during full-fledged excitation-evoked releases or Ca\textsuperscript{2+} waves (Fig. 3B). This result suggests that cisternal Ca\textsuperscript{2+} signaling operates virtually in an all-or-none fashion, due perhaps to the aforementioned diffusional strictures. The unexpectedly large jSR depletion during a spark further supports the notion for local store depletion to signal the termination of the elementary Ca\textsuperscript{2+} release events.

Once activated, a jSR undergoes refractoriness, as evidenced by the reduced amplitude of the succeeding sparks (Fig. 4A). We reasoned that local refractoriness and spark termination might share some common mechanisms. To delineate possible role of local store depletion in spark refractoriness, we measured the
restoration curve for spark amplitude. By using loose-seal patch clamp technique combined with confocal microscopy (26), sparks from subsurface jSR release units were activated by 400-ms patch membrane depolarization to 80 mV above the resting potential. To analyze restoration of local Ca\(^{2+}\) release after spark activation, we acquired recordings with multiple sparks activated in tandem during a single pulse by including the L-type Ca\(^{2+}\) channel agonist FPL641176 (10 \(\mu\)M) in the patch pipette to sustain the trigger Ca\(^{2+}\) influx. The local restitution of Ca\(^{2+}\) release was then indexed by the amplitude of the second spark relative to its corresponding first spark. It is noteworthy that, by using the spark amplitude ratio, this measurement of release recovery from refractoriness should be independent of L-type channel inactivation, because spark morphometrics are known to be unaffected by characteristics of the trigger Ca\(^{2+}\) signal (33, 34). Our results indicate that spark amplitude restitution exhibits a time constant of 187 ms, whereas local store 

**Fig. 5.** Visualization of complementary spark–blink signal pairs. (A) Simultaneous measurements of sparks and blinks. (Upper) Linescan images of repeated sparks (rhod-2, upper) and blinks (fluor-5N, lower). Dotted ellipses mark corresponding areas in the images. (Lower) Time courses of sparks (black, upper) and blinks (blue, lower). (B) Time courses of the spark (upper) and blink (lower) averaged from 86 event pairs in 9 cells. The fitted smooth curves (red) show sparks (upper) displaying \(\Delta F/F_0 = 0.30, t_{peak} = 21\) ms, and \(t_{recovery} = 43\) ms, and blinks (lower) displaying \(\Delta F/F_0 = 0.013, t_{nadir} = 32\) ms, and \(t_{recovery} = 23\) ms. Averaged spark obtained with rhod-2 alone (\(n = 22\) events) displayed \(\Delta F/F_0 = 0.41, t_{peak} = 19\) ms, and \(t_{recovery} = 31\) ms, suggesting that fluo-5N retention in the SR does not alter spark properties. (C) Averaged spatial profiles. Upper: Ca\(^{2+}\) spark (FWHM = 2.2 \(\mu\)m); lower: Ca\(^{2+}\) blink (FWHM = 1.0 \(\mu\)m). (D) Overview of the Ca\(^{2+}\) spark–blink duality with respect to transverse tubule (TT) and SR. LCC, L-type Ca\(^{2+}\) channel.

**Sparks-Blinks as Complementary Signaling Events.** To more rigorously demonstrate that blinks are nanoscopic store Ca\(^{2+}\) signaling events complementary to sparks, we measured simultaneously store and cytosolic Ca\(^{2+}\) by using high-speed dual-indicator confocal microscopy. As shown in Fig. 5A, Ca\(^{2+}\) sparks were well resolved with cytosolic rhod-2 (\(\Delta F/F_0 = 0.30, t_{peak} = 21\) ms, \(t_{recovery} = 43\) ms, FWHM = 2.2 \(\mu\)m). The companion

blinks obtained with SR-loaded fluo-5N, however, displayed degraded signal-to-noise property, because spectral separation of fluorescence has curtailed the blink signal (see Methods). To improve the blink measurement, we used signal averaging with sparks as a guide to align the corresponding areas in the rhod-2 and fluo-5N images. Results from 86 events in nine cells uncovered a clear spark-associated blink (Fig. 5B and C). The average blink closely resembles the blinks in terms of \(t_{nadir}\) (32 ms) and \(t_{recovery}\) (23 ms). The apparent blink amplitude, however, was reduced (\(\Delta F/F_0 = -0.013\)) and the width was slightly broadened (FWHM = 1.0 \(\mu\)m). This result was expected because sparks are many times bigger than blinks by spatial size, such that most of the underlying blinks would be out of focus when the companion sparks were captured (Fig. 7). As noted above, the \(t_{nadir}\) trails the \(t_{peak}\) by 11 ms. This delay reinforces the idea that the release flux in a spark or blink is a time-dependent declining function, consistent with cisternal Ca\(^{2+}\) depletion and release inactivation.

In summary, we have investigated Ca\(^{2+}\) signals within nanometer-sized Ca\(^{2+}\) stores in intact cells in real time, by combining measurements of cytosolic Ca\(^{2+}\), nanoscopic store Ca\(^{2+}\), and cellular ultrastructure. We demonstrated that large and rapid nanoscopic store Ca\(^{2+}\) depletions or Ca\(^{2+}\) sparks mirror Ca\(^{2+}\) sparks in the cytosol. Characterization of blinks affords unique insights into Ca\(^{2+}\)-induced Ca\(^{2+}\) release termination and refractoriness as well as intraorganelle Ca\(^{2+}\) regulation. The complementary spark–blink signal pairs in heart (Fig. 5D) may provide the prototype for similar reciprocal signals responsible for diverse store-dependent functions ranging from capacitative Ca\(^{2+}\) entry (5, 6) to store Ca\(^{2+}\)-dependent apoptotic signaling (10, 11). The highly sensitive measurement of nanoscopic store Ca\(^{2+}\) signal should make it possible to elucidate these signaling pathways with improved molecular and mechanistic resolution.
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